REDOX REGULATIE VAN INHIBITORY KAPPA B KINASE

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REDOX REGULATION OF INHIBITORY KAPPA B KINASE

OXIDANTS IN THE PATHOGENESIS OF INFLAMMATORY LUNG DISEASE

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de rector Magnificus Prof. mr. G.P.M.F. Mols, volgens het besluit van het College van Decanen, in het openbaar te verdedigen op donderdag 12 oktober 2006 om 14:00 uur

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Het denken mag zich nooit onderwerpen,
noch aan een dogma,
noch aan een partij,
noch aan een hartstocht,
noch aan een belang,
noch aan een vooroordeel,
noch aan om het even wat,
maar uitsluitend aan de feiten zelf,
want zich onderwerpen betekent het einde
van alle denken.

Henri Poincaré

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CHAPTER 1

General introduction

PROLOGUE

A necessary consequence of existence in an aerobic environment is the production of oxidants by all cell types. Generally, oxidants are believed to be harmful as they can cause damage to proteins, carbohydrates, lipids and DNA. Oxidants and the damage they cause have been tightly associated with a number of diseases, as well as ageing. However, oxidants are also toxic to pathogens and during infections intentional production of oxidants is paramount to fight and kill bacteria and viruses.
In recent years an entirely new function has been ascribed to oxidants. Evidence is now suggesting that when oxidants are produced in a controlled fashion and in modest quantities, they could actually play an important role in regulating normal cell and organ functions. Understanding the beneficial functions of oxidants and the fine balance that exists between the beneficial and adverse functions are the general concepts that will be investigated in this thesis.

BACKGROUND

Physiological oxidant generation and function

Oxidants are constantly produced as by-products of the process that generates energy within our cells. Adenosine triphosphate (ATP) is the molecular transporter of energy that is produced in the mitochondria. During the generation of ATP, the oxidant superoxide is formed as a by-product through the reduction of molecular oxygen. In addition, a number of metabolic enzyme systems also generate oxidants as by-products of their reactions.

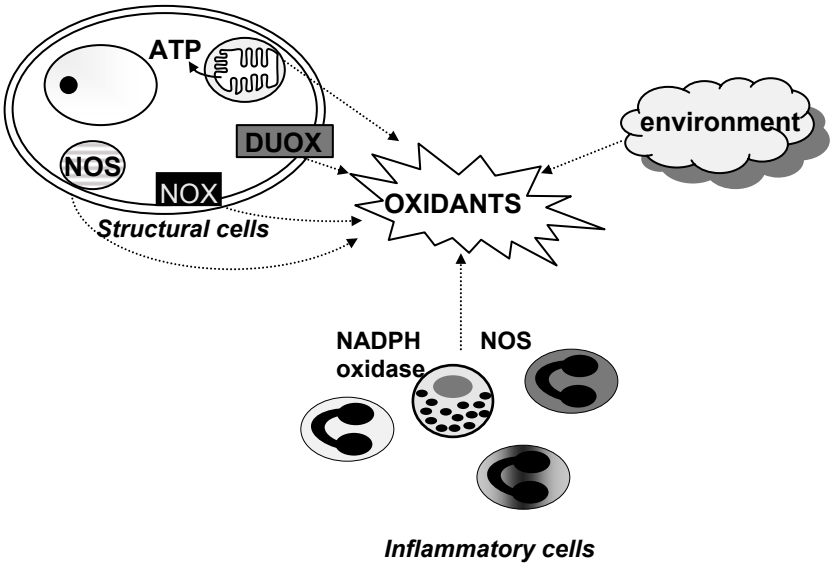

Figure 1 Oxidants can be produced through various enzymatic systems in resident as well as inflammatory cells. Oxidants can also be inhaled from the environment.

In addition, enzymes exist that function solely to produce oxidants in a regulated manner. The most well known deliberate producers of oxidants are nitric oxide synthases (NOS). Most cells contain three NOS isoforms that generate the oxidant nitric oxide ($NO^{\cdot}$) from L-arginine. NOS and $NO^{\cdot}$ will be introduced in greater detail in Chapter 2. Over the last 5 years additional enzymes have been discovered that exclusively produce oxidants. These enzymes are analogues of the phagocytic NADPH oxidases and are therefore termed non-phagocytic NADPH oxidase (NOX) or dual oxidase (DUOX). They use electrons from the electron-donating molecule NADPH to reduce molecular oxygen to form

superoxide. DUOX furthermore contains a peroxidase that converts superoxide into hydrogen peroxide (H_2O_2) (1, 2).

The discovery of enzymes that exclusively produce oxidants has led to the suggestion that oxidants play a critical role in maintaining homeostasis and are capable of regulating a number of physiological processes. The most significant data that support this hypothesis have come from studies that demonstrate that the response of cells to a number of growth factors and inflammatory proteins (cytokines) is dampened when cells are co-treated with antioxidants (3-7). These results indicate that the responses of cells are not only a result of the well known phosphorylation events that are triggered by these growth factors and cytokines, but are in addition caused by the generation of oxidants.

Many cellular functions are carried out by proteins, and the response of cells to growth factors and cytokines is usually the result of a change in the function of proteins. By modifying amino acids in proteins and thereby altering their activity and/or binding to other proteins, oxidants can affect cell responses. Protein alterations that occur after protein synthesis are termed posttranslational modifications and when they are carried out by oxidants they are referred to as redox-dependent posttranslational modifications.
The amino acid cysteine is one of the most rarely used amino acids in protein synthesis. The sulfhydryl group of cysteines is often very susceptible to oxidations, most notably when it carries a negative charge (thiolate form (8)). Furthermore, conserved cysteine residues play an important role in the structure, function and regulation of proteins. Therefore oxidation of these critical cysteines will likely result in altered protein function.
The most recognized redox-dependent posttranslational modification of cysteine residues by $NO^{\cdot}$ is termed S-nitrosylation. It has been shown to affect the function of a number of proteins and is introduced in greater detail in Chapter 2. H_2O_2 and other oxidants have furthermore been demonstrated to oxidize cysteine amino acids in proteins to sulfenic (-SOH), sulfinic ($-SO_2H$) sulfonic ($-SO_3H$) acids or induce the formation of disulfides. To various degrees these oxidations have been linked to altered protein functions. Additionally, H_2O_2 can induce the binding of the antioxidant tripeptide glutathione to cysteine residues in proteins. This is also a relatively well established redox-dependent posttranslational modification that is termed S-glutathionylation and can affect a number of proteins.

Pathological oxidant generation and function

In contrast to the normal physiological state, a number of pathological conditions, including asthma, are accompanied by an influx of inflammatory cells. In asthma, these inflammatory cells consist mainly of eosinophils. Inflammatory cells contain phagocytic NADPH oxidases will produce high fluxes of oxidants when they migrate into tissues and become activated. This process is termed the oxidative burst and serves to kill invading pathogens (9). In addition, asthma exacerbations are often caused by environmental agents that increase the oxidative burden in the lung, like tobacco smoke (10), ozone (11) and pollen (12). In this situation oxidant levels will be elevated, but oxidants will also be converted to more toxic reactive oxygen (ROS) and nitrogen species (RNS) that are known to cause damage to macromolecules. In patients with asthma evidence of increased oxidative stress in the lungs is found as levels of the oxidants H_2O_2 (13) and $NO^{\cdot}$ (14) are elevated in exhaled breath. In addition, markers of damage to lipids like 8-isoprostane (15) and malonyldialdehyde (16) as well as protein oxidation markers like nitrotyrosine (17) and protein carbonyls (18) can be found in samples of patient lungs.

Until recently, oxidative stress was considered to merely be a consequence of the disease process. Improvements of the measurement of oxidants and oxidative markers have made it possible to correlate their presence and quantities to disease parameters, mostly inflammation and severity. These developments have led to the opportunity to use the detection of oxidants to monitor therapies.
Irreversible oxidations that are the hallmarks of oxidative stress lead to the permanent loss of normal function of the targeted molecules. Irreversibly oxidized proteins will be targeted for degradation (19, 20) or will accumulate in the form of protein aggregates, as is described most commonly in neurodegenerative disorders (21, 22). ROS and RNS could therefore actually be contributing to the pathogenesis of asthma in multiple ways that could encompass both a loss of regulatory functions as well as an increase in damaging properties.

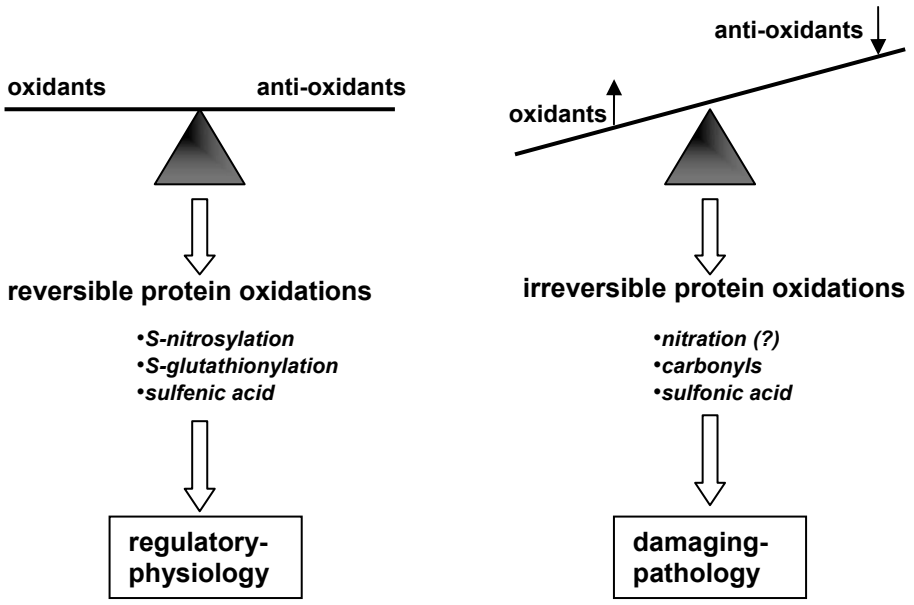

Figure 2 The oxidant-anti-oxidant imbalance that occurs in a number of inflammatory diseases can lead to damaging, irreversible protein oxidations. This can in addition cause a loss of reversible protein oxidations that are important in maintaining tissue homeostasis.

Antioxidant defenses

Oxidative stress as seen in many inflammatory diseases like asthma, can occur despite a battery of enzymatic as well as non-enzymatic antioxidant defenses. Because of the large surface area of the lungs that is in contact with potential harmful environmental agents, the lungs are well equipped with antioxidants to protect its resident cells. Non-enzymatic antioxidants include small molecules like the tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine) and vitamin C and E. On the other hand a number of enzyme systems are also present that detoxify oxidants. For instance, superoxide dismutase (SOD) will convert superoxide into H_2O_2. H_2O_2 can then be converted into water by catalase, glutathione peroxidase (GPx) or peroxiredoxins.
Glutathione is a key molecule in the maintenance of the normal redox balance in cells and tissues. In the lung, its concentration in the fluid that lines the epithelium (epithelial lining fluid) is typically one hundred-fold higher than in serum (23). When oxidant levels rise, GPx will utilize the oxidants to oxidize glutathione and reduce proteins. This results in the

formation of oxidized glutathione (GSSG, reaction 1) and reduced glutathione levels (GSH) can be restored by the glutathione reductase (GR) enzyme (reaction 2).

Oxidative stress is not only the result of an increased production of oxidants, but can also be the consequence of a decrease in antioxidant capacity. For instance, it is known that the levels of reduced glutathione (24, 25) as well as vitamin C (26) are decreased in the epithelial lining fluid of asthmatic patients. The activities of the antioxidant enzymes MnSOD (27) and Cu,ZnSOD (28), in addition have been demonstrated to be decreased in patients with asthma. Moreover, the blood of patients with asthma displays reduced levels of GPx activity when compared to control individuals (29). Given the indications of decreased antioxidant defenses, dietary antioxidants like selenium (30) and vitamin E and C (31-33) have been considered and tested for treatment of chronic inflammatory diseases like asthma, but have yielded variable levels of success.

The versatile transcription factor Nuclear factor kappa B (NF-κB)

NF-κB is a transcription factor that plays a pivotal role in a diversity of cellular processes, including cell survival, proliferation, differentiation and inflammation. It is often used as an example of a redox-sensitive transcription factor, and several key molecules/steps in its activation pathway have been demonstrated to be subject to regulation by ROS/RNS (34). Through targeting of different proteins in this signaling cascade, oxidants can influence the wide variety of (patho-) physiological processes that are regulated by NF-κB.

NF-κB is constituted by homo or heterodimers of the Rel family of proteins which include p50, p52, Rel A (p65), c-Rel and Rel B. NF-κB can be activated by a variety of agents ranging from viral and bacterial components to cytokines and UV light (35, 36). Under basal conditions, NF-κB is sequestered in the cytoplasm through binding to inhibitory kappa B proteins (IκB). Exposure to pro-inflammatory cytokines such as

tumor necrosis factor (TNF)α, will trigger the classical pathway of NF-κB activation, involving p50-p65 heterodimers (Figure 3). This pathway includes recruitment of the adaptor proteins TNF-receptor-associated death domain protein (TRADD), receptor-interacting protein (RIP) and TNF-receptor-associated factor 2 (TRAF2) to activated receptors at the cell membrane, which will in turn recruit and activate the IκB-kinase complex (IKK), also known as the IKK signalsome (37).

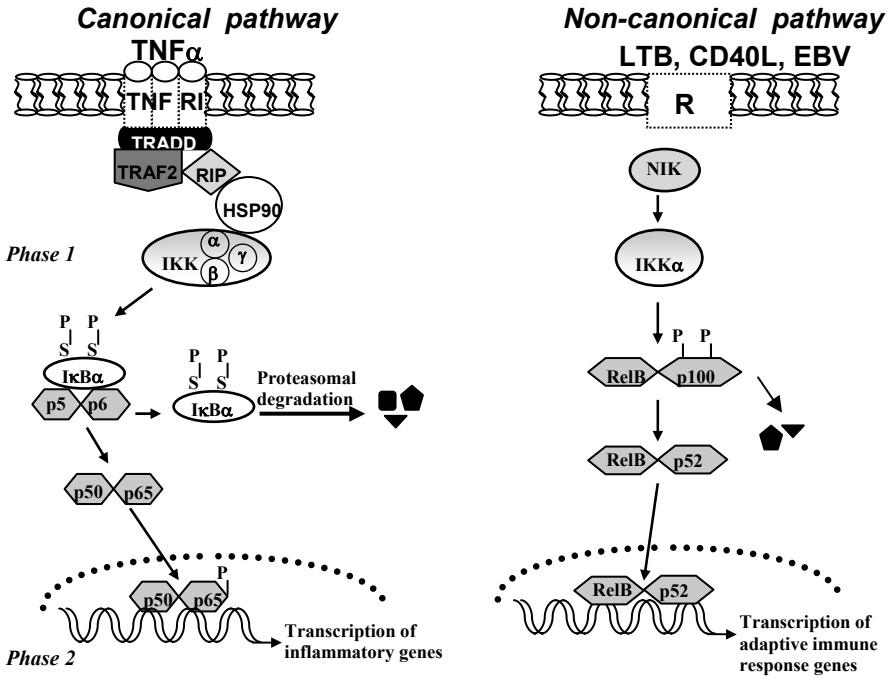

Figure 3 Canonical and non-canonical pathways of NF-κB activation. In the canonical pathway, TNFα binding to the TNF-R1 receptor leads to the recruitment of adapter proteins and the activation of the IKK complex. Subsequent phosphorylation and degradation of IκBα results in the translocation of p50-p65 dimers to the nucleus and the transcription of inflammatory genes. In the non-canonical pathway, NIK is responsible for activation of IKKα which leads to the processing of p100 to p52. RelB-p52 dimers translocate to the nucleus and activate the transcription of genes that are involved in adaptive immune responses.

The IKK signalsome exists as a large complex of proteins that amongst others include IKKα, IKKβ, IKKγ or NEMO, HSP90 and Cdc37. Through knock-out studies it has been demonstrated that IKKβ is the subunit that is absolutely required for the classical pathway of NF-κB activation through

phosphorylation of IκBα on serines 32 and 36, as well as IκBβ on serines 19 and 23 (38). These phosphorylation events induce the ubiquitinylation of the inhibitory proteins, leading to their degradation via the proteasome pathway and unmasking the nuclear localization sequence of NF-κB (39). NF-κB will be transported to the nucleus where it will bind κB sites in the promoter regions of genes, like TNFα, inducible Nitric Oxide Synthase (iNOS), inducible cyclooxygenase (COX-2) and Interleukins 6 and 8 (40). IKKs also contribute to transactivation directly by phosphorylating RelA as well as histone H3 (41-43). Subsequently, the transcription initiation complex will be assembled and these genes will be actively transcribed.

In addition to the classical pathway, the alternate or non-canonical pathway is triggered by cytokines such as lymphotoxin β (LTB, 44) and CD40 ligand (CD40L, 45), as well as viruses like Epstein-Barr (EBV, 46) and involves activation of NF-κB inducing kinase (NIK) and IKKα-mediated processing of p100 to p52 (47) (Figure 3). This will result in the activation of genes by p52-RelB heterodimers, like BLC, SLC and ELC, which are involved in peripheral lymphoid organogenesis (48). Lastly, activation of NF-κB has been described that is independent of IKK, but still involves phosphorylation dependent degradation of IκBα as well as nuclear translocation of p50-p65 heterodimers. Kinases that have been found to induce these phosphorylation events are the tyrosine kinase Syk (49) and the p38-activated serine casein kinase 2 (CK2) (50).

Enhanced activation of NF-κB has been demonstrated in a number of animal models as well as patients with asthma (51-54). These studies have identified airway epithelial and inflammatory cells as the main sites of activation of the transcription factor. Over the last decade the importance of NF-κB activation in asthma has been underscored by a number of studies that have utilized conditional knock-out, transgenic and pharmacological approaches to repress NF-κB activation (55-59). Collectively these various approaches demonstrated that repression of NF-κB activation in the lung causes a marked attenuation of the inflammatory component of asthma. In this regard it is also noteworthy to mention that corticosteroids which are the most effective treatment for asthma to date are potent inhibitors of NF-κB activity (60, 61).

THESIS OUTLINE

NF-κB activity is chronically augmented in asthma and gives rise to chronic inflammation, but the causes of this enhanced and prolonged activity are unknown. An intrinsic defect in negative feedback mechanisms or increased sensitivity to external stimuli like viruses or pollutants as well as genetic factors could be involved.
The working hypothesis for this thesis was that $NO^{\cdot}$ as well as H_2O_2, produced at physiological levels serve to modulate the activation of NF-κB in lung epithelial cells through posttranslational modifications of IKK. Therefore the aim of the first part of this thesis was to investigate the modulation of the activation of IKK and the transcription factor NF-κB by $NO^{\cdot}$ or H_2O_2. In the second part, the aims were to demonstrate the role of endogenously produced H_2O_2 in a mouse model of asthma, and to investigate the expression of the antioxidant enzyme glutaredoxin in this animal model.

In Chapter 2 an overview of the current literature is given with regard to the regulation of $NO^{\cdot}$ production and biochemical events by which $NO^{\cdot}$ transduces signals into cellular responses, with a particular focus on modulation of inflammation.

The repression of the binding of NF-κB to DNA by $NO^{\cdot}$ may be one of the mechanisms whereby $NO^{\cdot}$ can exert some of the anti-inflammatory properties it has been ascribed. Since IKK is the prerequisite enzyme complex that regulates the activation of NF-κB, in Chapter 3 we set out to determine if $NO^{\cdot}$ could modulate NF-κB activation at this more proximal step in the pathway. We furthermore investigated whether $NO^{\cdot}$ exerts its effects on IKK through S-nitrosylation, a $NO^{\cdot}$-dependent posttranslational modification that has been demonstrated to be an important mode of signal transduction for $NO^{\cdot}$.

Since the *in situ* detection of S-nitrosylated proteins is problematic due to the lack of reliable antibodies and the unstable nature of this modification, we adapted the biotin switch protocol published by Jaffrey et al (62) in Chapter 4 to visualize S-nitrosoproteins in intact cells and lung tissues using confocal microscopy.

Our laboratory had previously demonstrated that H_2O_2 can prevent the activation of IKK enzymatic activity in response to cytokines through non-

specified cysteine oxidation (63). In Chapter 5, we aimed to pin point the inhibitory effect of H_2O_2 to a specific cysteine and to determine the type of oxidation involved. We focused on H_2O_2's ability to induce the binding of glutathione to cysteine amino acids in proteins, also termed S-glutathionylation. This redox-dependent posttranslational modification had previously been described to limit NF-κB-DNA binding (64).

Because the lack of reliable techniques available to gain insights into the cellular and tissue distribution of S-glutathionylated proteins, we developed a technique that relies on the reduction of S-glutathionylation by glutaredoxin enzymes to visualize S-glutathionylated proteins *in situ* (Chapter 6).

Glutathione is one of the most important antioxidants of the lung. An extensive number of studies have shown how glutathione participates in the maintenance of the cellular redox state through the activity of GR and GPx. In addition, more recently the incorporation of glutathione into cysteine residues of proteins is thought to directly protect their sulfhydryl groups against irreversible oxidation. Glutaredoxin enzymes that regulate S-glutathionylation were investigated in a mouse model of asthma in Chapter 7.

Oxidative stress is one of the hallmarks of asthma and numerous studies have indicated an etiological role for oxidants in the disease process. To directly assess the role of endogenous H_2O_2 in asthma, we tested mice that overexpress the H_2O_2 detoxifying enzyme catalase in a model of asthma in Chapter 8.

Chapter 9 provides general discussion of this thesis and outlines some directions and thoughts for future research.

REFERENCES

1. Geiszt, M., J. Witta, J. Baffi, K. Lekstrom, and T. L. Leto. 2003. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *Faseb J 17:1502*.
2. Harper, R. W., C. Xu, J. P. Eiserich, Y. Chen, C. Y. Kao, P. Thai, H. Setiadi, and R. Wu. 2005. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett 579:4911*.
3. Bae, Y. S., S. W. Kang, M. S. Seo, I. C. Baines, E. Tekle, P. B. Chock, and S. G. Rhee. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem 272:217*.
4. Lee, S. R., K. S. Kwon, S. R. Kim, and S. G. Rhee. 1998. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem 273:15366*.
5. Sundaresan, M., Z. X. Yu, V. J. Ferrans, K. Irani, and T. Finkel. 1995. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science 270:296*.
6. Ohba, M., M. Shibanuma, T. Kuroki, and K. Nose. 1994. Production of hydrogen peroxide by transforming growth factor-beta 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. *J Cell Biol 126:1079*.
7. Meier, B., H. H. Radeke, S. Selle, M. Younes, H. Sies, K. Resch, and G. G. Habermehl. 1989. Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. *Biochem J 263:539*.
8. Forman, H. J., J. M. Fukuto, and M. Torres. 2004. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol 287:C246*.
9. Laroux, F. S., X. Romero, L. Wetzler, P. Engel, and C. Terhorst. 2005. Cutting Edge: MyD88 Controls Phagocyte NADPH Oxidase Function and Killing of Gram-Negative Bacteria. *J Immunol 175:5596*.
10. Eisner, M. D. 2002. Environmental tobacco smoke and adult asthma. *Clin Chest Med 23:749*.
11. Vagaggini, B., M. Taccola, S. Cianchetti, S. Carnevali, M. L. Bartoli, E. Bacci, F. L. Dente, A. Di Franco, D. Giannini, and P. L. Paggiaro. 2002. Ozone exposure increases eosinophilic airway response induced by previous allergen challenge. *Am J Respir Crit Care Med 166:1073*.
12. Boldogh, I., A. Bacsi, B. K. Choudhury, N. Dharajiya, R. Alam, T. K. Hazra, S. Mitra, R. M. Goldblum, and S. Sur. 2005. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest 115:2169*.
13. Emelyanov, A., G. Fedoseev, A. Abulimity, K. Rudinski, A. Fedoulov, A. Karabanov, and P. J. Barnes. 2001. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. *Chest 120:1136*.
14. Kharitonov, S. A., D. Yates, R. A. Robbins, R. Logan-Sinclair, E. A. Shinebourne, and P. J. Barnes. 1994. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet 343:133*.

15. Montuschi, P., M. Corradi, G. Ciabattoni, J. Nightingale, S. A. Kharitonov, and P. J. Barnes. 1999. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med 160:216*.
16. Hanta, I., S. Kuleci, N. Canacankatan, and A. Kocabas. 2003. The oxidant-antioxidant balance in mild asthmatic patients. *Lung 181:347*.
17. Kaminsky, D. A., J. Mitchell, N. Carroll, A. James, R. Soultanakis, and Y. Janssen. 1999. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J Allergy Clin Immunol 104:747*.
18. Nadeem, A., S. K. Chhabra, A. Masood, and H. G. Raj. 2003. Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol 111:72*.
19. Souza, J. M., I. Choi, Q. Chen, M. Weisse, E. Daikhin, M. Yudkoff, M. Obin, J. Ara, J. Horwitz, and H. Ischiropoulos. 2000. Proteolytic degradation of tyrosine nitrated proteins. *Arch Biochem Biophys 380:360*.
20. Grune, T., T. Reinheckel, and K. J. Davies. 1997. Degradation of oxidized proteins in mammalian cells. *Faseb J 11:526*.
21. Choi, J., H. D. Rees, S. T. Weintraub, A. I. Levey, L. S. Chin, and L. Li. 2005. Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. *J Biol Chem 280:11648*.
22. Paxinou, E., Q. Chen, M. Weisse, B. I. Giasson, E. H. Norris, S. M. Rueter, J. Q. Trojanowski, V. M. Lee, and H. Ischiropoulos. 2001. Induction of alpha-synuclein aggregation by intracellular nitrative insult. *J Neurosci 21:8053*.
23. Cantin, A. M., S. L. North, R. C. Hubbard, and R. G. Crystal. 1987. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol 63:152*.
24. Kelly, F. J., I. Mudway, A. Blomberg, A. Frew, and T. Sandstrom. 1999. Altered lung antioxidant status in patients with mild asthma. *Lancet 354:482*.
25. Dauletbaev, N., J. Rickmann, K. Viel, R. Buhl, T. O. Wagner, and J. Bargon. 2001. Glutathione in induced sputum of healthy individuals and patients with asthma. *Thorax 56:13*.
26. Misso, N. L., J. Brooks-Wildhaber, S. Ray, H. Vally, and P. J. Thompson. 2005. Plasma concentrations of dietary and nondietary antioxidants are low in severe asthma. *Eur Respir J 26:257*.
27. Comhair, S. A., W. Xu, S. Ghosh, F. B. Thunnissen, A. Almasan, W. J. Calhoun, A. J. Janocha, L. Zheng, S. L. Hazen, and S. C. Erzurum. 2005. Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. *Am J Pathol 166:663*.
28. De Raeve, H. R., F. B. Thunnissen, F. T. Kaneko, F. H. Guo, M. Lewis, M. S. Kavuru, M. Secic, M. J. Thomassen, and S. C. Erzurum. 1997. Decreased Cu,Zn-SOD activity in asthmatic airway epithelium: correction by inhaled corticosteroid in vivo. *Am J Physiol 272:L148*.
29. Mak, J. C., H. C. Leung, S. P. Ho, B. K. Law, W. K. Lam, K. W. Tsang, M. S. Ip, and M. Chan-Yeung. 2004. Systemic oxidative and antioxidative status in Chinese patients with asthma. *J Allergy Clin Immunol 114:260*.
30. Allam, M. F., and R. A. Lucane. 2004. Selenium supplementation for asthma. *Cochrane Database Syst Rev:CD003538*.
31. Pearson, P. J., S. A. Lewis, J. Britton, and A. Fogarty. 2004. Vitamin E supplements in asthma: a parallel group randomised placebo controlled trial. *Thorax 59:652*.
32. Kilic, F. S., and K. Erol. 2003. The effects of vitamin E in ovalbumin-sensitized guinea pigs. *Methods Find Exp Clin Pharmacol 25:27*.

33. Ram, F. S., B. H. Rowe, and B. Kaur. 2004. Vitamin C supplementation for asthma. *Cochrane Database Syst Rev:CD000993*.
34. Kabe, Y., K. Ando, S. Hirao, M. Yoshida, and H. Handa. 2005. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal 7:395*.
35. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol 16:225*.
36. Siebenlist, U., G. Franzoso, and K. Brown. 1994. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol 10:405*.
37. Devin, A., A. Cook, Y. Lin, Y. Rodriguez, M. Kelliher, and Z. Liu. 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity 12:419*.
38. May, M. J., and S. Ghosh. 1999. IkappaB kinases: kinsmen with different crafts. *Science 284:271*.
39. Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol 18:621*.
40. Wulczyn, F. G., D. Krappmann, and C. Scheidereit. 1996. The NF-kappa B/Rel and I kappa B gene families: mediators of immune response and inflammation. *J Mol Med 74:749*.
41. Sakurai, H., H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi. 1999. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem 274:30353*.
42. O'Mahony, A. M., M. Montano, K. Van Beneden, L. F. Chen, and W. C. Greene. 2004. Human T-cell lymphotropic virus type 1 tax induction of biologically Active NF-kappaB requires IkappaB kinase-1-mediated phosphorylation of RelA/p65. *J Biol Chem 279:18137*.
43. Yamamoto, Y., U. N. Verma, S. Prajapati, Y. T. Kwak, and R. B. Gaynor. 2003. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature 423:655*.
44. Dejardin, E., N. M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z. W. Li, M. Karin, C. F. Ware, and D. R. Green. 2002. The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity 17:525*.
45. Coope, H. J., P. G. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. Klaus, L. H. Johnston, and S. C. Ley. 2002. CD40 regulates the processing of NF-kappaB2 p100 to p52. *Embo J 21:5375*.
46. Eliopoulos, A. G., J. H. Caamano, J. Flavell, G. M. Reynolds, P. G. Murray, J. L. Poyet, and L. S. Young. 2003. Epstein-Barr virus-encoded latent infection membrane protein 1 regulates the processing of p100 NF-kappaB2 to p52 via an IKKgamma/NEMO-independent signalling pathway. *Oncogene 22:7557*.
47. Xiao, G., E. W. Harhaj, and S. C. Sun. 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell 7:401*.
48. Bonizzi, G., M. Bebien, D. C. Otero, K. E. Johnson-Vroom, Y. Cao, D. Vu, A. G. Jegga, B. J. Aronow, G. Ghosh, R. C. Rickert, and M. Karin. 2004. Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *Embo J 23:4202*.
49. Takada, Y., A. Mukhopadhyay, G. C. Kundu, G. H. Mahabeleshwar, S. Singh, and B. B. Aggarwal. 2003. Hydrogen peroxide activates NF-kappa B through tyrosine

phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. *J Biol Chem 278:24233*.
50. Kato, T., Jr., M. Delhase, A. Hoffmann, and M. Karin. 2003. CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. *Mol Cell 12:829*.
51. Hart, L. A., V. L. Krishnan, I. M. Adcock, P. J. Barnes, and K. F. Chung. 1998. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am J Respir Crit Care Med 158:1585*.
52. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. 2002. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol 160:1325*.
53. Stacey, M. A., G. Sun, G. Vassalli, M. Marini, A. Bellini, and S. Mattoli. 1997. The allergen Der p1 induces NF-kappaB activation through interference with IkappaB alpha function in asthmatic bronchial epithelial cells. *Biochem Biophys Res Commun 236:522*.
54. Zhao, S., Y. Qi, X. Liu, Q. Jiang, S. Liu, Y. Jiang, and Z. Jiang. 2001. Activation of NF-kappa B in bronchial epithelial cells from children with asthma. *Chin Med J (Engl) 114:909*.
55. Broide, D. H., T. Lawrence, T. Doherty, J. Y. Cho, M. Miller, K. McElwain, S. McElwain, and M. Karin. 2005. Allergen-induced peribronchial fibrosis and mucus production mediated by I{kappa}B kinase {beta}-dependent genes in airway epithelium. *Proc Natl Acad Sci U S A 102:17723*.
56. Poynter, M. E., R. Cloots, T. van Woerkom, K. J. Butnor, P. Vacek, D. J. Taatjes, C. G. Irvin, and Y. M. Janssen-Heininger. 2004. NF-kappa B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol 173:7003*.
57. Das, J., C. H. Chen, L. Yang, L. Cohn, P. Ray, and A. Ray. 2001. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat Immunol 2:45*.
58. Donovan, C. E., D. A. Mark, H. Z. He, H. C. Liou, L. Kobzik, Y. Wang, G. T. De Sanctis, D. L. Perkins, and P. W. Finn. 1999. NF-kappa B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J Immunol 163:6827*.
59. Yang, L., L. Cohn, D. H. Zhang, R. Homer, A. Ray, and P. Ray. 1998. Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med 188:1739*.
60. Scheinman, R. I., A. Gualberto, C. M. Jewell, J. A. Cidlowski, and A. S. Baldwin, Jr. 1995. Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol 15:943*.
61. Hart, L., S. Lim, I. Adcock, P. J. Barnes, and K. F. Chung. 2000. Effects of inhaled corticosteroid therapy on expression and DNA-binding activity of nuclear factor kappaB in asthma. *Am J Respir Crit Care Med 161:224*.
62. Jaffrey, S. R., and S. H. Snyder. 2001. The biotin switch method for the detection of s-nitrosylated proteins. *Sci STKE 2001:L1*.
63. Korn, S. H., E. F. Wouters, N. Vos, and Y. M. Janssen-Heininger. 2001. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. *J Biol Chem 276:35693*.
64. Pineda-Molina, E., and S. Lamas. 2002. S-glutathionylation of NF-kappa B subunit p50. *Methods Enzymol 359:268*.

CHAPTER 2

Nitric oxide and redox signaling in allergic airway inflammation

ABSTRACT

A number of diseases of the respiratory tract, as exemplified in this review by asthma, are associated with increased amounts of nitric oxide (NO) in the expired breath. Asthma is furthermore characterized by increased production of reactive oxygen species that scavenge NO to form more reactive nitrogen species as demonstrated by enhanced presence of nitrated proteins in the lungs of these patients. This increased oxidative metabolism leaves less bioavailable NO and coincides with lower amounts of S-nitrosothiols. In this review we speculate on mechanisms responsible for the increased amounts of NO in inflammatory airway disease and discuss the apparent paradox of higher levels of NO as opposed to decreased amounts of S-nitrosothiols. We will furthermore give an overview of the regulation of NO production and biochemical events by which NO transduces signals into cellular responses, with a particular focus on modulation of inflammation by NO. Lastly, difficulties in studying NO signaling and possible therapeutic uses for NO will be highlighted.

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Antioxidants & Redox Signaling

NO levels in exhaled breath of patients with asthma

Inflammatory diseases of the respiratory tract, such as asthma, bronchiectasis and acute lung injury, are commonly associated with enhanced local production of nitric oxide (NO). It has been presumed that the induction of type 2 NO synthase (NOS), the high-output form of NOS, is responsible for the increased levels of NO and its oxidation products in the expired breath (44, 85). Studies using selective NOS 2 inhibitors found exhaled NO levels to decrease up to 95% in asthmatics, suggesting that the majority of exhaled NO is derived from NOS2 (57, 164). As the selectivity of pharmacological inhibitors is variable, it remains to be determined if the induction of NOS2 in the airway epithelium and inflammatory cells is the only source of the increased amount of NO in expired breath as seen in asthma. Recently, this notion has also been brought to question based upon observations that exhaled NO levels remain increased in mice lacking NOS1 or 2, suggesting that NOS isoforms are redundant (18, 150). Other possible explanations for the elevated levels of NO in the expired air are the breakdown of S-nitrosothiols by S-nitrosoglutathione (GSNO) reductase, an enzyme shown to be more active in an ovalbumin-sensitization model of allergic airway disease (35), or the protonation of nitrite under acidic conditions, to form nitrous acid, which can decompose to release NO in the airways (110, 171). The pH of exhaled breath condensate of patients with acute asthma has been found to be as low as 5.2 (65), indicating that the lung surface of asthmatics is probably sufficiently acidic to cause formation of NO by this latter mechanism.
Measuring exhaled NO has been proposed as a noninvasive method to diagnose and monitor asthma as the levels closely correlate with markers of the disease, specifically eosinophilic inflammation and airflow parameters (75, 84). A recent study investigating the validity of exhaled NO for the diagnosis of asthma, found a 90% specificity in diagnosing asthma using exhaled NO levels, but a cutoff value for NO needs to be implemented in order for NO to be discriminatory for this disease (29).

NO has multiple functions in normal lung tissue; it participates in regulating airway and vascular tone, mucin secretion and influences mucociliary clearance. NO is also known for its bactericidal actions and provides additional host defenses against invading pathogens (34, 122), supported by studies demonstrating that NOS2-deficient mice are more susceptible to infection (122). Furthermore, NO may confer an early physiological defense against injury and inflammation by modulating the progression of the inflammatory process by affecting leukocyte adhesion

and migration into lung tissue (60, 104) and by inhibiting phagocyte oxidase activation (16). In addition, NO has been shown to alter pro-inflammatory cytokine production by affecting cell signaling pathways and transcription factor activation and thereby altering gene expression (66, 133, 151), as will be further discussed later on.

NO derivatives in asthma

S-nitrosothiols have been measured in exhaled breath condensates, tracheal aspirates and bronchoalveolar lavage fluids and normal values obtained from these studies vary markedly (from 80nM to 1μM (20, 45, 51)). This large variability could be due to the different ways of sampling, but it is also a consequence of the different specificities of the methods used to detect and quantify S-nitrosothiols. It is therefore hard to estimate the actual concentration of S-nitrosothiols in the epithelial lining fluid of the lungs, which will consist mainly of GSNO. However, intriguingly, most studies, report levels of S-nitrosothiols in above-mentioned samples from patients with asthma or cystic fibrosis (CF) to be markedly lower, or often undetectable, when compared to similar specimens from healthy subjects, despite normal or even enhanced airway NO production (45). These subnormal S-nitrosothiol levels could result from alterations in oxidative NO metabolism that would limit S-nitrosylation reactions, or accelerated oxidative or enzymatic degradation of S-nitrosothiols during inflammation. Indeed, oxidative metabolism has been presented by elevated protein tyrosine nitration in these conditions (78), which is largely a result of increased activation of the granulocyte peroxidases myeloperoxidase (MPO) and eosinophil peroxidase (EPO) (35). As mentioned earlier, GSNO reductase activity was found to be increased in an ovalbumin-sensitization model of asthma in guinea pigs. It was proposed that this GSNO reductase activity is leaking into the airway lining fluid from damaged airway epithelium (35). It is conceivable that decreased levels of S-nitrosothiols could contribute to asthma pathophysiology by minimizing the beneficial effects of S-nitrosothiols on airway smooth muscle tone and the antiinflammatory properties of these NO derivatives.

The increased presence of nitrotyrosines, on the other hand, may also have deleterious effects with regard to airway hyperresponsiveness and surfactant function (22). Overall, the decreased levels of S-nitrosothiols, accompanied by increased levels of nitrotyrosine in asthmatics, strongly

indicate altered or enhanced NO metabolism within the airways of these patients, which may have an impact on the overall pro-or anti-inflammatory properties of NO.

Regulation of NO production

NO is synthesized by three isoforms of NOS in a reaction that converts L-arginine to L-citrulline and NO, using oxygen and NADPH as cofactors. NOS3 (eNOS) was originally identified in endothelial cells and NOS1 (nNOS) in neuronal cells. A mitochondrial variant of NOS has recently been shown to be widely distributed among tissues (79), but there is no consensus as to whether this is a variant of one of the known constitutive NOS isozymes or whether it is an entirely different protein (96). NOS1 and 3 are both dependent on increases in intracellular calcium to bind calmodulin, in order to be active and produce low levels of NO. NOS2 (iNOS) is expressed by a variety of cells and is responsible for high-output production of NO, independent of elevations in intracellular calcium, due to the tight binding of calmodulin even at low levels of calcium. In contrast to NOS1 and 3, NOS2 is regulated at the level of transcription and its expression is induced by immunologic and inflammatory stimuli, including interferon-γ, tumor necrosis factor-α (TNFα), and lipopoly-saccharide (LPS).
NOS enzymes are also regulated at the level of translation (101), post-translation and degradation. Phosphorylation of NOS enzymes by Akt (25), calcium/calmodulin-dependent protein kinases (92) and members of the AMP-activated kinases PKA and G (14, 15) has been reported to both increase and decrease NOS activity, depending on the residue that is targeted by phosphorylation. In addition, NOS activity can be influenced by regulation of its proteolytic degradation. Following inhibition of the interaction between one of the chaperones and NOS or in response to treatment with guanidine compounds, NOS proteins are ubiquitinated and degraded via the proteasomal pathway (8, 91). Conditions that are accompanied by suboptimal amounts of the substrate for NOS, L-arginine, the essential cofactor tetrahydrobiopterin, or an altered composition of the NOS membrane complex can promote the generation of superoxide ($O_2^{\cdot -}$) by all isoforms (132, 158 24, 160). This cogeneration of NO and $O_2^{\cdot -}$ by NOS can lead to the formation of more reactive nitrogen species (RNS), like peroxynitrite ($ONOO^-$), and lesser availability of NO *per se*. This could be relevant to allergic airway disease, because cationic proteins like the eosinophil product major basic protein (MBP) can limit substrate availability for NOS, by inhibiting the cellular uptake of L-arginine (55).

NO signaling in the lung

The traditional view on signal transduction encompassed an image of free diffusing molecules within the cell, and NO, along this line of thinking has ideal properties as a signaling molecule, because it diffuses rapidly through aqueous phase and membranes. In recent years, however, this notion has been replaced with the appreciation that signaling takes place within confined subcellular compartments to insure specific targeting and propagation of signals (21). For NO, this implies that the source of NO and the target molecules should be colocalized. The three different isoforms of NOS enzymes and their multiple splice variants are indeed discretely localized within subcellular compartments and form complexes with different subsets of proteins through the interaction with scaffolding proteins, to enable specific targeting of proteins with distinct functions. All three isoforms are known to reside in caveolae, the microdomains of the plasmalemma that have been implicated in transcytosis and signal transduction events (128). NOS enzymes have been shown to interact directly with caveolins, the integral membrane proteins of caveolae under basal conditions, and their activity requires dissociation from caveolin (42, 46, 49, 93). This tight regulation of localization, production and activity makes NOS ideal for generating NO as a signaling molecule that can regulate physiological processes.

L-arginine availability is another regulator for the production of NO and is in part determined by its uptake by the cationic amino acid transporter 1 (CAT1). CAT1 is a member of the NOS membrane complex, ensuring direct delivery of L-arginine to NOS and optimizing NO release (116). In this regard, as mentioned earlier, cationic proteins like the eosinophil product MBP, can inhibit the cellular uptake of L-arginine and consequently inhibit NO production by alveolar macrophages and airway epithelial cells (55).

The availability of L-arginine, in addition to being regulated by uptake, is also determined by its consumption in an alternative reaction in which L-arginine is converted by arginase to form urea and L-ornithine, the latter being an essential precursor for the synthesis of polyamines and L-proline (145) (Fig. 1). Importantly, a recent study demonstrated no change in NOS2 expression, but found both arginase isoforms 1 and 2 to be up-regulated in animal models of allergic airway disease, as well as in patients with asthma (170). The induction of arginase could be due to Th2 cytokines, like interleukin (IL)-4 and IL-10, that are a characteristic feature of asthma (99, 121). This new finding is intriguing, as it implies lower availability of substrate for NOS and increased substrate for

collagen formation in the form of L-proline in allergic airways disease. In this regard, it is of interest to note that in a bleomycin-induced model of fibrosis of mouse lung, both arginase isoforms were found to be up-regulated (31).

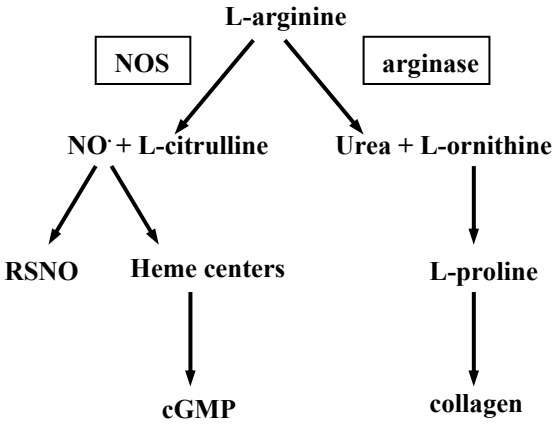

Figure 1 Schematic representation of L-arginine consuming pathways. L-arginine can be converted by NOS into NO and L-citrulline. NO can subsequently react with sulfhydryl groups of cysteine residues in proteins in a S-nitrosylation reaction or NO can react with heme centers and modify protein function. Alternatively, L-arginine can be converted by arginase to form urea and L-ornithine, the latter a precursor for L-proline and collagen synthesis.

Metabolism of NO to RNS

Oxidation of NO to RNS

A number of proinflammatory mediators that are present in asthmatic airways can cause an increase in the production of NO as well as reactive oxygen species. In conditions where NO and $O_2^{\cdot -}$ are produced at the same time and in close proximity, such as in inflammatory cells like eosinophils and neutrophils, which are known to infiltrate the lungs of asthmatics, the typical nitrating species, nitrogen dioxide ($NO_2^{\cdot}$) and $ONOO^-$, that can modify tyrosine residues in proteins are formed (69). Both eosinophils and neutrophils contain the enzyme NADPH oxidase, which converts O_2 into $O_2^{\cdot -}$, which in turn rapidly reacts with NO to form $ONOO^-$. Alternatively, the leukocyte peroxidases EPO and MPO, which are stored in granulae and released upon activation, catalyze the formation of $NO_2^{\cdot}$ from nitrite

(NO_2^-) and hydrogen peroxide (H_2O_2) (155, 159) (Figure 2). Studies using MPO and EPO knock out mice have confirmed the importance of these enzymes in the formation of nitrotyrosines *in vivo* (27, 67).
In addition to the formation of RNS by inflammatory cells, nonphagocytic cells are also a source of these oxidants. For instance, mitochondrial respiration, xanthine/xanthine oxidase, and nonphagocytic oxidases, represent important sources of $O_2^{\cdot -}$ (97), and as mentioned earlier, NOS isoforms are expressed in a variety of cells. Collectively, the biochemical pathways that are required for the formation of RNS are present in both inflammatory and noninflammatory settings (40). For review on oxidative events in asthma see Andreadis *et a*l. (4).

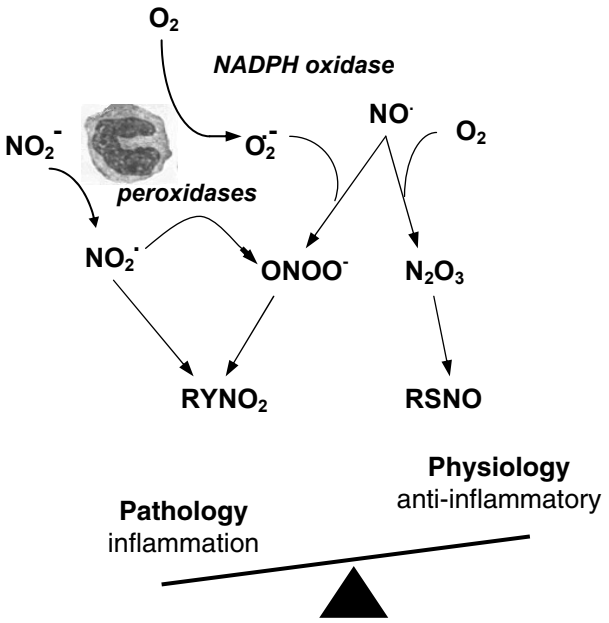

Figure 2 Oxidative metabolism of NO and the formation of RNS. Oxidation of NO is affected by the redox status of the cell, the presence of hydrophobic regions, and other reactants, such as oxygen and transition metals. Furthermore, activation of oxidases and peroxidases can decrease NO by causing the production of highly reactive RNS, like $ONOO^-$ and $NO_2^{\cdot}$.

Tyrosine nitration

Nitrotyrosine formation has been considered to be a footprint for the presence of $ONOO^-$ or NO_2. Increased nitrotyrosine reactivity has been detected in a variety of diseases of the respiratory tract, such as asthma, acute lung injury, CF, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (78, 119, 142, 169).
Although speculations exist that nitration of tyrosines impairs the ability of kinases to phosphorylate the same residues (107) or, on the other hand

that nitrotyrosines could mimic the tyrosine phosphorylation status (9), experimental evidence for these outcomes in diseases of the airways is lacking. Other data suggest that nitrated proteins are more susceptible to degradation by the proteasome (53, 54, 144), implying that removal of nitrated proteins may serve as a protective mechanism against nitrative stress. Evidence indicates that nitration is reversible and that nitrated proteins can be repaired by a so-called denitrase activity (22, 50). This activity was induced by LPS, destroyed by heat and trypsine, and importanty appeared to be substrate-dependent. However, the denitrase enzyme remains to be further purified and characterized in order to substantiate its relevance.

One of the proteins that has been shown to be nitrated and functionally impaired by the modification is IL-8, one of the chemokines for neutrophils. This cell type has been implicated in severe asthma (90) and in COPD. Studies have shown that the addition of $ONOO^-$ to IL-8 inhibits its binding to neutrophils and subsequently reduces its chemotactic potential *in vitro* (137). On the other hand, the antiinflammatory effects of the cytokine IL-10 *in vitro* are enhanced by $ONOO^-$ treatment (124). Furthermore, nitration of cytoskeletal proteins like actin and α-tubulin contributes to alterations in cell morphology, microtubular organization and causes intracellular redistribution of motor proteins (30). In the context of the lung, $ONOO^-$ has been shown to cause increased hyperresponsiveness to methacholine and to inhibit surfactant function through nitration of surfactant protein A (136). Although these findings are suggestive of an important role of tyrosine nitration of key target proteins in allergic airway diseases, a causal role for these events *in vivo* remains largely to be determined.

S-nitrosation / S-nitrosylation

Many of the biological actions of NO are mediated through the reaction with iron or heme-containing proteins such as guanylyl cyclase, cytochrome *c* oxidase (153), lipoxygenase (126), prostaglandin H synthase (125), and MPO (1, 7, 48). The binding of NO to heme iron of soluble guanylyl cyclase (sGC), causing its activation and subsequent rise in cyclic GMP (cGMP) production, was the first evidence that NO can act as a signaling molecule (61). cGMP acts as a second messenger, activating cGMP-dependent protein kinase, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase. Some of the effects of NO that are mediated in this manner include smooth muscle relaxation, neurotransmission and platelet aggregation and disaggregagation.

Approximately a decade ago, redox-sensitive protein cysteine residues were determined to represent alternative targets for NO. They can undergo so-called S-nitrosylation, the attachment of NO to sulfhydryl groups in proteins [whereas the attachment of NO to nucleophilic centers in general is called S-nitrosation (141)]. This results in changes in the tertiary structure and, in many cases, functional alterations of the proteins involved (148). S-nitrosylation has emerged as the prototypic redox-based posttranslational modification and has often been compared with phosphorylation (108). This analogy to other modes of signal transduction forms the main reason why the NO-dependent modification of sulfhydryl groups is called S-nitrosylation as opposed to S-nitrosation, which is the chemically correct term. The importance of this newly described posttranslational modification is emphasized by the large number of proteins from all major classes that have been characterized as potential targets for S-nitrosylation to date. These include channel/transporters, structural proteins, transport/storage proteins, metabolic enzymes, signaling proteins, and transcription factors (11) (Table 1).

TABLE 1. POTENTIAL TARGETS FOR S-NITROSYLATION

Protein	*Effect*	*References*
NMDA receptor	inhibition	Lipton *et al*., *Nature* 364: 626-632, 1993.
p21 H-ras	activation	Lander *et al*., *J Biol Chem* 272: 4323-4326, 1997.
ryanodine receptor	activation	Xu *et al*., *Science* 279: 234-237, 1998.
JNK	inhibition	Park *et al*., *Proc Natl Acad Sci* 97: 14382-14387, 2000.
Caspase 3	suppresses activity	Mannick *et al*., *J Cell Biol* 154: 1111-1116, 2001.
p50	inhibits DNA binding	Marshall *et al*., *Biochemistry* 40: 1688-1693, 2001.
thioredoxin	required for activity	Haendeler *et al*., *Nat Cell Biol* 4: 743-749, 2002.
MMP9	activation	Gu *et al*., *Science* 297: 1186-1190, 2002.
IKKβ	inhibition	Reynaert *et al*., *Proc Natl Acad Sci* 101: 8945-8950, 2004.

S-nitrosylation requires (enzymatic) activation of NO to an NO^+ equivalent product [N_2O_3 (64), iron NO^+ complexes, perhaps $ONOO^-$] (156), because NO itself does not react with thiols directly. A nitroso functional group, that is, NO^+, can also be transferred from a nitrosothiol to another cysteine residue in a transnitrosation reaction (63) (Fig. 3). Intriguingly, not all cysteine residues are equally susceptible to S-nitrosylation. In fact, the majority of proteins that have been shown to be targeted by S-nitrosylation are only modified at a single critical cysteine residue. This cysteine is generally located within an acid-base and overall

hydrophobic structural motif that may only be apparent from the tertiary or quaternary structure of the protein (149). The exact biochemical pathways involved in S-nitrosylation *in vivo* are still unknown, but appear to depend mainly on the microenvironment of the protein, which determines the p*K*a of the cysteine being targeted (6) and the accessibility to nitrosating intermediates.

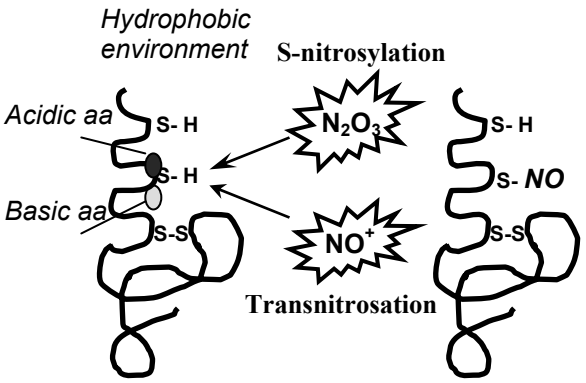

Figure 3 S-nitrosylation of proteins. S-nitrosylation of cysteine residues in proteins is believed to occur within an acid-base motif and mainly hydrophobic environment where NO rapidly reacts with oxygen to form the S-nitrosating species N_2O_3. NO can also be transferred from one thiol to another in a transnitrosation reaction.

Conversely, S-nitrosylation of proteins is a transient modification, and can be reversed by several nonenzymatic pathways, such as reactions with other cellular thiols (11, 64, 76), ascorbate (81), various heme proteins (146), copper ions (168) and light (140). Furthermore, enzymatic pathways that break down S-nitrosothiols also have been described *in vitro* and *in vivo*. They include thioredoxin reductase (123), xanthine oxidase (154), Cu/Zn superoxide dismutase (77), and a recently identified cellular GSNO reductase, which utilizes NADH and glutathione (GSH) as co-factors (Table 2). It should be noted that the latter enzyme is otherwise known as GSH-dependent formaldehyde dehydrogenase or alcohol dehydrogenase class III (105). Further studies are required to characterize the role of this enzyme in the metabolism of S-nitrosothiols and to investigate if other dehydrogenases share this SNO reductase activity.

Overall, the various biochemical and enzymatic pathways that regulate S-nitrosylation and denitrosylation indicate a tight regulation of these events, and provide strong arguments for S-nitrosylation as an important biochemical posttranslational modification associated with NO. Furthermore, S-nitrosothiols can serve as a systemic reservoir of NO (45), tightly regulating the location and amount of NO that is necessary for the control of protein function and signal transduction.

TABLE 2. DEGRADATION OF *S*-NITROSOTHIOLS

Enzymatic:
Alcohol dehydrogenase class III
Xanthine oxidase
Thioredoxin reductase
Cu/Zn superoxide dismutase
Non-enzymatic:
Ascorbate
Ligh
Heme proteins
Low molecular weight thiols
Transition metal ions

Investigations into the role of NO in asthma

Approaches to determine the involvement of NO in allergic airway disease have included pharmacological inhibition of one or more NOS isoforms and the use of mice containing targeted deletions of NOS isozymes. Because of the suggested involvement of NOS2, studies of airway inflammation have mostly focused on NOS2-knockout mice or NOS2 inhibitors and have suggested that NOS2-derived NO contributes to lung inflammation, lung injury and mortality in influenza virus pneumonitis (2), acute lung injury by LPS or ozone inhalation (83, 87, 127), as well as eosinophilic infiltration following ovalbumin immunization and challenge (23, 162). Nevertheless, several other reports have failed to demonstrate a contribution of NOS2 to allergic airway inflammation (23, 36), and in some studies of oxidant-induced lung injury, NOS2 deficiency actually resulted in increases in neutrophil influx (83, 89), which is more consistent with the known antiinflammatory properties of NO. Furthermore, whereas NOS2 inhibition or deficiency only seemed to affect inflammation and

protein nitration, it appeared to have no effect on other features of allergic airways disease, such as airway hyperreactivity, airway cell proliferation and eosinophilia (23, 33, 88), although conflicting reports exist (33, 68). However, recent pharmacological studies have suggested a role for the constitutive isoforms of NOS rather than NOS2 in eosinophilic inflammation in models of allergic airways inflammation (10), and the constitutive NOS isozymes have been associated with changes in responsiveness to methacholine challenge (37). For example, airway hyperreactivity in a mouse model of allergic airway disease was suppressed in animals deficient in NOS1, but increased in NOS3-deficient mice (23). These previous findings are also consistent with recent linkages of NOS1 and NOS3 gene polymorphisms with human asthma and elevated levels of exhaled NO (52, 157).
One of the main issues in interpreting results obtained from generic NOS knockout mice is that the three different isoforms may be redundant. For instance, mice lacking either NOS1 or 3 display increased exhaled NO levels (150). Additionally, NO affects immune functions and loss of NOS2 has been suggested to alter immunization *per se*, which compromises the studies evaluating NO in allergic airways disease (82). Thus, improved mouse models that use conditional or tissue-specific targeting approaches will be required to elucidate the role of various NOS isoforms in allergic airway inflammation.

NO and inflammation

NF-κB

NF-κB is a versatile transcription factor that plays a pivotal role in inflammation, cell survival and proliferation. NF-κB can be induced by >150 different stimuli and participates in the transcription of >150 different genes. Proinflammatory mediators induced by NF-κB include adhesion molecules (intercellular adhesion molecule 1), enzymes (NOS2, cyclooxygenase-2), cytokines (TNFα, IL1-β) and chemokines (eotaxin, RANTES) (129).
NF-κB is maintained in a latent form in the cytoplasm via sequestration by IκB inhibitory proteins. Canonical NF-κB activating stimuli cause the inducible degradation of IκB proteins, unmasking the nuclear localization signal of NF-κB, resulting in its nuclear translocation, binding to NF-κB motifs, and the activation of gene transcription (47, 80, 114). The enzyme

complex responsible for phosphorylation of IκB on specific serine residues is inhibitory kappa B kinase (IKK), which consists of at least three subunits: IKKα, IKKβ and IKKγ (the latter also known as NF-κB essential modulator) (166). Although IKKα and IKKβ are both catalytically active, studies in knockout mice have demonstrated that IKKβ is responsible for degradation of IκB in response to many signals (47, 80, 103, 115). In contrast, IKKα activation requires the NF-κB inducing kinase (161), which is activated by a different subset of stimuli, such as CD40 ligand (19). IKKα plays an important role in the transcriptional activation of NF-κB responsive genes by inducing the processing of p100 to p52 (139) and by phosphorylation of histone H3 (5, 163). IKKγ is the regulatory subunit responsible for stabilizing the IKK complex and allowing interaction with upstream regulatory proteins (135).

NF-κB and asthma

As reviewed earlier, considerable evidence exists that NO plays a role in the inflammatory process associated with allergic airway disease. One of the mechanisms by which NO can regulate inflammation is by affecting signaling pathways that culminate in the activation of NF-κB, a transcription factor that is believed to play a central role in the pathogenesis of asthma. Excessive activation of NF-κB has been shown to occur in alveolar macrophages obtained from induced sputum, as well as in airway epithelial cells obtained from bronchial brushings from asthmatics as compared to normal individuals (58). Furthermore, allergens that are associated with allergic asthma (131, 147) as well as viral infections and ozone that cause asthma exacerbations, are known to activate NF-κB *in vitro* (74, 113). Lastly, corticosteroids that are the most effective treatment for asthma exert their antiinflammatory effects in part by preventing the activation of NF-κB (56).

NO and NF-κB

Cellular studies have suggested several mechanisms by which NO regulates NF-κB (for review, see 73 (Fig. 4)). First, it is believed that a feedback mechanism exists by which NF-κB mediated induction of NOS2 produces NO, which in turn inhibits NF-κB and consequently suppresses the inflammatory response. Mechanisms by which NO represses NF-κB include the increased transcription of IκBα, the stabilization of the

mRNA, and inhibition of the proteolytic degradation of IκBα (130). S-nitrosylation of cysteine residue 62 within the p50 subunit of NF-κB has furthermore been shown to prevent DNA binding activity and subsequent inhibition of the expression of NF-κB related proteins (109, 111). Recent studies have also demonstrated that IKKβ is susceptible to inhibition by oxidants (94, Reynaert *et al., Proc Natl Acad Sci USA* 101: 8945-8950, 2004). Marshall and Stamler furthermore have alluded to a target for NO-mediated repression of NF-κB upstream of the degradation of IκBα in Jurkat T-cells, which could represent IKK. In contrast, NO-mediated repression of NF-κB in lung epithelial cells occurred predominately at the level of DNA binding, and thus could reflect S-nitrosylation of p50 (112).

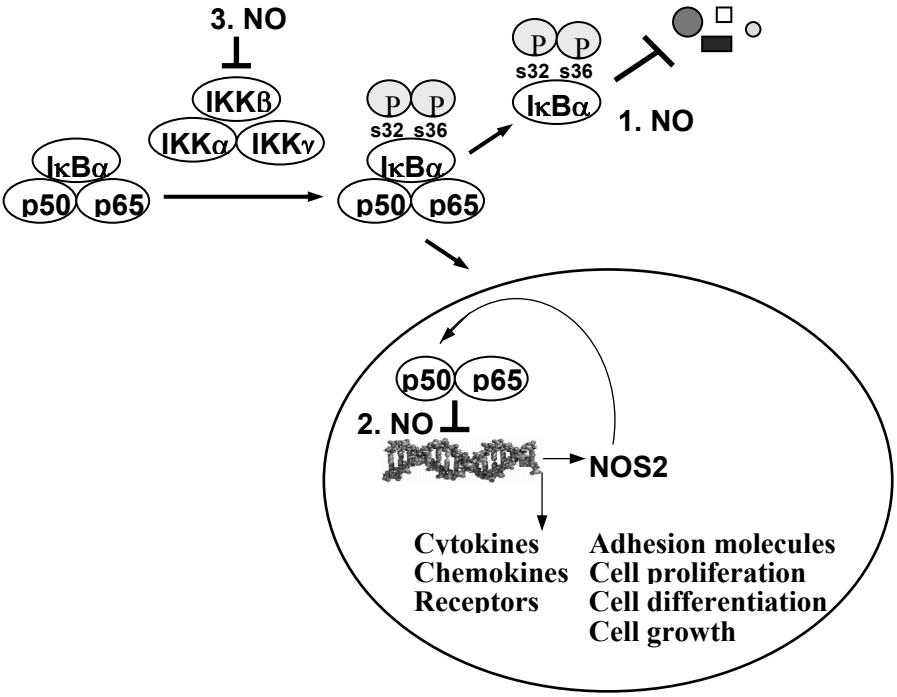

Figure 4 Influence of NO on the NF-κB signaling pathway. NO has been shown to inhibit the proteasomal degradation of IκBα, increase transcription of IκBα, and stabilize the mRNA (1). DNA binding activity of NF-κB in addition, is also inhibited by S-nitrosylation of cysteine residue 62 within the p50 subunit (2). IKK also is susceptible to repression by NO (3). Collectively, a feedback mechanism appears to exist by which NF-κB mediated induction of NOS2 and the consequent rise in NO production, represses NF-κB.

These findings suggest that the mode of inhibition of NF-κB by NO may be cell type-dependent. The recent finding that stimulation of cells with TNFα, which activates IKK and NF-κB, reduces overall protein S-nitrosylation (62), is certainly consistent with an inhibitory effect of S-nitrosylation on IKK and NF-κB activation, although no specific targets for denitrosylation were identified. In addition to inhibitory effects of NO on NF-κB, several studies have also reported stimulatory effects of NO on NF-κB, potentially related to S-nitrosylation of upstream factors such as p21Ras (98). Collectively, ample evidence exists in support of a regulatory role of NO on NF-κB activation. It appears that this regulation occurs at various levels, in many cases through S-nitrosylation of selected cellular targets, and that the overall effects on NF-κB depend strongly on the cell type, the localization and extent of NOS induction, and/or activation of NF-κB (17). Whether alterations in S-nitrosylation are also involved in regulating NF-κB or other transcription factors in airway inflammation *in vivo* remains unclear. Overall effects of such NO-dependent regulation of NF-κB on inflammation may not be easy to predict, as NF-κB activation also regulates the production of antiinflammatory cytokines and apoptosis of granulocytes, thereby controlling later resolution stages of inflammation (41, 95, 100), in addition to its well known involvement in the initiation of inflammation.

NO and apoptosis

Asthma is commonly associated with impaired apoptosis of inflammatory cells in lung tissue, specifically eosinophils, leading to the accumulation of these cells and their potent mediators, aggravating the inflammatory state as opposed to restoring normal tissue homeostasis. The lack of clearance of eosinophils may be correlated with the severity of the disease (28), and it has been demonstrated that glucocorticoids exert their antiinflammatory effects in part by targeting inflammatory cells to undergo apoptosis (26). Asthmatic lung epithelium, on the other hand, is more susceptible to oxidant-induced apoptosis, and this can lead to sloughing of airway epithelial cells and consequently a loss of barrier and regulatory function (13).

An extensive number of reports have clearly demonstrated that NO and its derivatives are involved in regulating cell death. In the context of asthma, NO and S-nitrosothiols have been shown to inhibit eosinophil apoptosis *in vitro*. For instance, NO has been shown to disrupt Fas-induced apoptosis of eosinophils at the level of, or proximal to, activation of c-jun-N-

terminal kinase (JNK) (59), and inhibition of endogenous NO production was found to increase the expression of Bcl-2 in eosinophils isolated from blood of asthmatics through effects on the mitogen activated protein kinases ERK (extracellular signal-regulated kinase) and p38, indicating that endogenous NO can serve to promote apoptosis via suppression of Bcl-2 expression (106).
S-nitrosylation of caspases, a family of cysteine proteases that are responsible for many proteolytic events that occur during apoptosis, has received much attention as a NO-dependent inhibitory mechanism of cellular apoptosis. For instance, Fas-induced apoptosis not only requires the cleavage of caspase-3 zymogens into their active from, but it is also associated with denitrosylation of the catalytic-site cysteine (32). Ligation of Fas concurrently induces heme iron nitrosation of cytochrome *c* and its subsequent release from the mitochondria into the cytoplasm, where it participates in the formation of the apoptosome. It was proposed that Fas stimulation induces a subtle conformational change in cytochrome *c*, that would increase the reactivity of its heme iron to NO that is locally produced in the mitochondria (138). Caspases 9 and 8 have also been shown to be inhibited by S-nitrosylation directly (86, 152), and additional evidence exists that NO prevents the correct assembly of the apoptosome and subsequent processing of procaspases 9, 3 and 8 (167). In addition, endogenous NO was shown to control cell cycle and prevent apoptosis of pulmonary epithelial cells, as depletion of NO in these cells caused alterations in cell cycle and increased apoptosis in a cGMP-dependent fashion (72).

Proapoptotic effects of NO may be exerted by its local production in the mitochondria, where it has the potential to regulate respiration and affect mitochondrion-regulated apoptosis. For instance, NO can induce necrotic cell death by inhibiting mitochondrial respiration through the inhibition of cytochrome *c* oxidase, by binding to either the heme iron or the copper center and competing with oxygen (12). This will lead to a depletion of energy and consequently to necrosis. Inhibition of glyceraldehyde-3-phosphate-dehydrogenase by NO via S-nitrosylation of the active-site cysteine (117, 118) represses glycolysis, leading to a depletion of ATP production, and subsequently causes necrosis.
As an example of NO induced apoptosis, both NO gas and GSNO have been shown to cause apoptosis of neutrophils, another cell type that has been suggested to play a role in severe asthma (90). The interaction of NO with $O_2^{\cdot-}$ and the formation of $ONOO^-$ was shown to be partially responsible for the induction of apoptosis, as the effects were mitigated by

treatment with superoxide dismutase (38, 39), although the precise site of action of NO in the apoptotic cascade was not shown.

Collectively, these reports demonstrate that NO and its derivatives play a role in regulating cell death. NO-induced cell death, whether it is in the form of apoptosis or necrosis, may serve to kill invading pathogens and limit the extent of inflammation by enhancing the removal of inflammatory cells. It should be noted that necrotic cell death can actually enhance inflammation and NF-κB activation (102). The effects of NO on cell death are complex and may be difficult to predict *in vivo*. In physiological conditions, the antiapoptotic effects of NO and S-nitrosothiols in particular may prevail, whereas superphysiological concentrations of NO may lead to death of host cells.

Studies of NO function in vivo

In order to determine the involvement of NO in the regulation of airway inflammation *in vivo* at the molecular level, and to identify the proteins that are regulated by NO, improved techniques will be required to demonstrate the involvement of S-nitrosylation in disease states and identify and localize specific S-nitrosylated proteins. The majority of proposed targets for S-nitrosylation have been investigated *in vitro*. In fact, rarely has an endogenous source of NO been directly linked to this posttranslational event. A major difficulty in assessing the role of endogenous protein S-nitrosylation has been the reliable detection of this modification within proteins *in vivo* and a lack of quantitative information regarding S-nitrosylation, which is mainly due to the unstable nature of S-nitrosothiols in the presence of metal ions. In addition, antibodies developed against the S-nitrosocysteine moiety have been used with variable success due to the small size of the epitope and its well known instability. The most widely used and accepted technique to date to demonstrate S-nitrosylation of proteins is chemiluminescence, coupled to either chemical reduction or photolysis of the S-NO bond. Recently developed strategies to selectively derivatize and isolate S-nitrosothiols from cell lysates and tissue homogenates (70, 71) (Fig. 5) will allow a more systematic approach to identify major cellular targets for protein S-nitrosylation. However, great care needs to be taken to avoid false positive and negative outcomes, including the complete blocking of all reduced sufhydryl groups and the presence of metal chelators to prevent transnitrosation or denitrosylation reactions. Nonetheless, this approach

may aid in elucidating the role of S-nitrosylated proteins in inflammation in general, and in the pathology of airway diseases such as asthma specifically.

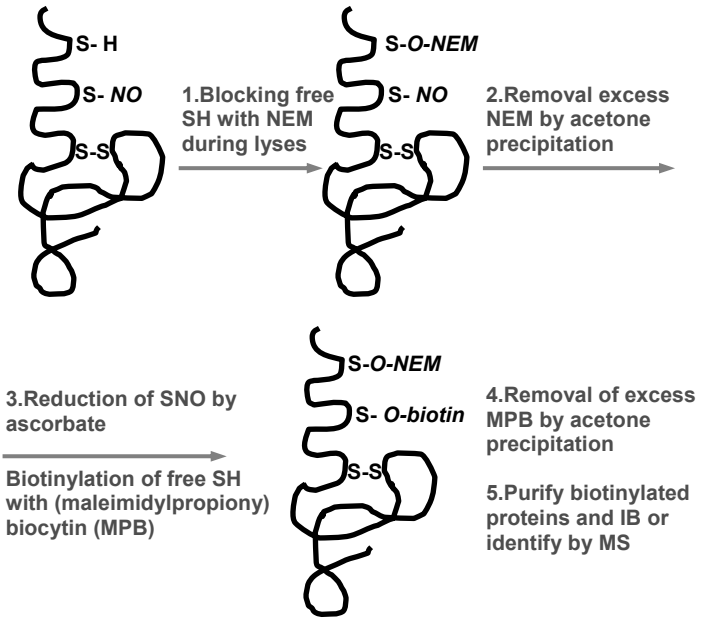

Figure 5 Principle of biotin derivatization to identify S-nitrosylated proteins. In the first step, free sulfhydryl groups are blocked, after which the S-NO bond is reduced by ascorbate, and newly formed sulfhydryls finally labeled with biotin. Biotinylated proteins can subsequently be purified on streptavidin columns and identified by western blotting for a specific protein or by mass spectrometry. NEM, *N*-ethylmaleimide Adapted from Wu *et al. (J Biol Chem* 273: 18898-18905, 1998) and Jaffrey and Snyder (69).

The identification of nitrated proteins may be less challenging as the nitrotyrosine moiety is a chemically more stable event, which makes the production of antibodies directed against nitrotyrosine an easier process, although specificity could still be problematic. A wide variety of target proteins have been identified *in vitro*, as well as *in vivo*. Establishing tyrosine nitration as an important posttranslational event, linking the modification directly to a change in protein function *in vivo*, however, remains an important challenge in this aspect of NO research.

Can NO be used as a therapeutic?

The inhalation of NO is already being used in the treatment of respiratory diseases, such as pulmonary hypertension of newborns, but concerns exist about its toxicity (134). For lung diseases such as asthma that appear to be associated with impaired NO functions, which may affect the regulation of airway tone and mucociliary clearance, a rationale for NO inhalation therapy would be apparent, although administered NO will most likely undergo the same fate as endogenously produced NO. As many of the beneficial actions of NO are attributed to S-nitrosothiols, these compounds could represent a more appropriate therapy. In this regard, GSNO represents an endogenous bronchodilator that is two log orders more potent then theophylline (43). Furthermore, in CF patients, aerosolized GSNO acutely increased oxygen saturation, and CF cells treated with GSNO treatment *in vitro* showed enhanced maturation of the CF transmembrane regulator and restoration of normal functionality (3, 143, 165). In addition, the S-nitrosylating gas, *O*-nitrosoethanol, which does not react with oxygen, improved ventilation to perfusion matching in an animal model of pulmonary hypertension (120). Whether S-nitrosothiols could improve asthma by relaxing airway smooth muscles and act to repress airway inflammation remains to be determined, because indications exist that the enzyme that metabolizes GSNO is elevated in allergic airway disease (35), potentially limiting the beneficial effects of this strategy.

Future directions to study NO signaling in the lung

Compelling evidence exists that a number of respiratory diseases have features of altered NO metabolism that may be expected to affect its biological properties, as has been reviewed here. Over the years, NO has firmly been established as potent second messenger molecule, influencing a myriad of physiological and pathophysiological processes, through cGMP-dependent pathways and S-nitrosylation reactions. Linking the alterations in NO metabolism observed in different disease states to altered posttranslational modifications of proteins by NO is the main challenge that lies ahead in this research field. Due to the unstable nature of S-nitrosylation, identifying targets in an *in vivo* setting represents a major difficulty that will need to be overcome. Derivatization techniques and advances in mass spectrometry should help to accomplish this goal. An improved understanding of the biochemical pathways that dictate NO availability will also be required in order to optimize the beneficial

functions of NO, while limiting the formation of more damaging RNS. Lastly, an improvement of the available mouse models will be required to overcome the problems and controversies obtained from the use of generic knockout mice. This includes models that would overexpress or be deficient in NOS in selective compartments of the lung, like airway epithelium, smooth muscle, vessels and inflammatory cells, to dissect out the relative contributions of NO generated therein. Given the rapid technical advancements in these areas, it is likely that better insights into the role of NO in pulmonary diseases will be forthcoming.

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ABBREVIATIONS

CAT1, cationic amino acid transporter 1; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; EPO, eosinophil peroxidase; GSH, glutathione; GSNO, S-nitrosoglutathione; IKK, inhibitory kappa B kinase; IL, interleukine; JNK, c-jun-N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MBP, major basic protein; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; NO, nitric oxide; $NO_2^{\cdot}$, nitrogen dioxide; NOS, nitric oxide synthase; $O_2^{\cdot -}$, superoxide; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; TNFα, tumor necrosis factor α

REFERENCES

1. Abu-Soud, H. M., M. Y. Khassawneh, J. T. Sohn, P. Murray, M. A. Haxhiu, and S. L. Hazen. Peroxidases inhibit nitric oxide (NO) dependent bronchodilation: development of a model describing NO-peroxidase interactions. *Biochemistry* 40(39):11866-75, 2001.
2. Akaike, T., Y. Noguchi, S. Ijiri, K. Setoguchi, M. Suga, Y. M. Zheng, B. Dietzschold, and H. Maeda. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A* 93(6):2448-53, 1996.
3. Andersson, C., B. Gaston, and G. M. Roomans. S-Nitrosoglutathione induces functional DeltaF508-CFTR in airway epithelial cells. *Biochem Biophys Res Commun* 297(3):552-7., 2002.
4. Andreadis, A. A., S. L. Hazen, S. A. Comhair, and S. C. Erzurum. Oxidative and nitrosative events in asthma. *Free Radic Biol Med* 35(3):213-25, 2003.
5. Anest, V., J. L. Hanson, P. C. Cogswell, K. A. Steinbrecher, B. D. Strahl, and A. S. Baldwin. A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. *Nature* 423(6940):659-63, 2003.
6. Ascenzi, P., M. Colasanti, T. Persichini, M. Muolo, F. Polticelli, G. Venturini, D. Bordo, and M. Bolognesi. Re-evaluation of amino acid sequence and structural consensus rules for cysteine-nitric oxide reactivity. *Biol Chem* 381(7):623-7., 2000.
7. Baldus, S., J. P. Eiserich, A. Mani, L. Castro, M. Figueroa, P. Chumley, W. Ma, A. Tousson, C. R. White, D. C. Bullard, M. L. Brennan, A. J. Lusis, K. P. Moore, and B. A. Freeman. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest* 108(12):1759-70, 2001.
8. Bender, A. T., D. R. Demady, and Y. Osawa. Ubiquitination of neuronal nitric-oxide synthase in vitro and in vivo. *J Biol Chem* 275(23):17407-11, 2000.
9. Berlett, B. S., R. L. Levine, and E. R. Stadtman. Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: both modifications mimic effects of adenylylation. *Proc Natl Acad Sci U S A* 95(6):2784-9, 1998.
10. Birrell, M. A., K. McCluskie, B. Haddad el, C. H. Battram, S. E. Webber, M. L. Foster, M. H. Yacoub, and M. G. Belvisi. Pharmacological assessment of the nitric-oxide synthase isoform involved in eosinophilic inflammation in a rat model of sephadex-induced airway inflammation. *J Pharmacol Exp Ther* 304(3):1285-91, 2003.
11. Broillet, M. C. S-nitrosylation of proteins. *Cell Mol Life Sci* 55(8-9):1036-42., 1999.
12. Brookes, P. S., A. L. Levonen, S. Shiva, P. Sarti, and V. M. Darley-Usmar. Mitochondria: regulators of signal transduction by reactive oxygen and nitrogen species. *Free Radic Biol Med* 33(6):755-64, 2002.
13. Bucchieri, F., S. M. Puddicombe, J. L. Lordan, A. Richter, D. Buchanan, S. J. Wilson, J. Ward, G. Zummo, P. H. Howarth, R. Djukanovic, S. T. Holgate, and D. E. Davies. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol* 27(2):179-85, 2002.
14. Butt, E., M. Bernhardt, A. Smolenski, P. Kotsonis, L. G. Frohlich, A. Sickmann, H. E. Meyer, S. M. Lohmann, and H. H. Schmidt. Endothelial nitric-oxide

synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J Biol Chem* 275(7):5179-87, 2000.
15. Chen, Z. P., K. I. Mitchelhill, B. J. Michell, D. Stapleton, I. Rodriguez-Crespo, L. A. Witters, D. A. Power, P. R. Ortiz de Montellano, and B. E. Kemp. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* 443(3):285-9, 1999.
16. Clancy, R. M., J. Leszczynska-Piziak, and S. B. Abramson. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J Clin Invest* 90(3):1116-21, 1992.
17. Connelly, L., M. Palacios-Callender, C. Ameixa, S. Moncada, and A. J. Hobbs. Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol* 166(6):3873-81, 2001.
18. Cook, S., P. Vollenweider, B. Menard, M. Egli, P. Nicod, and U. Scherrer. Increased eNO and pulmonary iNOS expression in eNOS null mice. *Eur Respir J* 21(5):770-3, 2003.
19. Coope, H. J., P. G. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. Klaus, L. H. Johnston, and S. C. Ley. CD40 regulates the processing of NF-kappaB2 p100 to p52. *Embo J* 21(20):5375-85, 2002.
20. Csoma, Z., A. Bush, N. M. Wilson, L. Donnelly, B. Balint, P. J. Barnes, and S. A. Kharitonov. Nitric oxide metabolites are not reduced in exhaled breath condensate of patients with primary ciliary dyskinesia. *Chest* 124(2):633-8, 2003.
21. Davare, M. A., V. Avdonin, D. D. Hall, E. M. Peden, A. Burette, R. J. Weinberg, M. C. Horne, T. Hoshi, and J. W. Hell. A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. *Science* 293(5527):98-101, 2001.
22. Davis, I. C., S. Zhu, J. B. Sampson, J. P. Crow, and S. Matalon. Inhibition of human surfactant protein A function by oxidation intermediates of nitrite. *Free Radic Biol Med* 33(12):1703-13, 2002.
23. De Sanctis, G. T., J. A. MacLean, K. Hamada, S. Mehta, J. A. Scott, A. Jiao, C. N. Yandava, L. Kobzik, W. W. Wolyniec, A. J. Fabian, C. S. Venugopal, H. Grasemann, P. L. Huang, and J. M. Drazen. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J Exp Med* 189(10):1621-30, 1999.
24. Delgado-Esteban, M., A. Almeida, and J. M. Medina. Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia. *J Neurochem* 82(5):1148-59, 2002.
25. Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A. M. Zeiher. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399(6736):601-5, 1999.
26. Druilhe, A., S. Letuve, and M. Pretolani. Glucocorticoid-induced apoptosis in human eosinophils: mechanisms of action. *Apoptosis* 8(5):481-95, 2003.
27. Duguet, A., H. Iijima, S. Y. Eum, Q. Hamid, and D. H. Eidelman. Eosinophil peroxidase mediates protein nitration in allergic airway inflammation in mice. *Am J Respir Crit Care Med* 164(7):1119-26., 2001.
28. Duncan, C. J., A. Lawrie, M. G. Blaylock, J. G. Douglas, and G. M. Walsh. Reduced eosinophil apoptosis in induced sputum correlates with asthma severity. *Eur Respir J* 22(3):484-90, 2003.

29. Dupont, L. J., M. G. Demedts, and G. M. Verleden. Prospective evaluation of the validity of exhaled nitric oxide for the diagnosis of asthma. *Chest* 123(3):751-6, 2003.
30. Eiserich, J. P., A. G. Estevez, T. V. Bamberg, Y. Z. Ye, P. H. Chumley, J. S. Beckman, and B. A. Freeman. Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc Natl Acad Sci U S A* 96(11):6365-70, 1999.
31. Endo, M., S. Oyadomari, Y. Terasaki, M. Takeya, M. Suga, M. Mori, and T. Gotoh. Induction of arginase I and II in bleomycin-induced fibrosis of mouse lung. *Am J Physiol Lung Cell Mol Physiol* 285(2):L313-21, 2003.
32. Eu, J. P., L. Liu, M. Zeng, and J. S. Stamler. An apoptotic model for nitrosative stress. *Biochemistry* 39(5):1040-7., 2000.
33. Eynott, P. R., N. Paavolainen, D. A. Groneberg, A. Noble, M. Salmon, P. Nath, S. Y. Leung, and K. F. Chung. Role of nitric oxide in chronic allergen-induced airway cell proliferation and inflammation. *J Pharmacol Exp Ther* 304(1):22-9, 2003.
34. Fang, F. C. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 99(12):2818-25, 1997.
35. Fang, K., R. Johns, T. Macdonald, M. Kinter, and B. Gaston. S-nitrosoglutathione breakdown prevents airway smooth muscle relaxation in the guinea pig. *Am J Physiol Lung Cell Mol Physiol* 279(4):L716-21., 2000.
36. Feder, L. S., D. Stelts, R. W. Chapman, D. Manfra, Y. Crawley, H. Jones, M. Minnicozzi, X. Fernandez, T. Paster, R. W. Egan, W. Kreutner, and T. T. Kung. Role of nitric oxide on eosinophilic lung inflammation in allergic mice. *Am J Respir Cell Mol Biol* 17(4):436-42, 1997.
37. Feletou, M., M. Lonchampt, F. Coge, J. P. Galizzi, C. Bassoullet, C. Merial, P. Robineau, J. A. Boutin, P. L. Huang, P. M. Vanhoutte, and E. Canet. Regulation of murine airway responsiveness by endothelial nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol* 281(1):L258-67, 2001.
38. Fortenberry, J. D., M. L. Owens, and L. A. Brown. S-nitrosoglutathione enhances neutrophil DNA fragmentation and cell death. *Am J Physiol* 276(3 Pt 1):L435-42., 1999.
39. Fortenberry, J. D., M. L. Owens, M. R. Brown, D. Atkinson, and L. A. Brown. Exogenous nitric oxide enhances neutrophil cell death and DNA fragmentation. *Am J Respir Cell Mol Biol* 18(3):421-8., 1998.
40. Fries, D. M., E. Paxinou, M. Themistocleous, E. Swanberg, K. K. Griendling, D. Salvemini, J. W. Slot, H. F. Heijnen, S. L. Hazen, and H. Ischiropoulos. Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species. *J Biol Chem* 278(25):22901-7, 2003.
41. Fujihara, S., C. Ward, I. Dransfield, R. T. Hay, I. J. Uings, B. Hayes, S. N. Farrow, C. Haslett, and A. G. Rossi. Inhibition of nuclear factor-kappaB activation un-masks the ability of TNF-alpha to induce human eosinophil apoptosis. *Eur J Immunol* 32(2):457-66, 2002.
42. Fulton, D., J. P. Gratton, and W. C. Sessa. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther* 299(3):818-24, 2001.

43. Gaston, B., J. M. Drazen, A. Jansen, D. A. Sugarbaker, J. Loscalzo, W. Richards, and J. S. Stamler. Relaxation of human bronchial smooth muscle by S-nitrosothiols in vitro. *J Pharmacol Exp Ther* 268(2):978-84, 1994.
44. Gaston, B., J. M. Drazen, J. Loscalzo, and J. S. Stamler. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 149(2 Pt 1):538-51., 1994.
45. Gaston, B., S. Sears, J. Woods, J. Hunt, M. Ponaman, T. McMahon, and J. S. Stamler. Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet* 351(9112):1317-9., 1998.
46. Gath, I., J. Ebert, U. Godtel-Armbrust, R. Ross, A. B. Reske-Kunz, and U. Forstermann. NO synthase II in mouse skeletal muscle is associated with caveolin 3. *Biochem J* 340 (Pt 3):723-8, 1999.
47. Ghosh, S., and M. Karin. Missing pieces in the NF-kappaB puzzle. *Cell* 109 Suppl:S81-96, 2002.
48. Glover, R. E., V. Koshkin, H. B. Dunford, and R. P. Mason. The reaction rates of NO with horseradish peroxidase compounds I and II. *Nitric Oxide* 3(6):439-44, 1999.
49. Goligorsky, M. S., H. Li, S. Brodsky, and J. Chen. Relationships between caveolae and eNOS: everything in proximity and the proximity of everything. *Am J Physiol Renal Physiol* 283(1):F1-10, 2002.
50. Gow, A. J., D. Duran, S. Malcolm, and H. Ischiropoulos. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett* 385(1-2):63-6, 1996.
51. Grasemann, H., B. Gaston, K. Fang, K. Paul, and F. Ratjen. Decreased levels of nitrosothiols in the lower airways of patients with cystic fibrosis and normal pulmonary function. *J Pediatr* 135(6):770-2., 1999.
52. Grasemann, H., C. N. Yandava, K. Storm van's Gravesande, A. Deykin, A. Pillari, J. Ma, L. A. Sonna, C. Lilly, M. J. Stampfer, E. Israel, E. K. Silverman, and J. M. Drazen. A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. *Biochem Biophys Res Commun* 272(2):391-4, 2000.
53. Grune, T., and K. J. Davies. Breakdown of oxidized proteins as a part of secondary antioxidant defenses in mammalian cells. *Biofactors* 6(2):165-72, 1997.
54. Grune, T., T. Reinheckel, and K. J. Davies. Degradation of oxidized proteins in mammalian cells. *Faseb J* 11(7):526-34, 1997.
55. Hammermann, R., J. Hirschmann, C. Hey, J. Mossner, G. Folkerts, F. P. Nijkamp, I. Wessler, and K. Racke. Cationic proteins inhibit L-arginine uptake in rat alveolar macrophages and tracheal epithelial cells. Implications for nitric oxide synthesis. *Am J Respir Cell Mol Biol* 21(2):155-62., 1999.
56. Hancox, R. J., D. A. Stevens, I. M. Adcock, P. J. Barnes, and D. R. Taylor. Effects of inhaled beta agonist and corticosteroid treatment on nuclear transcription factors in bronchial mucosa in asthma. *Thorax* 54(6):488-92, 1999.
57. Hansel, T. T., S. A. Kharitonov, L. E. Donnelly, E. M. Erin, M. G. Currie, W. M. Moore, P. T. Manning, D. P. Recker, and P. J. Barnes. A selective inhibitor of inducible nitric oxide synthase inhibits exhaled breath nitric oxide in healthy volunteers and asthmatics. *Faseb J* 17(10):1298-300, 2003.
58. Hart, L. A., V. L. Krishnan, I. M. Adcock, P. J. Barnes, and K. F. Chung. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am J Respir Crit Care Med* 158(5 Pt 1):1585-92, 1998.

59. Hebestreit, H., B. Dibbert, I. Balatti, D. Braun, A. Schapowal, K. Blaser, and H. U. Simon. Disruption of fas receptor signaling by nitric oxide in eosinophils. *J Exp Med* 187(3):415-25, 1998.
60. Hickey, M. J., K. A. Sharkey, E. G. Sihota, P. H. Reinhardt, J. D. Macmicking, C. Nathan, and P. Kubes. Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. *Faseb J* 11(12):955-64, 1997.
61. Hobbs, A. J., and L. J. Ignarro. Nitric oxide-cyclic GMP signal transduction system. *Methods Enzymol* 269:134-48, 1996.
62. Hoffmann, J., J. Haendeler, A. M. Zeiher, and S. Dimmeler. TNFalpha and oxLDL reduce protein S-nitrosylation in endothelial cells. *J Biol Chem* 276(44):41383-7., 2001.
63. Hogg, N. Biological chemistry and clinical potential of S-nitrosothiols. *Free Radic Biol Med* 28(10):1478-86., 2000.
64. Hogg, N. The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol* 42:585-600, 2002.
65. Hunt, J. F., K. Fang, R. Malik, A. Snyder, N. Malhotra, T. A. Platts-Mills, and B. Gaston. Endogenous airway acidification. Implications for asthma pathophysiology. *Am J Respir Crit Care Med* 161(3 Pt 1):694-9, 2000.
66. Idriss, S. D., T. Gudi, D. E. Casteel, V. G. Kharitonov, R. B. Pilz, and G. R. Boss. Nitric oxide regulation of gene transcription via soluble guanylate cyclase and type I cGMP-dependent protein kinase. *J Biol Chem* 274(14):9489-93, 1999.
67. Iijima, H., A. Duguet, S. Y. Eum, Q. Hamid, and D. H. Eidelman. Nitric oxide and protein nitration are eosinophil dependent in allergen- challenged mice. *Am J Respir Crit Care Med* 163(5):1233-40., 2001.
68. Iijima, H., A. Duguet, S. Y. Eum, Q. Hamid, and D. H. Eidelman. Nitric oxide and protein nitration are eosinophil dependent in allergen-challenged mice. *Am J Respir Crit Care Med* 163(5):1233-40, 2001.
69. Ischiropoulos, H., L. Zhu, and J. S. Beckman. Peroxynitrite formation from macrophage-derived nitric oxide. *Arch Biochem Biophys* 298(2):446-51, 1992.
70. Jaffrey, S. R., H. Erdjument-Bromage, C. D. Ferris, P. Tempst, and S. H. Snyder. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3(2):193-7., 2001.
71. Jaffrey, S. R., and S. H. Snyder. The biotin switch method for the detection of s-nitrosylated proteins. *Sci STKE* 2001(86):L1., 2001.
72. Janssen, Y. M., R. Soultanakis, K. Steece, E. Heerdt, R. J. Singh, J. Joseph, and B. Kalyanaraman. Depletion of nitric oxide causes cell cycle alterations, apoptosis, and oxidative stress in pulmonary cells. *Am J Physiol* 275(6 Pt 1):L1100-9, 1998.
73. Janssen-Heininger, Y. M., M. E. Poynter, and P. A. Baeuerle. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med* 28(9):1317-27, 2000.
74. Jaspers, I., E. Flescher, and L. C. Chen. Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells. *Am J Physiol* 272(3 Pt 1):L504-11, 1997.
75. Jatakanon, A., S. Kharitonov, S. Lim, and P. J. Barnes. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 54(2):108-14, 1999.

76. Ji, Y., T. P. Akerboom, H. Sies, and J. A. Thomas. S-nitrosylation and S-glutathiolation of protein sulfhydryls by S- nitroso glutathione. *Arch Biochem Biophys* 362(1):67-78., 1999.
77. Jourd'heuil, D., F. S. Laroux, A. M. Miles, D. A. Wink, and M. B. Grisham. Effect of superoxide dismutase on the stability of S-nitrosothiols. *Arch Biochem Biophys* 361(2):323-30, 1999.
78. Kaminsky, D. A., J. Mitchell, N. Carroll, A. James, R. Soultanakis, and Y. Janssen. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J Allergy Clin Immunol* 104(4 Pt 1):747-54., 1999.
79. Kanai, A. J., L. L. Pearce, P. R. Clemens, L. A. Birder, M. M. VanBibber, S. Y. Choi, W. C. de Groat, and J. Peterson. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. *Proc Natl Acad Sci U S A* 98(24):14126-31, 2001.
80. Karin, M., and Y. Ben-Neriah. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 18:621-63, 2000.
81. Kashiba-Iwatsuki, M., M. Yamaguchi, and M. Inoue. Role of ascorbic acid in the metabolism of S-nitroso-glutathione. *FEBS Lett* 389(2):149-52, 1996.
82. Kenyon, N. J., K. Gohil, and J. A. Last. Susceptibility to ovalbumin-induced airway inflammation and fibrosis in inducible nitric oxide synthetase-deficient mice: mechanisms and consequences. *Toxicol Appl Pharmacol* 191(1):2-11, 2003.
83. Kenyon, N. J., A. van der Vliet, B. C. Schock, T. Okamoto, G. M. McGrew, and J. A. Last. Susceptibility to ozone-induced acute lung injury in iNOS-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 282(3):L540-5, 2002.
84. Kharitonov, S. A., and P. J. Barnes. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 163(7):1693-722, 2001.
85. Kharitonov, S. A., D. Yates, R. A. Robbins, R. Logan-Sinclair, E. A. Shinebourne, and P. J. Barnes. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 343(8890):133-5, 1994.
86. Kim, P. K., Y. G. Kwon, H. T. Chung, and Y. M. Kim. Regulation of caspases by nitric oxide. *Ann N Y Acad Sci* 962:42-52., 2002.
87. Kleeberger, S. R., S. P. Reddy, L. Y. Zhang, H. Y. Cho, and A. E. Jedlicka. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol* 280(2):L326-33., 2001.
88. Koarai, A., M. Ichinose, H. Sugiura, M. Tomaki, M. Watanabe, S. Yamagata, Y. Komaki, K. Shirato, and T. Hattori. iNOS depletion completely diminishes reactive nitrogen-species formation after an allergic response. *Eur Respir J* 20(3):609-16, 2002.
89. Kobayashi, H., R. Hataishi, H. Mitsufuji, M. Tanaka, M. Jacobson, T. Tomita, W. M. Zapol, and R. C. Jones. Antiinflammatory properties of inducible nitric oxide synthase in acute hyperoxic lung injury. *Am J Respir Cell Mol Biol* 24(4):390-7, 2001.
90. Koh, Y. Y., R. Dupuis, M. Pollice, K. H. Albertine, J. E. Fish, and S. P. Peters. Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. *Am J Respir Cell Mol Biol* 8(5):493-9, 1993.
91. Kolodziejski, P. J., A. Musial, J. S. Koo, and N. T. Eissa. Ubiquitination of inducible nitric oxide synthase is required for its degradation. *Proc Natl Acad Sci U S A* 99(19):12315-20, 2002.

92. Komeima, K., Y. Hayashi, Y. Naito, and Y. Watanabe. Inhibition of neuronal nitric-oxide synthase by calcium/ calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108-15 neuronal cells. *J Biol Chem* 275(36):28139-43, 2000.
93. Kone, B. C., T. Kuncewicz, W. Zhang, and Z. Y. Yu. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol* 285(2):F178-90, 2003.
94. Korn, S. H., E. F. Wouters, N. Vos, and Y. M. Janssen-Heininger. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. *J Biol Chem* 276(38):35693-700., 2001.
95. Kupfner, J. G., J. J. Arcaroli, H. K. Yum, S. G. Nadler, K. Y. Yang, and E. Abraham. Role of NF-kappaB in endotoxemia-induced alterations of lung neutrophil apoptosis. *J Immunol* 167(12):7044-51, 2001.
96. Lacza, Z., J. A. Snipes, J. Zhang, E. M. Horvath, J. P. Figueroa, C. Szabo, and D. W. Busija. Mitochondrial nitric oxide synthase is not eNOS, nNOS or iNOS. *Free Radic Biol Med* 35(10):1217-28, 2003.
97. Lambeth, J. D., G. Cheng, R. S. Arnold, and W. A. Edens. Novel homologs of gp91phox. *Trends Biochem Sci* 25(10):459-61, 2000.
98. Lander, H. M., D. P. Hajjar, B. L. Hempstead, U. A. Mirza, B. T. Chait, S. Campbell, and L. A. Quilliam. A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem* 272(7):4323-6, 1997.
99. Lang, R., D. Patel, J. J. Morris, R. L. Rutschman, and P. J. Murray. Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* 169(5):2253-63, 2002.
100. Lawrence, T., D. W. Gilroy, P. R. Colville-Nash, and D. A. Willoughby. Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 7(12):1291-7, 2001.
101. Lee, J., H. Ryu, R. J. Ferrante, S. M. Morris, Jr., and R. R. Ratan. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. *Proc Natl Acad Sci U S A* 100(8):4843-8, 2003.
102. Li, M., D. F. Carpio, Y. Zheng, P. Bruzzo, V. Singh, F. Ouaaz, R. M. Medzhitov, and A. A. Beg. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. *J Immunol* 166(12):7128-35, 2001.
103. Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J Exp Med* 189(11):1839-45, 1999.
104. Lin, H. C., C. H. Wang, C. T. Yu, K. S. Hwang, and H. P. Kuo. Endogenous nitric oxide inhibits neutrophil adherence to lung epithelial cells to modulate interleukin-8 release. *Life Sci* 69(11):1333-44, 2001.
105. Liu, L., A. Hausladen, M. Zeng, L. Que, J. Heitman, and J. S. Stamler. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 410(6827):490-4., 2001.
106. Maa, S. H., C. H. Wang, C. Y. Liu, H. C. Lin, K. H. Huang, and H. P. Kuo. Endogenous nitric oxide downregulates the Bcl-2 expression of eosinophils through mitogen-activated protein kinase in bronchial asthma. *J Allergy Clin Immunol* 112(4):761-7, 2003.

107. Mallozzi, C., A. M. Di Stasi, and M. Minetti. Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *Faseb J* 11(14):1281-90, 1997.
108. Mannick, J. B., and C. M. Schonhoff. Nitrosylation: the next phosphorylation? *Arch Biochem Biophys* 408(1):1-6., 2002.
109. Marshall, H. E., K. Merchant, and J. S. Stamler. Nitrosation and oxidation in the regulation of gene expression. *Faseb J* 14(13):1889-900., 2000.
110. Marshall, H. E., and J. S. Stamler. NO waiting to exhale in asthma. *Am J Respir Crit Care Med* 161(3 Pt 1):685-7., 2000.
111. Marshall, H. E., and J. S. Stamler. Inhibition of NF-kappa B by S-nitrosylation. *Biochemistry* 40(6):1688-93., 2001.
112. Marshall, H. E., and J. S. Stamler. Nitrosative Stress-induced Apoptosis through Inhibition of NF-kappa B. *J Biol Chem* 277(37):34223-8., 2002.
113. Mastronarde, J. G., B. He, M. M. Monick, N. Mukaida, K. Matsushima, and G. W. Hunninghake. Induction of interleukin (IL)-8 gene expression by respiratory syncytial virus involves activation of nuclear factor (NF)-kappa B and NF-IL-6. *J Infect Dis* 174(2):262-7, 1996.
114. May, M. J., and S. Ghosh. Signal transduction through NF-kappa B. *Immunol Today* 19(2):80-8, 1998.
115. May, M. J., and S. Ghosh. IkappaB kinases: kinsmen with different crafts. *Science* 284(5412):271-3, 1999.
116. McDonald, K. K., S. Zharikov, E. R. Block, and M. S. Kilberg. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *J Biol Chem* 272(50):31213-6, 1997.
117. Mohr, S., H. Hallak, A. de Boitte, E. G. Lapetina, and B. Brune. Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 274(14):9427-30, 1999.
118. Mohr, S., J. S. Stamler, and B. Brune. Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by S-nitrosylation and subsequent NADH attachment. *J Biol Chem* 271(8):4209-14, 1996.
119. Morrissey, B. M., K. Schilling, J. V. Weil, P. E. Silkoff, and D. M. Rodman. Nitric oxide and protein nitration in the cystic fibrosis airway. *Arch Biochem Biophys* 406(1):33-9, 2002.
120. Moya, M. P., A. J. Gow, T. J. McMahon, E. J. Toone, I. M. Cheifetz, R. N. Goldberg, and J. S. Stamler. S-nitrosothiol repletion by an inhaled gas regulates pulmonary function. *Proc Natl Acad Sci U S A* 98(10):5792-7., 2001.
121. Munder, M., K. Eichmann, J. M. Moran, F. Centeno, G. Soler, and M. Modolell. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol* 163(7):3771-7, 1999.
122. Nathan, C. Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 100(10):2417-23, 1997.
123. Nikitovic, D., and A. Holmgren. S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem* 271(32):19180-5, 1996.
124. Numanami, H., D. K. Nelson, J. C. Hoyt, J. L. Freels, M. Habib, J. Amano, M. Haniuda, S. Koyama, and R. A. Robbins. Peroxynitrite enhances interleukin-10 reduction in the release of neutrophil chemotactic activity. *Am J Respir Cell Mol Biol* 29(2):239-44, 2003.

125. O'Donnell, V. B., B. Coles, M. J. Lewis, B. C. Crews, L. J. Marnett, and B. A. Freeman. Catalytic consumption of nitric oxide by prostaglandin H synthase-1 regulates platelet function. *J Biol Chem* 275(49):38239-44, 2000.
126. O'Donnell, V. B., K. B. Taylor, S. Parthasarathy, H. Kuhn, D. Koesling, A. Friebe, A. Bloodsworth, V. M. Darley-Usmar, and B. A. Freeman. 15-Lipoxygenase catalytically consumes nitric oxide and impairs activation of guanylate cyclase. *J Biol Chem* 274(29):20083-91, 1999.
127. Okamoto, T., K. Gohil, E. I. Finkelstein, P. Bove, T. Akaike, and A. Van Der Vliet. Multiple contributing roles for NOS2 in LPS-induced acute airway inflammation in mice. *Am J Physiol Lung Cell Mol Physiol*, 2003.
128. Okamoto, T., A. Schlegel, P. E. Scherer, and M. P. Lisanti. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem* 273(10):5419-22, 1998.
129. Pahl, H. L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18(49):6853-66, 1999.
130. Peng, H. B., P. Libby, and J. K. Liao. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem* 270(23):14214-9., 1995.
131. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol* 160(4):1325-34, 2002.
132. Pritchard, K. A., Jr., A. W. Ackerman, E. R. Gross, D. W. Stepp, Y. Shi, J. T. Fontana, J. E. Baker, and W. C. Sessa. Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. *J Biol Chem* 276(21):17621-4., 2001.
133. Raychaudhuri, B., R. Dweik, M. J. Connors, L. Buhrow, A. Malur, J. Drazba, A. C. Arroliga, S. C. Erzurum, M. S. Kavuru, and M. J. Thomassen. Nitric oxide blocks nuclear factor-kappaB activation in alveolar macrophages. *Am J Respir Cell Mol Biol* 21(3):311-6., 1999.
134. Richardson, G., and N. Benjamin. Potential therapeutic uses for S-nitrosothiols. *Clin Sci (Lond)* 102(1):99-105., 2002.
135. Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* 395(6699):297-300, 1998.
136. Sadeghi-Hashjin, G., G. Folkerts, P. A. Henricks, A. K. Verheyen, H. J. van der Linde, I. van Ark, A. Coene, and F. P. Nijkamp. Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* 153(5):1697-701, 1996.
137. Sato, E., K. L. Simpson, M. B. Grisham, S. Koyama, and R. A. Robbins. Reactive nitrogen and oxygen species attenuate interleukin- 8-induced neutrophil chemotactic activity in vitro. *J Biol Chem* 275(15):10826-30, 2000.
138. Schonhoff, C. M., B. Gaston, and J. B. Mannick. Nitrosylation of Cytochrome c during Apoptosis. *J Biol Chem* 278(20):18265-70., 2003.
139. Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S. C. Sun, and M. Karin. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* 293(5534):1495-9, 2001.

140. Sexton, D. J., A. Muruganandam, D. J. McKenney, and B. Mutus. Visible light photochemical release of nitric oxide from S-nitrosoglutathione: potential photochemotherapeutic applications. *Photochem Photobiol* 59(4):463-7, 1994.
141. Simon, D. I., M. E. Mullins, L. Jia, B. Gaston, D. J. Singel, and J. S. Stamler. Polynitrosylated proteins: characterization, bioactivity, and functional consequences. *Proc Natl Acad Sci U S A* 93(10):4736-41., 1996.
142. Sittipunt, C., K. P. Steinberg, J. T. Ruzinski, C. Myles, S. Zhu, R. B. Goodman, L. D. Hudson, S. Matalon, and T. R. Martin. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 163(2):503-10, 2001.
143. Snyder, A. H., M. E. McPherson, J. F. Hunt, M. Johnson, J. S. Stamler, and B. Gaston. Acute effects of aerosolized S-nitrosoglutathione in cystic fibrosis. *Am J Respir Crit Care Med* 165(7):922-6., 2002.
144. Souza, J. M., I. Choi, Q. Chen, M. Weisse, E. Daikhin, M. Yudkoff, M. Obin, J. Ara, J. Horwitz, and H. Ischiropoulos. Proteolytic degradation of tyrosine nitrated proteins. *Arch Biochem Biophys* 380(2):360-6, 2000.
145. Spector, E. B., S. C. Rice, S. Moedjono, B. Bernard, and S. D. Cederbaum. Biochemical properties of arginase in human adult and fetal tissues. *Biochem Med* 28(2):165-75, 1982.
146. Spencer, N. Y., H. Zeng, R. P. Patel, and N. Hogg. Reaction of S-nitrosoglutathione with the heme group of deoxyhemoglobin. *J Biol Chem* 275(47):36562-7, 2000.
147. Stacey, M. A., G. Sun, G. Vassalli, M. Marini, A. Bellini, and S. Mattoli. The allergen Der p1 induces NF-kappaB activation through interference with IkappaB alpha function in asthmatic bronchial epithelial cells. *Biochem Biophys Res Commun* 236(2):522-6, 1997.
148. Stamler, J. S., S. Lamas, and F. C. Fang. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 106(6):675-83., 2001.
149. Stamler, J. S., E. J. Toone, S. A. Lipton, and N. J. Sucher. (S)NO signals: translocation, regulation, and a consensus motif. *Neuron* 18(5):691-6., 1997.
150. Steudel, W., M. Kirmse, J. Weimann, R. Ullrich, J. Hromi, and W. M. Zapol. Exhaled nitric oxide production by nitric oxide synthase-deficient mice. *Am J Respir Crit Care Med* 162(4 Pt 1):1262-7, 2000.
151. Thomassen, M. J., and M. S. Kavuru. Human alveolar macrophages and monocytes as a source and target for nitric oxide. *Int Immunopharmacol* 1(8):1479-90, 2001.
152. Torok, N. J., H. Higuchi, S. Bronk, and G. J. Gores. Nitric oxide inhibits apoptosis downstream of cytochrome C release by nitrosylating caspase 9. *Cancer Res* 62(6):1648-53., 2002.
153. Torres, J., M. A. Sharpe, A. Rosquist, C. E. Cooper, and M. T. Wilson. Cytochrome c oxidase rapidly metabolises nitric oxide to nitrite. *FEBS Lett* 475(3):263-6, 2000.
154. Trujillo, M., M. N. Alvarez, G. Peluffo, B. A. Freeman, and R. Radi. Xanthine oxidase-mediated decomposition of S-nitrosothiols. *J Biol Chem* 273(14):7828-34, 1998.
155. van der Vliet, A., J. P. Eiserich, B. Halliwell, and C. E. Cross. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272(12):7617-25, 1997.

156. van der Vliet, A., P. A. Hoen, P. S. Wong, A. Bast, and C. E. Cross. Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J Biol Chem* 273(46):30255-62, 1998.
157. Van's Gravesande, K. S., M. E. Wechsler, H. Grasemann, E. S. Silverman, L. Le, L. J. Palmer, and J. M. Drazen. Association of a Missense Mutation in the NOS3 Gene with Exhaled Nitric Oxide Levels. *Am J Respir Crit Care Med* 168(2):228-31, 2003.
158. Vasquez-Vivar, J., B. Kalyanaraman, P. Martasek, N. Hogg, B. S. Masters, H. Karoui, P. Tordo, and K. A. Pritchard, Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 95(16):9220-5, 1998.
159. Wu, W., Y. Chen, and S. L. Hazen. Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem* 274(36):25933-44, 1999.
160. Xia, Y., and J. L. Zweier. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A* 94(13):6954-8, 1997.
161. Xiao, G., E. W. Harhaj, and S. C. Sun. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 7(2):401-9, 2001.
162. Xiong, Y., G. Karupiah, S. P. Hogan, P. S. Foster, and A. J. Ramsay. Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J Immunol* 162(1):445-52, 1999.
163. Yamamoto, Y., U. N. Verma, S. Prajapati, Y. T. Kwak, and R. B. Gaynor. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* 423(6940):655-9, 2003.
164. Yates, D. H., S. A. Kharitonov, P. S. Thomas, and P. J. Barnes. Endogenous nitric oxide is decreased in asthmatic patients by an inhibitor of inducible nitric oxide synthase. *Am J Respir Crit Care Med* 154(1):247-50, 1996.
165. Zaman, K., M. McPherson, J. Vaughan, J. Hunt, F. Mendes, B. Gaston, and L. A. Palmer. S-nitrosoglutathione increases cystic fibrosis transmembrane regulator maturation. *Biochem Biophys Res Commun* 284(1):65-70., 2001.
166. Zandi, E., and M. Karin. Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. *Mol Cell Biol* 19(7):4547-51, 1999.
167. Zech, B., R. Kohl, A. von Knethen, and B. Brune. Nitric oxide donors inhibit formation of the Apaf-1/caspase-9 apoptosome and activation of caspases. *Biochem J* 371(Pt 3):1055-64, 2003.
168. Zeng, H., N. Y. Spencer, and N. Hogg. Metabolism of S-nitrosoglutathione by endothelial cells. *Am J Physiol Heart Circ Physiol* 281(1):H432-9., 2001.
169. Zhu, S., L. B. Ware, T. Geiser, M. A. Matthay, and S. Matalon. Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. *Am J Respir Crit Care Med* 163(1):166-72, 2001.
170. Zimmermann, N., N. E. King, J. Laporte, M. Yang, A. Mishra, S. M. Pope, E. E. Muntel, D. P. Witte, A. A. Pegg, P. S. Foster, Q. Hamid, and M. E. Rothenberg. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J Clin Invest* 111(12):1863-74, 2003.
171. Zweier, J. L., A. Samouilov, and P. Kuppusamy. Non-enzymatic nitric oxide synthesis in biological systems. *Biochim Biophys Acta* 1411(2-3):250-62., 1999.

CHAPTER 3

Nitric oxide represses inhibitory κB kinase through S-nitrosylation

ABSTRACT

Nitric oxide (NO) possesses antiinflammatory effects, which may be exerted via its ability to inhibit the transcription factor, NF-κB. A commonly proposed mode of action for inhibition of NF-κB by NO involves interference with NF-κB binding to DNA. Because activation of Inhibitory kappa B kinase (IKK), the prerequisite enzyme complex necessary to induce NF-κB, is subject to redox regulation, we assessed whether IKK could present a more proximal target for NO to inhibit NF-κB activation. We demonstrate here that S-nitrosothiols (SNO) caused a dose-dependent inhibition of the enzymatic activity of IKK, in lung epithelial cells and in Jurkat T cells, which was associated with S-nitrosylation of the IKK complex. Using biotin derivatization of SNO, we revealed that IKKβ, the catalytic subunit required for NF-κB activation, was a direct target for S-nitrosylation. A mutant version of IKKβ containing a Cys-179-to-Ala mutation was refractory to inhibition by SNO or to increases in S-nitrosylation, in contrast to wild-type IKKβ, demonstrating that Cys-179 is the main target for attack by SNO. Importantly, inhibition of NO synthase activity in Jurkat T cells resulted in activation of IKK, in association with its denitrosylation. Moreover, NO synthase inhibition enhanced the ability of tumor necrosis factor α to activate IKK, illustrating the importance of endogenous NO in regulating the extent of NF-κB activation by cytokines. Collectively, our findings demonstrate that IKKβ is an important target for the redox regulation of NF-κB by endogenous or exogenous NO, providing an additional mechanism for its antiinflammatory properties.

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INTRODUCTION

Nitric oxide (NO, nitrogen monoxide) is a pleiotropic short-lived free radical that participates in diverse biological processes such as the regulation of vessel and airway tone, inflammation, neurotransmission and apoptosis. Although interactions with heme groups (such as in guanylyl cyclase) are the most recognized events associated with the signaling activities of NO, it is increasingly becoming appreciated that nitrosylation of protein sulfhydryl groups represents an important NO-dependent post-translational modification that impinges upon signal transduction cascades (1). Numerous proteins have been identified as targets for S-nitrosylation, including H-Ras (2), caspases (3) c-Jun-N-terminal kinase (JNK) (4), and ornithine decarboxylase (5), among others. In fact, the inhibition of JNK by NO was recently described as a potential antiinflammatory mechanism (4).

NF-κB is a transcription factor that plays a pivotal role in inflammation, cell survival and proliferation. NF-κB is maintained in a latent form in the cytoplasm via sequestration by inhibitory κB (IκB) proteins. NF-κB activating stimuli cause the inducible degradation of IκB proteins, unmasking the nuclear localization signal of NF-κB, resulting in its nuclear translocation, binding to NF-κB motifs and activation of gene transcription (6, 7). The enzyme complex responsible for phosphorylation of IκB on specific serine residues is IκB kinase (IKK) which consists of at least 3 subunits: IKKα, IKKβ and IKKγ. Although IKKα and IKKβ are both catalytically active, studies in knockout mice have demonstrated that IKKβ is responsible for degradation of IκB in response to many, but not all, signals (6, 8). In contrast, IKKα plays an important role in transcriptional activation of NF-κB responsive genes by phosphorylating histone H3 (9, 10). IKKγ is the regulatory subunit responsible for stabilizing the IKK complex and allowing interaction with upstream regulatory proteins (11).

Because the activation of IKK is essential to induce NF-κB, IKK would also be an ideal target for negative regulation in order to prevent the activation of NF-κB. Indeed, arsenite (12), cyclopentenone prostaglandins (13), hydrogen peroxide (14), and 4-hydroxy-2-nonenal (15) are all capable of inhibiting IKKβ via targeting of cysteine residue(s) of IKKβ, resulting in a failure to activate NF-κB. Numerous studies have reported that NO is capable of modulating the activation of NF-κB (for review, see

ref. 16). The inhibitory effect of NO on NF-κB is believed to play an important role in negative feed back regulation of NO production. The NOS2 gene promoter contains NF-κB regulatory sequences required for maximal gene activation (17, 18) and inhibition of NF-κB therefore decreases NOS2 gene activation (19), decreasing further production of NO. Multiple mechanisms have been described by which NO inhibits NF-κB. For instance, NO has been demonstrated to stabilize IκB (20, 21), induce IκBα mRNA (20) and prevent nuclear translocation of NF-κB (22). Recent emphasis has been focused on S-nitrosylation of Cys-62 of the p50 subunit, which is known to inhibit the ability of NF-κB to bind DNA (23-25).

Despite the current knowledge that the IKK complex is an important regulatory step in the activation of NF-κB by many stimuli (6, 13, 26), and its known sensitivity to redox stress (12, 14, 15), it is unknown whether IKK represents a direct target for inactivation by NO. If NO indeed is an important regulatory molecule in preventing the activation of NF-κB under (patho)physiological conditions, it is plausible that it might inhibit the NF-κB cascade upstream of the degradation of IκB, in addition to inhibiting the NF-κB DNA binding activity, providing more powerful means to prevent NF-κB activation. Therefore, the studies described herein were undertaken to assess whether IKK represents a direct target for S-nitrosylation and inactivation by NO.

MATERIALS AND METHODS

Cell Culture and Reagents. A line of spontaneously transformed mouse alveolar type II epithelial cells (C10) (27) was propagated in cell culture media-1066 medium containing 50 units/ml penicilline and 50 μg/ml streptomycin (P/S), 2 mM L-glutamine and 10% FBS, all from GIBCO/BRL. Jurkat T cells were cultured in DMEM high-glucose medium, supplemented with P/S and 10% FBS. At least 1 h before adding the test agents, the cells were switched to phenol red-free DMEM/F12 containing P/S and 0.5% FBS, except for incubations with N^G-monomethyl-L-arginine (L-NMMA). Tumor necrosis factor (TNF)α, L-NMMA, S-nitrosoglutathione (GSNO), S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and S-nitrosocysteine antibody were purchased from Calbiochem. The JNK1 and IKKβ and γ antibodies were purchased from Santa Cruz Biotechnology, the IκBα antibody from Cell Signaling

Technology (Beverly, MA), the phospho-IκBα antibody was purchased from BD Biosciences (Bedford, MA), the phosphoserine antibody was purchased from Zymed and clasto lactacystin β-lactone was purchased from Sigma (St.Louis, MO). CSNO was prepared fresh before every experiment, as described in ref. 28.

Kinase Assays. Cells were exposed to test agents and, at indicated times, transferred to ice, washed twice with cold PBS, and lysed in buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 10 μg/ml leupeptin, 1% aprotinin, 250 μM DTT and 100 μM NaF (14). Lysates were cleared by centrifugation at 16,000 x g for 10 min at 4°C. Protein concentrations were determined, and the IKK complex was immunoprecipitated with an IKKγ antibody (Santa Cruz Biotechnology) at 4°C for 1.5 h by using protein G agarose beads. Precipitates were washed once with lysis buffer and twice with kinase buffer (20 mM HEPES/ 20 mM β-glycerolphosphate/ 1 mM $MnCl_2$/ 5 mM $MgCl_2$/ 2 mM NaF/ 250 μM DTT). The kinase reaction was performed by using 1 μg of GST-IκBα as a substrate, provided by Rosa Ten (Mayo Clinic, Rochester, MN), and 5 μCi (1 Ci= 37 GBq) of [γ^{32}P]adenosine triphosphate at 30°C for 30 min. Kinase assays were performed in presence of 250 μM DTT, the minimal concentration necessary to maintain maximal TNFα-stimulated activity (14). Reactions were stopped by the addition of 2x Laemmli sample buffer. Samples were boiled and separated on 15% polyacrylamide gel, and gels were dried and examined by autoradiography. In separate experiments, the immunoprecipitated IKK complex or JNK1 from TNFα-stimulated cells was exposed to SNAP or GSNO for 15 min in lysis buffer *in vitro* before assessment of kinase activity. The kinase reaction for JNK was performed by using 1 μg GST-c-Jun as a substrate.

Transfection. C10 cells were transfected (Lipofectamine Plus, Invitrogen) by using 2 μg of plasmid [hemeagglutinin-tagged IKKβ wild type (wt HA-IKKβ) or HA-tagged IKKβ C179A; gifts of Michael Karin, University of California at San Diego, La Jolla], for 3 h, washed, and used in experiments 24 h later. The transfection efficiency using this procedure approximates 30% (data not shown). No effects of empty vector were observed.

Detection of S-nitrosylation using biotin derivatization coupled to western blotting. Detection of S-nitrosylated proteins was performed via

the biotin switch method (29) with the following modifications. After treatments, cells were rinsed two times with PBS containing 0.1 mM EDTA and 0.01 mM neocuproine and lysed in HEN buffer (25 mM HEPES, pH 7.7/ 0.1 mM EDTA/ 0.01 mM neocuproine) containing 0.5% 3-[(3-cholamididopropyl)dimethylammonio]-1-propanesulfonate, 0.1% SDS and 20 mM *N*-ethylmaleimide (NEM) at 4°C for 30 min to block free thiols. Lysates were cleared by centrifugation at 16,000 x g for 10 min at 4°C, and excess NEM was removed by protein precipitation by using cold acetone. Protein pellets were resuspended in HENS buffer (HEN 1% SDS), SNO bonds were decomposed by adding 20 mM sodium ascorbate and the resulting free thiols were reacted with 0.05 mM sulfhydryl-specific biotinylating agent, *N*-(3-malemidylpropionyl)biocytin (MPB, Molecular Probes) for 30 min at room temperature (RT), resulting in biotinylation of SNO. After removal of excess MPB by another protein precipitation using acetone, IKK was immunoprecipitated by using IKKβ or HA (12CA5, Roche Diagnostics) antibodies. Immunoprecipitates were washed three times with HEN buffer and resuspended in 50 μl of HEN containing Laemmli sample buffer, boiled at 95°C for 5 min, loaded on 10% acrylamide gels, and transferred to nitrocellulose. Biotinylated IKKβ was detected on the membrane by using horseradish peroxidase-linked streptavidin. To confirm equal amounts of IKKβ, biotinylated lysates were also subjected to Western blotting for IKKβ or HA. To confirm the specificity of SNO labeling, addition of MPB or reduction by ascorbate was omitted in some samples. All procedures until biotinylation were performed in the dark.

Western Blots. A fraction of the lysates used for *in vitro* kinase assays, biotin derivatization or chemiluminescence was mixed with 2x Laemmli sample buffer, and samples were boiled and loaded on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and membranes blocked in 5% milk in Tris-buffered saline (TBS). After two washes in TBS containing 0.05% Tween 20 (TBS-Tween), membranes were incubated with primary antibodies against HA, IKKγ, IKKβ, JNK1, IκBα, phospho-IκBα, or phosphoserine for 1h at RT. Membranes were washed three times for 20 min in TBS-Tween, and incubated with a peroxidase-conjugated secondary antibody for 1h at RT. After three 15-min washes with TBS-Tween, conjugated peroxidase was detected by chemiluminescence according to the manufacturer's instructions (Kirkegaard & Perry Laboratories).

Measurement of SNO content by chemiluminescence. The total cellular SNO concentration (protein-bound plus free) was measured in lysates of cells treated with SNO in the presence or absence of L-or D-cys. After three washes with PBS, cells were lysed in the same buffer as was used for the biotin switch method, nitrate was quenched with 0.6% sulfanilamide in 1 M HCl for 10 min at RT, and samples were injected into 5 ml of a solution of 45 mM KI and 10 mM I_2 in glacial acetic acid at 60°C, contained within a purge vessel and connected to a NO chemiluminescence analyzer (Ionics, Boulder, CO) (30). The amount of NO released from samples was estimated from a standard curve generated by injection of L-CSNO stock solutions. IKKβ was immunoprecipitated from Jurkat T cell lysates by using a monoclonal IKKβ antibody and protein G agarose beads. After washing the immunoprecipitates three times with HEN buffer (25 mM Hepes, pH 7.7/ 0.1 mM EDTA/ 0.01 mM neocuproine) containing 50 mM NaCl to minimize coassociating proteins, antigen-antibody complexes were removed from the beads by three 10-min incubations in 50 μl of 100 mM glycine, pH 3.0 at 4°C. The eluates were treated with 0.6% sulfanilamide before the assessment of the SNO content via chemiluminescence, as described. As a control, some lysates or immunoprecipitates were treated with 4.4 mM $HgCl_2$ for 10 min at RT, followed by 20-min incubation at 4ºC and 10-min incubation with sulfanilamide at RT. To confirm that IKKβ was the predominant protein immunoprecipitated under these conditions, Laemmli sample buffer was added to the immunoprecipitate, and samples were boiled and evaluated on a silver-stained gel. All experiments were repeated at least two times, and similar results were obtained.

RESULTS

***In Vitro* effects of SNO on the enzymatic activity of IKK.** We first determined whether SNAP or GSNO were capable of inactivating isolated active IKK. For that purpose, C10 cells were stimulated with TNFα for 5 min to induce maximal enzymatic activity (14). The IKK complex was then immunoprecipitated from cell lysates by using an IKKγ antibody and exposed to SNAP or GSNO for 15 min at concentrations ranging from 100 μM to1 mM before the kinase reaction. Both SNAP (Fig. 1*A*) and GSNO (not shown) caused a dose-dependent decrease in IKK enzymatic activity. For comparison, we verified whether these SNO also were capable of inhibiting the activity of JNK, another serine-directed kinase that was

recently demonstrated to be sensitive to inhibition by NO (4). In contrast to these previous observations, results in Fig.1*B* demonstrate that concentrations of SNAP or GSNO up to 1 mM failed to inhibit JNK enzymatic activity. These results demonstrate that SNO inhibit the enzymatic activity of IKK, but not JNK *in vitro*.

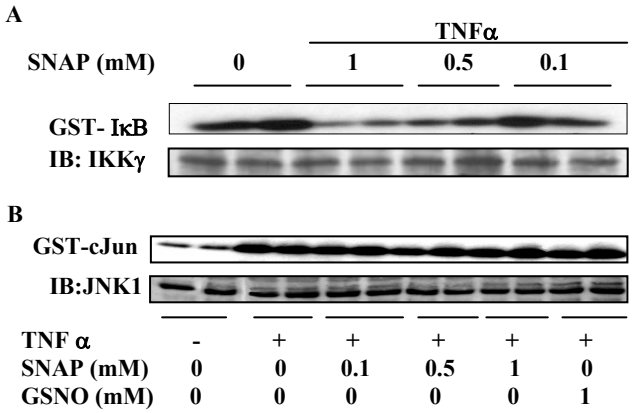

Figure 1 Inactivation of IKK but not JNK by SNAP *in vitro*. (*A*) C10 cells were exposed to 10 ng/ml TNFα for 5 min to induce IKK activity. Immunoprecipiated IKK was then exposed to indicated concentrations of SNAP for 15 min, and an *in vitro* kinase assay was performed by using GST-IκB as a substrate. IB, Western blotting for IKKγ. (*B*) C10 cells were treated with 10 ng/ml TNFα for 15 min to activate JNK, and JNK1 was immunoprecipitated from lysates. After a 15-min exposure to the indicated concentrations of SNAP or GSNO, an *in vitro* kinase was performed using GST-c-Jun as a substrate. IB, Western blotting for JNK1.

Inhibition of IKK by SNO in intact cells. We next established whether SNO are also capable of inhibiting IKK activity in intact cells. TNFα-induced IKK activity was not inhibited after administration of SNAP or GSNO alone, at the doses and time point selected, but was markedly inhibited in intact C10 (Fig. 2*A Left*) and Jurkat T cells (data not shown) in presence of L-cys, whereas L-cys itself had no effect. On the other hand, L-CSNO inhibited IKK activity in the absence of extra L-cys in Jurkat T cells (Fig. 2*A Rightl*) and C10 cells (data not shown). In contrast, D-CSNO or GSNO/D-cys failed to inhibit IKK activity (Fig. 2*B*). Although exposure to GSNO increased the SNO content in TNFα–stimulated cells (TNFα: 0.19 ± 0.01 pmol/μg protein; TNFα + GSNO: 5.78 ± 0.05 pmol/μg protein) this increase was more pronounced in the presence of L-cys (13.65 ± 0.82 pmol/μg protein), but not in the presence of D-cys (8.36 ± 0.66 pmol/μg protein). Collectively, these

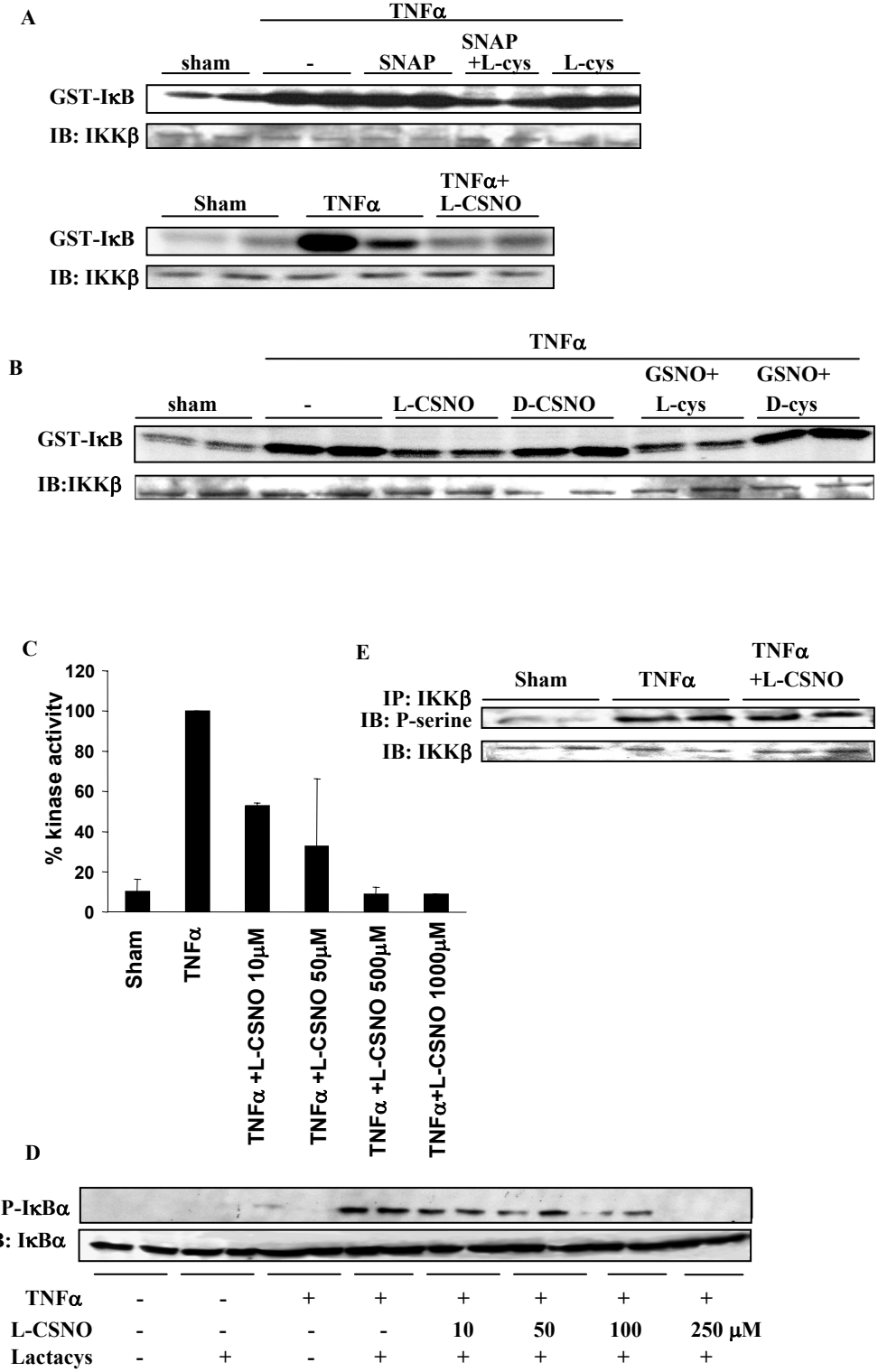
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Figure 2 Repression of IKK activity in intact cells by exposure to SNO. (*A. Leftl*) C10 cells were treated with 1 mM SNAP in presence or absence of 1 mM L-cys for 15 min before exposure to 10 ng/ml TNFα for 5 min, and an *in vitro* kinase assay was performed. IB, Western blot of IKKβ. (*Rightl*) Jurkat T cells were treated with 500 μM L-CSNO for 30 min and subsequently with 10 ng/ml TNFα for 10 min, and an *in vitro* kinase assay was performed. (*B*) C10 cells were treated with 250 μM L- or D-CSNO, or 500 μM GSNO in presence of 500 μM of L-or D-cys for 15 min, before stimulation with 10 ng/ml TNFα for 5 min. The activity of IKK was assessed in an *in vitro* kinase assay. IB, Western blot of IKKβ. (*C*) C10 cells were treated with indicated concentrations of L-CSNO for 15 min and subsequently with 10 ng/ml TNFα for 5 min. The activity of IKK was assessed in an *in vitro* kinase assay. Results were quantified by phospho-image analysis and expressed as the percent kinase activity compared with TNFα–only-treated cells. (*D*) C10 cells were incubated with 2.5 μM clasto lactacystin β-lactone for 30 min to block proteasomal degradation of proteins and then exposed to L-CSNO for 15 min, followed by a 5-min incubation with 10 ng/ml TNFα. The amount of phosphorylated IκBα (*p*-IκBα, *Upper*) and total IκBα (IκBα, *Lower*) was assessed by Western blotting. (*E*) C10 cells were treated with 250 μM L-CSNO for 15 min and subsequently with 10 ng/ml TNFα for 5 min. IKKβ was immunoprecipitated from lysates, and phosphoserine content was assessed by Western blot by using a phosphoserine antibody. IB, Western blot of IKKβ.

findings indicate that L-cys mainly facilitates the cellular uptake of NO from SNAP or GSNO through intermediate formation of L-CSNO, thereby promoting inhibition of IKK. Dose-response analyses revealed that inhibition of TNFα-induced activation of IKK (Fig. 2*C*) or phosphorylation of IκBα (Fig. 2*D*) was detectable at concentrations of L-CSNO as low as 10 μM, which are believed to reflect patho-physiological amounts of extracellular SNO (31).

The possibility exists that SNO inhibit IKK activity by interfering with its phosphorylation, which is required for kinase activity. Results in Fig. 2*E* demonstrate that exposure of cells to SNO did not interfere with TNFα-induced serine phosphorylation of IKKβ, suggesting that SNO do not act upstream in the activation pathway of IKK but rather inhibit the phosphorylated enzyme directly, consistent with our findings in Fig.1*A* demonstrating that SNO can readily inhibit active IKK *in vitro*.

IKKβ is a target for S-nitrosylation. Because the kinase activity of the β subunit of the IKK complex is responsible for phosphorylation of IκBα in response to TNFα, and this was inhibited upon treatment with SNO (Figs. 1*A* and 2), we next wanted to assess whether IKKβ represents a direct

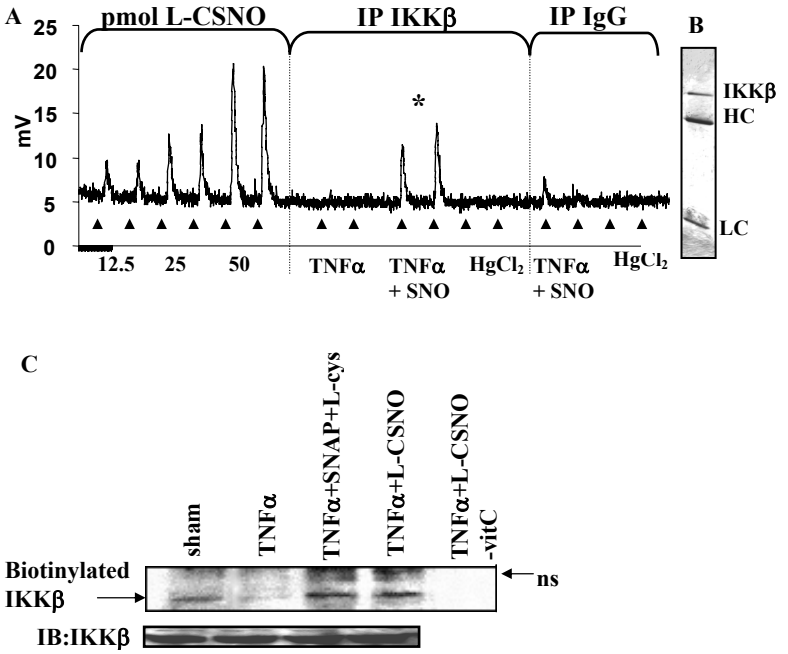

Figure 3 S-nitrosylation of IKKβ. (*A*) Jurkat T cells were treated with 1 mM L-CSNO for 30 min and subsequently with TNFα for 10 min. IKKβ was immunoprecipitated from lysates containing 2.2 mg of protein by using an IKKβ antibody. A control immunoprecipitation was performed by using an isotype-matched Ig (IgG). After immunoprecipitation, selected samples were treated with $HgCl_2$, and all samples were treated with sulfanilamide to ensure specificity. S-nitrosylation of IKKβ was assessed by chemiluminescence. * $p < 0.05$ (Student's *t* test) compared with NO signal obtained in the TNFα + L-CSNO subjected to immunoprecipitation with the IgG control antibody. (*B*) IKKβ was immunoprecipitated from untreated Jurkat T cells, samples boiled in sample buffer, separated on SDS/PAGE gel, and silver stained. The location of IKKβ is indicated. HC, antibody heavy chain; LC, antibody light chain. (*C*) Cells were treated as in *A*, and lysates were subjected to biotin derivatization. Biotinylation of IKKβ was detected after immunoprecipitation of the IKKβ-containing complex and Western blotting using streptavidin-horseradish peroxidase. In control samples, reduction by ascorbate (- vitC) was omitted. IB, anti-IKKβ immunoblot; ns, non-specific reactivity.

target for S-nitrosylation. For this purpose, we immunopurified IKKβ from TNFα-stimulated Jurkat T cells treated with SNO and performed chemiluminescence analysis to determine the SNO content of IKKβ. While we could not detect a SNO signal from IKKβ immunopurified from lysates of untreated cells (data not shown) or cells stimulated with TNFα (Fig. 3*A*), IKKβ obtained from cells exposed to L-CSNO and TNFα demonstrated a marked increase in SNO content. The observed chemiluminescence signal was not due to contaminating nitrite, because samples were treated with sulfanilamide to quench nitrite. In addition, the chemiluminescence signal was completely ablated after $HgCl_2$ treatment, indicating that the measured NO is derived from SNO within the immunopurified protein. Control immunoprecipitations with equal amounts of an isotype-matched control antibody resulted in a barely detectable signal. Analysis of the immunoprecipitate by silver staining (Fig. 3*B*) revealed that IKKβ was the major detectable protein under the conditions used here, confirming that the measured NO was likely derived from SNO bonds in IKKβ and not from other coimmunoprecipitated proteins.

Alternatively, we employed the biotin derivatization method to assess whether the IKKβ subunit is directly targeted by S-nitrosylation. For this purpose, Jurkat T cells were treated as described, and cell lysates were derivatized in order to selectively biotinylate SNO moieties. IKKβ was subsequently immunoprecipitated from the lysates, and its biotinylation was assessed by using streptavidin-horseradish peroxidase on a Western blot. Results in Fig. 3*C* demonstrate detectable endogenous S-nitrosylation of IKKβ in untreated cells, which was decreased in response to TNFα. Furthermore, SNAP/L-cys or L-CSNO caused an increase in S-nitrosylation of IKKβ. Biotin labeling was absent when ascorbate was omitted in the derivatization procedure, showing the specificity of biotinylation for detection of SNO.

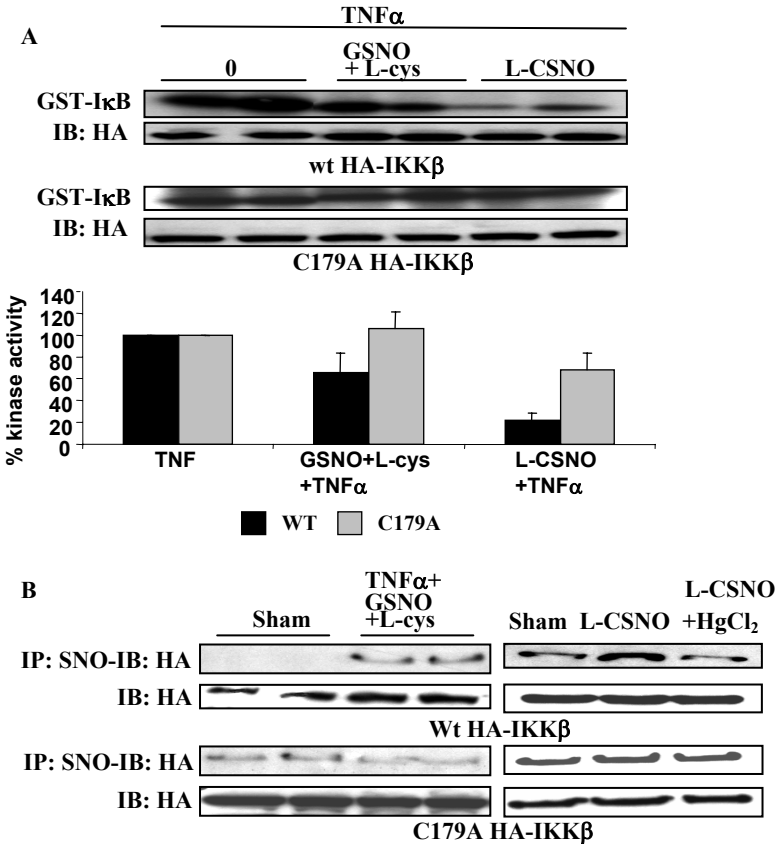

Figure 4 Cys-179 of IKKβ is target for S-nitrosylation. (*A*) C10 cells were transfected with wt or C179A HA-IKKβ, treated with 1 mM GSNO/L-cys or L-CSNO for 15 min, before exposure to 10 ng/ml TNFα for 5 min. IKK activity was assessed in an *in vitro* kinase assay, after immunoprecipitation with an HA antibody. IB; anti-HA immunoblot. (*Bottom*) Quantitation by phosphoimage analysis. Results are expressed as percentage of IKK activity compared with TNFα-only treated cells. (*B Left*) wt (*Upper*) or C179A HA-IKKβ transfected C10 cells (*Lower*) were treated with 1 mM GSNO/L-cys for 15 min before exposure to 10 ng/ml TNFα for 5 min. S-nitrosylated proteins were immunoprecipitated, by using a S-nitrosocysteine antibody (IP SNO) and IKKβ detected by detection of HA by Western blotting. (*Lower*) HA Western blots on total cell lysates. (*B Rights*) Assessment of specificity of the S-nitrosocysteine antibody. wt (*Upper*) or C179AHA-IKKβ transfected cells (*Lower*) were left untreated (left lane), treated with 1 mM L-CSNO for 15 min (middle lane) or treated with 1 mM L-CSNO for 15 min followed by incubation with $HgCl_2$ (right lane) before immunoprecipitation. S-nitrosylated proteins were then immunoprecipitated by using a S-nitrosocysteine antibody (IP: SNO) and IKKβ detected by Western blotting for HA; (*Lower*) HA Western blots on total cell lysates.

Cysteine residue 179 of IKKβ is a target for S-nitrosylation. Cys-179 is located in between Ser-177 and -181, which are required for activation of IKKβ by TNFα. Because it has been shown that Cys-179 is a target for inhibition by arsenite (12), we wanted to investigate if this residue was specifically targeted by S-nitrosylation. For this purpose, wt HA-IKKβ or Cys-179-to-Ala mutated HA-tagged IKKβ (C179A HA-IKKβ) were transfected into C10 cells, which were then treated with SNO to assess the extent of inhibition of TNFα-stimulated enzymatic activity. As is apparent from Fig. 4*A,* both GSNO/L-cys as well as L-CSNO inhibited the activity of wt HA-IKKβ. In contrast, C179A HA-IKKβ was largely refractory to inhibition by SNO. Accordingly, treatment of cells with GSNO/L-cys increased S-nitrosylation of wt HA-IKKβ but not of C179A HA-IKKβ, as revealed by immunoprecipitation using an antibody directed against SNO and Western blotting for HA (Fig. 4*B Left*). The selectivity of the SNO antibody was demonstrated by incubating lysates of L-CSNO treated cells with $HgCl_2$ before immunoprecipitation with the SNO antibody, which resulted in a markedly lower amount of wt HA-IKKβ recovered by immunoprecipitation (Fig. 4*B Right Upper*), whereas $HgCl_2$ did not affect the amount of C179A HA-IKKβ immunoprecipitated with SNO antibody (Fig. 4*B Right Lower*), illustrating some nonspecific reactivity of this antibody. Collectively, these data demonstrate that Cys-179 of IKKβ is a major target for S-nitrosylation and inhibition by SNO.

Repression of IKK enzymatic activity by endogenous NOS activity. If NO is an important negative regulator of IKK in intact cells, inhibition of endogenous NOS activity might relieve this repression, resulting in activation of IKK or enhanced IKK activation by TNFα. To address this question, Jurkat T cells were incubated with 1 mM of the NOS inhibitor, L-NMMA. Results in Fig. 5*A* demonstrate that 4 h after addition of L-NMMA, IKK activity was markedly enhanced. Furthermore, L-NMMA also potentiated the ability of TNFα to induce IKK activity. Consistent with these findings, inhibition of NOS caused a decrease in SNO-dependent biotinylation of IKKβ, indicating a lesser extent of S-nitrosylation. As described above, omission of ascorbate or the biotin label markedly attenuated biotin reactivity in the sham sample, indicating specific detection of S-nitrosothiols. These results illustrate that endogenous NOS activity is an important repressor of IKK activity in Jurkat T cells.

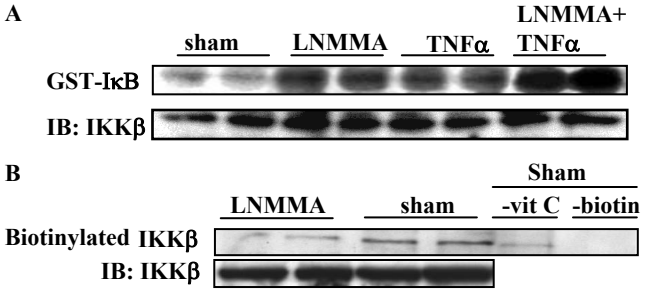

Figure 5 Repression of IKK activity in intact cells by endogenous NOS activity. Jurkat T cells were treated with 1 mM L-NMMA for 4 h followed by stimulation with 10 ng/ml TNFα for 10 min or mock manipulations. Selected dishes were treated with TNFα alone. (*A*) IKK activity was assessed in an *in vitro* kinase assay. IB, anti-IKKβ immunoblot. (*B*) Lysates were subjected to biotin derivatization, and biotinylated IKKβ was detected after immunoprecipitation of IKKβ and Western blotting using streptavidin-horseradish peroxidase. In control samples, reduction by ascorbate (-vit C) or labeling with N-(3-malemidylpropionyl)biocytin (-biotin) was omitted. IB, anti-IKKβ immunoblot.

DISCUSSION

In the present study, we demonstrate that IKKβ is a direct target for S-nitrosylation. This repression of the NF-κB pathway proximal to DNA binding ensures adequate inhibition of NF-κB activation by NO. The inhibitory action of SNO is not general to all serine-directed kinases. When we evaluated the inhibitory action of SNO on JNK, reported to be inhibited by S-nitrosylation (4), we failed to observe inhibitory effects of SNO. The discrepancy with published work and our observations is puzzling and may be related to the differences in cell types and species investigated. Furthermore, the previous study used anisomycin or UV to activate JNK (4), whereas in the current study, we utilized TNFα. Nonetheless, our work suggests that the antiinflammatory role of NO previously attributed to inhibition of JNK (4) may also be due to its ability to inhibit IKK.

In cells exposed to SNO, a transnitrosation reaction, i.e., the transfer of NO^+ from the SNO to IKK, likely is responsible for the decrease in kinase activity. This explains the relative lack of inactivation of IKK in C10 lung epithelial cells exposed to SNAP and GSNO alone, which cannot readily enter the intact cells (Fig. 2*A* and *B*). In contrast, coincubation of SNAP or GSNO with L-cys results in a transnitrosation reaction to form L-CSNO

(28, 32), which is taken up by cells via the L-cys amino acid transport system (33). The transnitrosation mechanism is supported by our observations demonstrating that in the presence of L-cys, but not D-cys, the SNO content of cells exposed to GSNO increased, consistent with inactivation of IKK. Furthermore, direct administration of L-CSNO, but not D-CSNO, inhibited IKK enzymatic activity in intact cells. Although cys may convert S-nitrosothiols to NO extracellularly and thereby promote the formation of N_2O_3, which would diffuse into the cells and result in S-nitrosylation (34), the observed stereoselective effects of cys argue against such a mechanism, and illustrate the importance of cellular SNO uptake in IKK inhibition.

The activity of the IKK complex is markedly inhibited in intact cells by concentrations of SNO as low as 10 μM. Levels of SNO up to 1 μM have been measured in exhaled breath condensate of normal individuals (31). Furthermore, a number of disease states, like pneumonia (35) and inflammatory lung disease (36), have been reported to be accompanied by increased amounts of SNO. The doses of SNO used to detect S-nitrosylation of IKK were higher, likely due to limits of detection imposed by the assays that were used. Importantly, our studies in Jurkat T cells point to a crucial role of endogenous NO in repressing baseline IKK activation and in regulating the magnitude of IKK activation by cytokines. Consequently, inhibition of NOS activity is sufficient to activate IKK in Jurkat T cells. However, incubation of C10 cells with L-NMMA failed to influence IKK enzymatic activity (data not shown), consistent with a recent observation demonstrating that the mode of inhibition of NF-κB by NO is cell type dependent (37). Nonetheless, our observations may have important ramifications in inflammatory conditions were the concentrations of NO and the sources of its production are known to be altered. The consumption of NO by peroxidases (38) may lower the concentrations of bioavailable NO and thereby minimize its ability to repress IKK, resulting in chronic activation of NF-κB, which accompanies many inflammatory diseases. Alternatively, direct metabolism of SNO by a recently identified GSNO reductase, which is conserved from bacteria to humans, may lower bioavailable SNO (39). In support of the latter, increased activity of GSNO reductase has recently been demonstrated in allergic airways inflammation (40).

Structural factors that influence the susceptibility to S-nitrosylation include neighboring or surrounding amino acids that affect cysteine reactivity and the presence of a hydrophobic environment that promotes

the formation of S-nitrosylating species via the reaction between O_2 and NO (34). This 'motif' can be apparent from the primary structure of the protein but also as a result of its 3D conformation or protein-protein interactions (41). This makes it difficult to predict the cysteines in IKKβ subject to S-nitrosylation, given that its structure has not been solved. Additionally, the organization of the IKK complex is highly complex and consists of many protein subunits that can associate and dissociate, including Hsp90 and Cdc37 (42, 43). It is furthermore of importance to consider that endothelial NOS associates with HSP90 (44), thereby directly localizing an endogenous source of NO to the IKK complex.

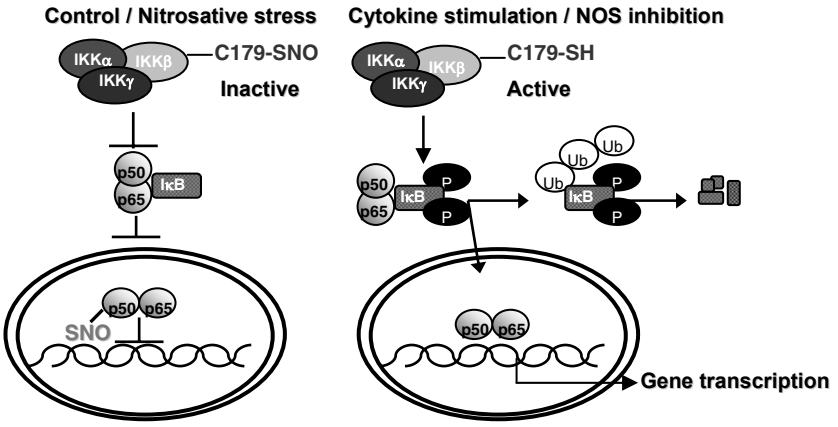

Figure 6 A model whereby *S*-nitrosylation of inhibitory κB kinase (IKK)β at Cys-179 inhibits the NF-κ B pathway in Jurkat T cells. Under baseline conditions, *S*-nitrosylation of IKKβprevents NF-κ B activation (*Left*). Consequently, after NO synthase (NOS) inhibition IKKβ becomes denitrosylated, which promotes the activation of IKK and NF-κB and enhances the ability of tumor necrosis factor (TNF)α to activate IKKβ (*Right*). Under conditions of nitrosative stress, *S*-nitrosothiols can inhibit cytokine-stimulated IKKβ activation, providing a potent feedback regulatory mechanism by which the NF-κB pathway is repressed, proximally to the degradation of inhibitory κB (IκB)α. Although not addressed in this paper, it should be noted that *S*-nitrosylation of p50, the DNA-binding subunit of NF-κ B, also interferes with the ability of NF-κB to bind DNA (*Left Bottom*), illustrating that multiple sites of the NF-κB pathway are regulated by *S*-nitrosylation.

Although Cys-179 is not an apparent target for S-nitrosylation based upon its primary sequence (41), our data demonstrate that this residue is the main target for oxidative modification by SNO. We have shown that the C179A mutant is refractory to inhibition by SNO and that treatment with

SNO did not induce enhanced S-nitrosylation of this form of IKKβ. Cys-179 has previously been shown to be redox sensitive and to be oxidized by arsenite (12) and alkylated by cyclopentenone prostaglandins (13). Cys-179 is strategically located in between the 2 serines that are required for activation of IKK by TNFα. It is conceivable that the TNFα–induced phosphorylation of Ser-177 and -181, which creates a more negative charge surrounding Cys-179, promotes its susceptibility to trans-nitrosation. However, additional experiments are clearly needed to elucidate the interplay between phosphorylation and S-nitrosylation of IKKβ, as well as the mechanism by which S-nitrosylation impacts on IKK activity.

The identification of IKKβ as a target for S-nitrosylation provides new insights into the mechanisms of inhibition of NF-κB by NO, and a proposed model of this mechanism is presented (Fig.6, which is published as supporting information on the PNAS web site). These observations may provide strategies aimed at enhancing the inhibitory effect of NO at a level before DNA-binding activity, to augment its antiinflammatory mode of action.

ACKNOWLEDGEMENTS

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REFERENCES

1. Stamler, J. S., Lamas, S. & Fang, F. C. (2001) *Cell* 106, 675-83.
2. Mallis, R. J., Buss, J. E. & Thomas, J. A. (2001) *Biochem J* 355, 145-53.
3. Mannick, J. B., Hausladen, A., Liu, L., Hess, D. T., Zeng, M., Miao, Q. X., Kane, L. S., Gow, A. J. & Stamler, J. S. (1999) *Science* 284, 651-4.
4. Park, H. S., Huh, S. H., Kim, M. S., Lee, S. H. & Choi, E. J. (2000) *Proc Natl Acad Sci U S A* 97, 14382-7.
5. Bauer, P. M., Buga, G. M., Fukuto, J. M., Pegg, A. E. & Ignarro, L. J. (2001) *J Biol Chem* 276, 34458-64.
6. Karin, M. & Ben-Neriah, Y. (2000) *Annu Rev Immunol* 18, 621-63.
7. May, M. J. & Ghosh, S. (1998) *Immunol Today* 19, 80-8.
8. May, M. J. & Ghosh, S. (1999) *Science* 284, 271-3.
9. Anest, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D. & Baldwin, A. S. (2003) *Nature* 423, 659-63.
10. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T. & Gaynor, R. B. (2003) *Nature* 423, 655-9.
11. Rothwarf, D. M., Zandi, E., Natoli, G. & Karin, M. (1998) *Nature* 395, 297-300.
12. Kapahi, P., Takahashi, T., Natoli, G., Adams, S. R., Chen, Y., Tsien, R. Y. & Karin, M. (2000) *J Biol Chem* 275, 36062-6.
13. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M. & Santoro, M. G. (2000) *Nature* 403, 103-8.
14. Korn, S. H., Wouters, E. F., Vos, N. & Janssen-Heininger, Y. M. (2001) *J Biol Chem* 276, 35693-700.
15. Ji, C., Kozak, K. R. & Marnett, L. J. (2001) *J Biol Chem* 276, 18223-8.
16. Janssen-Heininger, Y. M., Poynter, M. E. & Baeuerle, P. A. (2000) *Free Radic Biol Med* 28, 1317-27.
17. Xie, Q. W., Kashiwabara, Y. & Nathan, C. (1994) *J Biol Chem* 269, 4705-8.
18. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W. & Murphy, W. J. (1993) *Proc Natl Acad Sci U S A* 90, 9730-4.
19. Griscavage, J. M., Wilk, S. & Ignarro, L. J. (1996) *Proc Natl Acad Sci U S A* 93, 3308-12.
20. Peng, H. B., Libby, P. & Liao, J. K. (1995) *J Biol Chem* 270, 14214-9.
21. Katsuyama, K., Shichiri, M., Marumo, F. & Hirata, Y. (1998) *Arterioscler Thromb Vasc Biol* 18, 1796-802.
22. Okamoto, T., Valacchi, G., Gohil, K., Akaike, T. & van der Vliet, A. (2002) *Am J Respir Cell Mol Biol* 27, 463-73.
23. Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R. & Hay, R. T. (1996) *Nucleic Acids Res* 24, 2236-42.
24. delaTorre, A., Schroeder, R. A., Bartlett, S. T. & Kuo, P. C. (1998) *Surgery* 124, 137-41; discussion 141-2.
25. Marshall, H. E. & Stamler, J. S. (2001) *Biochemistry* 40, 1688-93.
26. Rothwarf, D. M. & Karin, M. (1999) *Sci STKE* 1999, RE1.
27. Malkinson, A. M., Dwyer-Nield, L. D., Rice, P. L. & Dinsdale, D. (1997) *Toxicology* 123, 53-100.
28. Mallis, R. J. & Thomas, J. A. (2000) *Arch Biochem Biophys* 383, 60-9.
29. Jaffrey, S. R. & Snyder, S. H. (2001) *Sci STKE* 2001, L1.
30. Feelisch, M., Rassaf, T., Mnaimneh, S., Singh, N., Bryan, N. S., Jourd'Heuil, D. & Kelm, M. (2002) *Faseb J* 16, 1775-85.

31. Csoma, Z., Bush, A., Wilson, N. M., Donnelly, L., Balint, B., Barnes, P. J. & Kharitonov, S. A. (2003) *Chest* 124, 633-8.
32. Padgett, C. M. & Whorton, A. R. (1997) *Am J Physiol* 272, C99-108.
33. Nemoto, T., Shimma, N., Horie, S., Saito, T., Okuma, Y., Nomura, Y. & Murayama, T. (2003) *Eur J Pharmacol* 458, 17-24.
34. Hogg, N. (2002) *Annu Rev Pharmacol Toxicol* 42, 585-600.
35. Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J. & et al. (1993) *Proc Natl Acad Sci U S A* 90, 10957-61.
36. Corradi, M., Montuschi, P., Donnelly, L. E., Pesci, A., Kharitonov, S. A. & Barnes, P. J. (2001) *Am J Respir Crit Care Med* 163, 854-8.
37. Marshall, H. E. & Stamler, J. S. (2002) *J Biol Chem* 277, 34223-8.
38. Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W., Zhang, C., Tousson, A., Castro, L., Lusis, A. J., Nauseef, W. M., White, C. R. & Freeman, B. A. (2002) *Science* 296, 2391-4.
39. Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J. & Stamler, J. S. (2001) *Nature* 410, 490-4.
40. Fang, K., Johns, R., Macdonald, T., Kinter, M. & Gaston, B. (2000) *Am J Physiol Lung Cell Mol Physiol* 279, L716-21.
41. Hess, D. T., Matsumoto, A., Nudelman, R. & Stamler, J. S. (2001) *Nat Cell Biol* 3, E46-9.
42. Chen, G., Cao, P. & Goeddel, D. V. (2002) *Mol Cell* 9, 401-10.
43. Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A. M., Schirle, M., Schlegl, J., Schwab, M., Stein, M. A., Bauer, A., Casari, G., Drewes, G., Gavin, A. C., Jackson, D. B., Joberty, G., Neubauer, G., Rick, J., Kuster, B. & Superti-Furga, G. (2004) *Nat Cell Biol* 6, 97-105.
44. Gratton, J. P., Fontana, J., O'Connor, D. S., Garcia-Cardena, G., McCabe, T. J. & Sessa, W. C. (2000) *J Biol Chem* 275, 22268-72.

CHAPTER 4

In situ detection and visualization of S-nitrosylated proteins following chemical derivatization: identification of Ran GTPase as a target for S-nitrosylation

ABSTRACT

The formation of S-nitrosylated proteins is a nitric oxide-dependent post-translational modification important in signal transduction, yet the *in situ* detection of S-nitrosylated proteins remains problematic. In this study, we adapted a recently developed biotin derivatization approach to visualize S-nitrosylated proteins in intact cells. This strategy circumvents the use of antibodies directed against S-nitrosocysteine, which may have problematic specificity, due to epitope instability. Endogenous protein S-nitrosylation could be observed in intact cells and in mouse lung sections using fluorophore-conjugated streptavidin and confocal microscopy, and was enhanced by S-nitrosothiols and reduced following treatment with the nitric oxide synthase inhibitor, L-*N*-monomethyl arginine. Intriguingly, protein S-nitrosylation was detected mainly in the nuclear compartment of cells under baseline conditions and was enhanced when nuclear export was blocked with leptomycin B. We also determined that the small GTPase Ran, a key regulator of nucleocytoplasmic transport, is a target for S-nitrosylation. These findings demonstrate that biotin derivatization is a useful approach to detect S-nitrosylated proteins *in situ* in cellular compartments or tissues, and will be useful in the assessment of altered S-nitrosylation in pathological conditions.

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INTRODUCTION

Nitric oxide (NO) is an important signaling molecule, playing significant roles in physiology and pathophysiology [1]. The cellular responses to NO are transduced via multiple chemical reactions, including direct reactions with heme centers, metalloproteins, and indirect biochemical reactions after oxidative and other metabolism to various reactive nitrogen species (RNS). In particular, the reactions of RNS with protein cysteine thiols that result in [1]S-nitrosylation [2] have received a great deal of recent attention, because they represent an important post-translational modification that may transduce NO-dependent signals [3,4]. To date, a large group of proteins have been characterized as targets for S-nitrosylation, including metabolic, structural, cytoskeletal, ion channel, and signaling proteins, and S-nitrosylation in many cases is believed to regulate protein activity and function (reviewed in [3]). For example, S-nitrosylation has been shown to inhibit the activity of caspases [5] and denitrosylation is required for enzymatic activity of these enzymes. Similarly, S-nitrosylation of Inhibitory Kappa B Kinase (IKK) [6] and the p50 subunit of the transcription factor nuclear factor kappa B (NF-κB), are believed to be responsible for the NO-induced inhibition of NF-κB [7,8]**.** A recent study also demonstrated that S-nitrosylation of parkin inhibits its ubiquitin E3 ligase activity, and its protective function, providing a potential mechanism by which S-nitrosylation could contribute to neuro-degenerative processes [9].

Many of the proteins targeted by S-nitrosylation were originally identified using exogenous NO donors and it has not always been established whether S-nitrosylation of these proteins is associated with endogenous NOS activity. This is primarily due to the technical limitations in methodology to detect S-nitrosylated proteins in intact cells. Recently, Jaffrey et al. [10] described a novel approach to assess S-nitrosylation, using a selective biotin switch method.

[1]The attachment of NO to sulfhydryl groups in proteins is referred to as S-nitrosylation in this manuscript, whereas the attachment of NO to nucleophilic centers in general is called nitrosation. S-nitrosylation has emerged as the prototypic redox-based post-translational modification and has often been compared with phosphorylation. This analogy to other modes of signal transduction forms the main reason why the NO-dependent modification of sulfhydryl groups is called S-nitrosylation as opposed to S-nitrosation, which is the chemically correct term.

This method offers significant advantages in that biotinylated proteins can be detected on Western blots following incubation with anti-biotin antibodies or recognition via streptavidin. Moreover, biotinylated proteins can be captured on streptavidin matrixes for identification by mass spectrometry [10].
Although valuable information can be gained from the identification of S-nitrosylated proteins in cell lysates or tissue homogenates, the ability to visualize them in intact cells or tissues under physiological or pathological conditions will provide additional insights into the spatial relationship between S-nitrosylation patterns, NOS expression/activity, and pathophysiological alterations. Unfortunately, antibodies directed against S-nitrosocysteine may suffer from a lack of specificity, as the S-nitroso moiety is highly labile. Therefore, the objective of the present study was to determine whether biotin derivatization can be used in order to detect S-nitrosylated proteins *in situ*, and to visualize their subcellular localization in intact cells or tissues, using streptavidin-conjugated fluorophores and confocal microscopy. Using this approach, we are able to detect S-nitrosylation in intact lung epithelial cells or in lung tissues, and this was decreased after NOS inhibition and enhanced following incubation with S-nitrosothiols or NOS2 induction. Surprisingly, marked S-nitrosylation was observed in the nucleus, and this was enhanced after nuclear export was blocked. The small G protein, RanGTPase, an important regulator of nuclear trafficking, was found to be a direct target for S-nitrosylation, pointing to a potential role of S-nitrosylation in the regulation of nucleocytoplasmic transport.

EXPERIMENTAL PROCEDURES

Cell culture and exposure to test agents. A line of spontaneously transformed mouse lung alveolar type II epithelial cells (C10) was used in all experiments [11]. C10 cells were cultured in CMRL1066 medium containing 10% fetal bovine serum (FBS, Gibco). In the experiments involving microscopic analysis, the cells were grown on glass coverslips. One hour prior to exposure to test agents, the medium was switched to phenol red free DMEM/F12 containing 0.5% FBS. Cells were exposed to S-nitroso-glutathione (GSNO) or S-nitroso N-acetyl penicillamine (SNAP) for 1h, in presence of an equimolar concentration of freshly prepared L-cysteine which was added immediately after the S-nitrosothiols in order to promote the uptake of SNO [12, 6]. To monitor S-nitrosylation in response to NOS2 activation, cells were treated with 10 ng/mL TNFα and 100

U/mL IFNγ, for 24 h. Incubation with L-N-monomethyl arginine (L-NMMA) for 16 h was used to inhibit endogenous NO production and D-NMMA was used as a control. Nuclear export was blocked by leptomycin B (5 ng/mL, for 12h). All experiments were performed in duplicate and were repeated at least three times.

Measurement of SNO content by chemiluminescence. S-nitrosothiols were detected in cell lysates or immunoprecipitated protein by chemiluminescence, using a nitric oxide analyzer (Ionics, Boulder, CO). Cells were lysed in HEN buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 0.01 mM neocuproine) containing 0.1% SDS, and 0.5% CHAPS. Lysates were centrifuged, protein concentrations determined, and supernatants were incubated 15 min in the presence or absence of sulfanilamide (0.5%). Fifty microliters of supernatants (normalized to contain containing 12 μg protein), was injected into a purge vessel containing 5 mL of 45 mM potassium iodide (KI) and 10 mM iodine (I_2) in glacial acetic acid, at 60^{o}C, purged continuously with nitrogen [13]. As a complimentary approach to estimate the protein S-nitrosothiol content of the cell, proteins were precipitated with acetone for 20 min at –20^{o}C, in order to eliminate low molecular weight nitrothiols, and avoid quenching with sulfanilamide. After centrifugation, the pellet was ressuspended in lysis buffer, and 30 μL of this suspension was injected in the purge vessel, under the same conditions described above. As a control for detection of S-nitrosylated proteins, lysates were incubated with 4.5 mM $HgCl_2$ for 10 min at RT followed by 20 min incubation at 4^{o}C, before acetone precipitation. Ran was immunoprecipitated from equivalent amounts of cell lysates, using a Ran goat polyclonal IgG (Santa Cruz) and protein G-agarose beads for 1h and 30 min, at 4^{o}C. Immunoprecipitates were washed three times with HEN buffer and the immunocomplexes bound to the beads were injected into a purge vessel for NO analysis. The entire lysis and immunoprecipitation protocol prior to analysis by chemiluminescence took approximately 2 hours and 30 min, and avoided exposure to direct light.

Assessment of S-nitrosylation using chemical derivatization coupled to immunoprecipitation and Western blotting. The biotin labeling of S-nitrosylated proteins in lysates was based on previously described procedures [14,15]. At the selected time points, cells were rinsed with PBS containing 0.1 mM EDTA and 0.01 mM neocuproine and lysed in HEN buffer containing 0.1% SDS, 0.5% CHAPS, and 20 mM NEM (*N*-ethylmalemide) by rocking for 30 min, at 4^{o}C. Lysates were centrifuged at

14,000*g*, 4°C for 10 min, and excess of NEM used to block sulfhydryl groups (SH), was removed by protein precipitation with acetone. Pellets were resuspended in HEN buffer containing 1% SDS (HENS), and S-nitrosothiols were reduced and biotinylated by the simultaneous addition of 10 mM sodium ascorbate and 0.05 mM of the sulfhydryl-specific biotinylating agent, MPB [*N*-(3-maleimidylpropionyl) biocytin, Molecular Probes], for 1 h at RT. The extra label was removed by a second acetone precipitation and proteins resuspended in HENS buffer for immuno-precipitation of RanGTPase as described above. Immunoprecipitates were washed three times with 500 μL each of HEN buffer and resuspended in 25 μL of HEN followed by addition of 25 μL of 2x Laemmli sample buffer, boiled at 95°C for 5 min., separated by SDS-PAGE, and transferred to nitrocellulose membranes. Biotinylation of Ran was detected using streptavidin-HRP. Control experiments were performed in which sodium ascorbate was omitted, preventing the reduction of S-nitrosothiols [15]. Lastly, to assess the contribution of endogenous biotinylated proteins, MPB was omitted in some samples. Samples were protected from light during all procedures prior to electrophoresis. Control dishes were subjected to mock manipulations and are referred to as sham controls.

Assessment of S-nitrosylation in intact cells using biotin derivatization and analysis by confocal microscopy. To evaluate the localization of S-nitrosylated proteins in intact cells, the biotin derivatization method was used in combination with fluorophore labeling and visualization by confocal microscopy. Cells grown on coverslips were rinsed with PBS containing 0.1 mM EDTA and 0.01 mM neocuproine, and fixed in 4% PFA for 20 min at RT. Cells were permeabilized and SH groups blocked in HEN buffer containing 2.5% SDS, and 20 mM MMTS (methylmethanethiosulfonate) for 20 min at 55°C, with constant shaking. After removal of MMTS, cells were incubated with 1 mM sodium ascorbate and 0.4 mM biotin-HPDP, for 1h at RT and coverslips were incubated overnight with streptavidin-FITC, at 4°C. Alternatively, blocking with 20 mM NEM for 30 min at 4°C, followed by incubation with 0.05 mM MPB plus 1 mM sodium ascorbate for 1 h at RT resulted in identical staining patterns (see below). Nuclei were stained with 10 μg/mL PI, for 30 min at RT, cells were analyzed by confocal microscopy (magnification 40x) using an Olympus BX50 microscope coupled to a Bio-Rad (Hercules CA) MRC 1024 confocal scanning laser microscope system. Control experiments were performed in which blocking of SH groups was omitted, or in which 100 μM $HgCl_2$ was added prior to blocking of SH groups with

MMTS or NEM. To assess the contribution of endogenous biotinylated proteins, the biotin-label was omitted in some control coverslips. A diagram summarizing the procedure is shown in Fig 1. All manipulations were performed avoiding direct light (see above).

Assessment of S-nitrosylation in frozen lung tissue using biotin derivatization and analysis by confocal microscopy. Balb/c mice were anesthetized with 400 mg/kg Avertin (2,2,2-tribromoethanol) via intraperitoneal injection and 50 μL of 10 mM GSNO or PBS were administered intratracheally. Two hours later, mice were euthanized by a lethal dose of pentobarbital via intraperitoneal injection. The lungs were instilled with PBS containing 0.1 mM EDTA and 0.01 mM neocuproine for 10 minutes at a pressure of 25 cm H_2O and put in a cassette, embedded in medium for frozen tissue specimens (OCT solution, Sakura Finetek, USA) and immediately frozen in isopentane cooled in liquid nitrogen for preparation of sections. Tissues were then cut in 10 μm sections, affixed to glass microscope slides, and prepared for biotin derivatization. The sections were washed in PBS containing 0.1 mM EDTA and 0.01 mM neocuproine, fixed in PFA (4%), for 20 min, at RT, and permeabilized with PBS containing 1% triton, 0.1 mM EDTA, 0.01 mM neocuproine, and 20 mM NEM to simultaneously block SH groups, for 30 min, at 4°C. After removal of permeabilizing and blocking solution, sections were incubated with 1 mM sodium ascorbate and 0.05 mM MPB for 1h at RT and then were incubated overnight with streptavidin-FITC at 4°C. Nuclei were stained with 10 μg/mL PI, for 30 min at RT, and sections were scanned using an Olympus BX50 upright microscope configured to a Bio-Rad MRX 1024 confocal scanning laser microscope system. The Institutional Animal Care and Use Committee granted approval for this study.

RESULTS

NO detection by chemiluminescence. We first confirmed that treatment with exogenous S-nitrosothiols resulted in an increase in the SNO content of cells. Mouse lung epithelial (C10) cells were incubated with 1 mM GSNO for 1h, lysed, and the NO released following reduction in acidic KI/I_2 was detected by chemiluminescence. As demonstrated in Fig. 2A, the total NO content in lysates from cells treated with GSNO was increased when compared with lysates from non-treated cells, and a fraction of the NO signal observed is resistant to quenching by

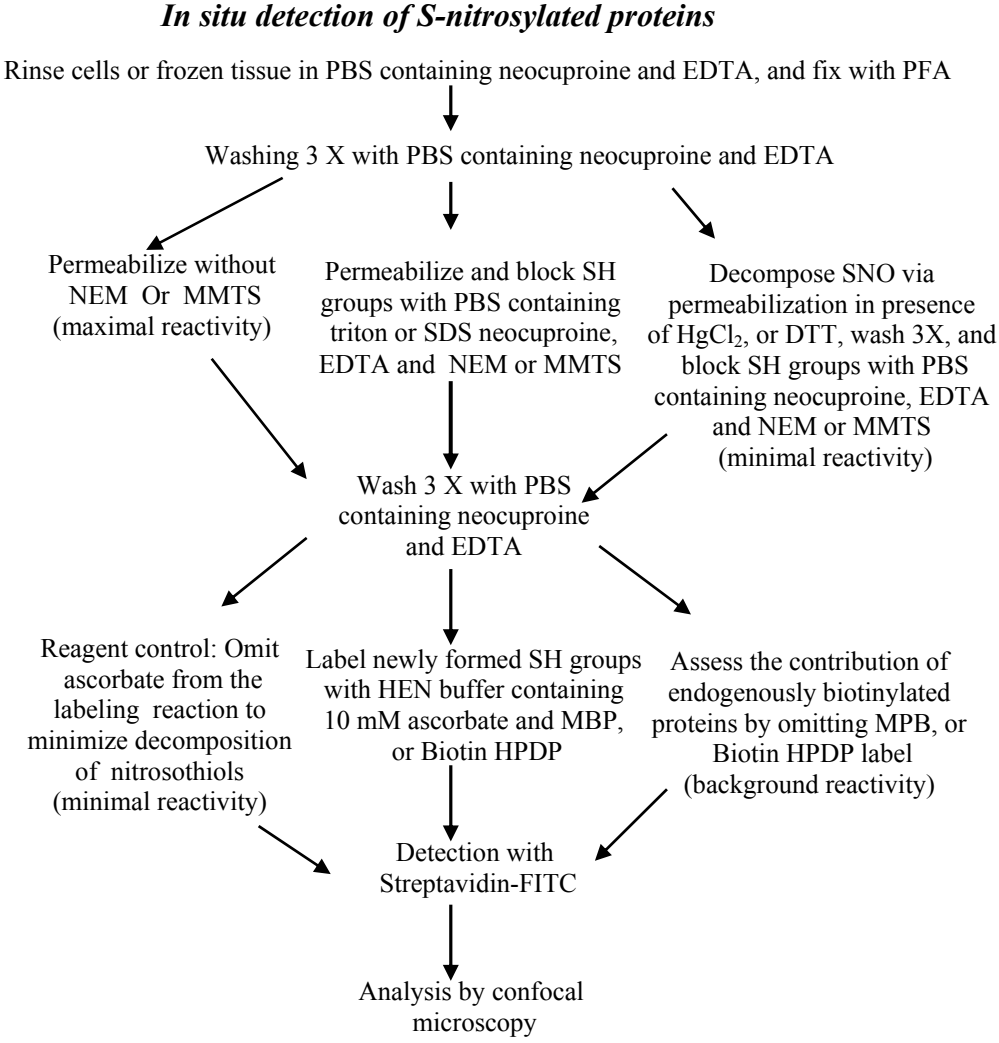
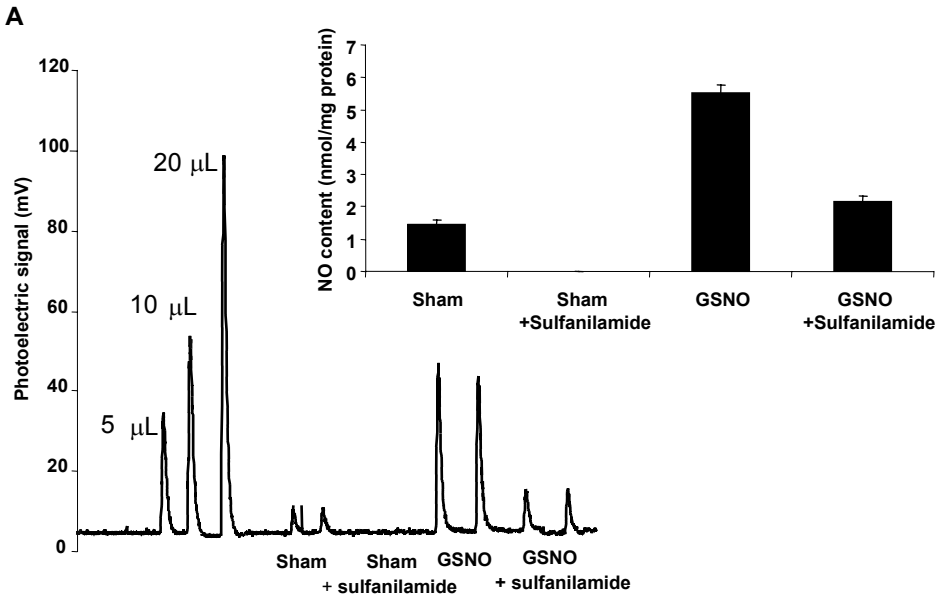


Figure 1 Flow chart of the biotin derivatization for the detection of S-nitrosylated proteins *in situ.* SDS is used as a detergent when MMTS is used as blocking agent. MMTS blocking is performed at 55°C, whereas NEM blocking is performed at 4°C. These two agents can be used interchangeably in fixed cells. NEM blocking is the preferred method for blocking sulfhydryls in frozen tissues, due to loss of tissue following incubation and rocking of the tissue at 55°C.

sulfanilamide (Fig. 2A), indicative of S-nitrosothiols. Although sulfanilamide has been used to remove nitrite in acid conditions, the concern has been reported that sulfanilamide might in fact affect the homeostasis of S-nitrosothiols [16], leading to a potential underestimation of the SNO content. Therefore, as an alternative approach to assess increases in the SNO content in cells exposed to S-nitrosothiols, whole cell lysates were incubated in presence or absence of $HgCl_2$, and subsequently subjected to acetone precipitation. Results in Fig. 2B demonstrate increases in the NO signal in cells exposed to GSNO, in a dose dependent manner, which are partially sensitive to decomposition with $HgCl_2$, consistent with the formation of S-nitrosylated proteins. Using either approach, we were not able to detect S-nitrosothiols in sham controls (< 5 pmol).



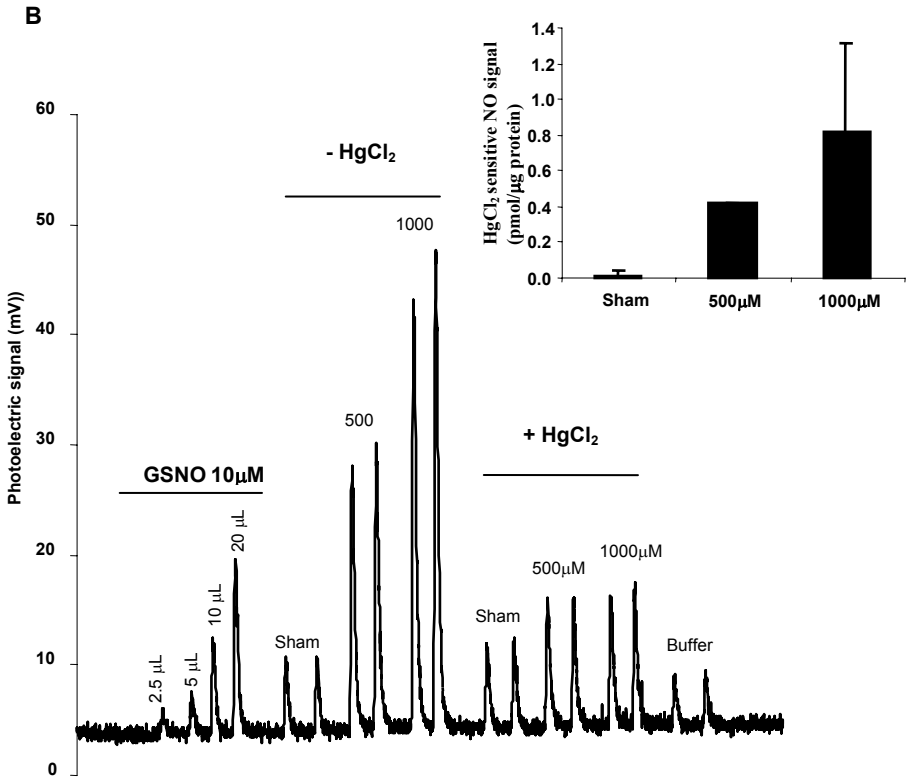


Figure 2 SNO content was determined using chemiluminecence detection. A: Whole cell lysates were incubated 15 min in the presence or absence of sulfanilamide and 50 μL of lysate was injected into a purge vessel containing KI/I_2 system for the detection of NO. B: Whole cell lysates were incubated with $HgCl_2$ to decompose S-nitrosothiols, or left untreated, followed by acetone precipitation. Resuspended precipitates were injected into the purge vessel under the same conditions described before. A 10 μM stock of GSNO was used to generate a standard curve. Inset: Quantitation of the $HgCl_2$ sensitive NO signal, reflecting S-nitrosothiol containing proteins, in cells treated with 500 μM or 1 mM GSNO.

Detection of S-nitrosylated proteins in situ. We next assessed whether biotin derivatization, which originally was described for cell lysates [14], could be adapted in order to detect S-nitrosylation in intact cells. This procedure would be an attractive alternative to immunochemical detection using antibodies and might offer the advantage in avoiding problems related to antibody specificity. Our data in Fig. 3 demonstrate that we can detect S-nitrosylated proteins in intact C10 cells using biotinylation, streptavidin-FITC and analysis by confocal laser scanning microscopy. In non-treated cells (sham), some endogenous reactivity was observed. This reactivity was abolished upon the addition of $HgCl_2$, which decomposes SNO, prior to blocking with methyl methamethiosulfonate (MMTS). Omission of ascorbate, which reduces SNO bonds, prior to the addition of the biotin-HPDP label also markedly, decreased the observed reactivity. Furthermore, fluorescence was also not apparent when the biotin-HPDP label was omitted [17], indicating that the observed fluorescence is due to derivatization of S-nitrosylated proteins, and not endogenous biotin (Fig. 3). In contrast, the omission of the SH blocking agent, MMTS, greatly enhanced biotinylation by biotin-HPDP, as a result of labeling of all available reduced cysteines (Fig. 3). As an additional reagent control, the treatment of cells with 500 μM H_2O_2 failed to alter the staining pattern, illustrating that cysteine oxidation per se does not promote biotinylation (Fig 3).

In accordance with our observations in Fig. 2, biotinylation was enhanced in cells treated with GSNO or SNAP for 1 h. Furthermore, treatment with L-NMMA, substantially reduced biotinylation, whereas the inactive isomer D-NMMA did not affect the SNO pattern, demonstrating that the observed signals are dependent on endogenous NOS activity, and can be detected by the *in situ* method (Fig 3).

We also verified whether induction of NO production in C10 cells would enhance S-nitrosylation of proteins. C10 cells were stimulated simultaneously with tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), in order to induce NOS2 and elevate production of NO, which can be detected in the medium as nitrite. Administration of these cytokines caused nitrite accumulation in the medium over 24 h (Sham; 0.57 μM, TNFα + IFNγ; 7.95 μM), which was prevented with L-NMMA for 16 h (1.1 μM). Results in Fig. 4A demonstrate that the overall levels of S-nitrosylated proteins increased in cells stimulated with a combination of these cytokines, compared to sham, as determined by the enhanced streptavidin-FITC reactivity (Fig 4A, middle panel).

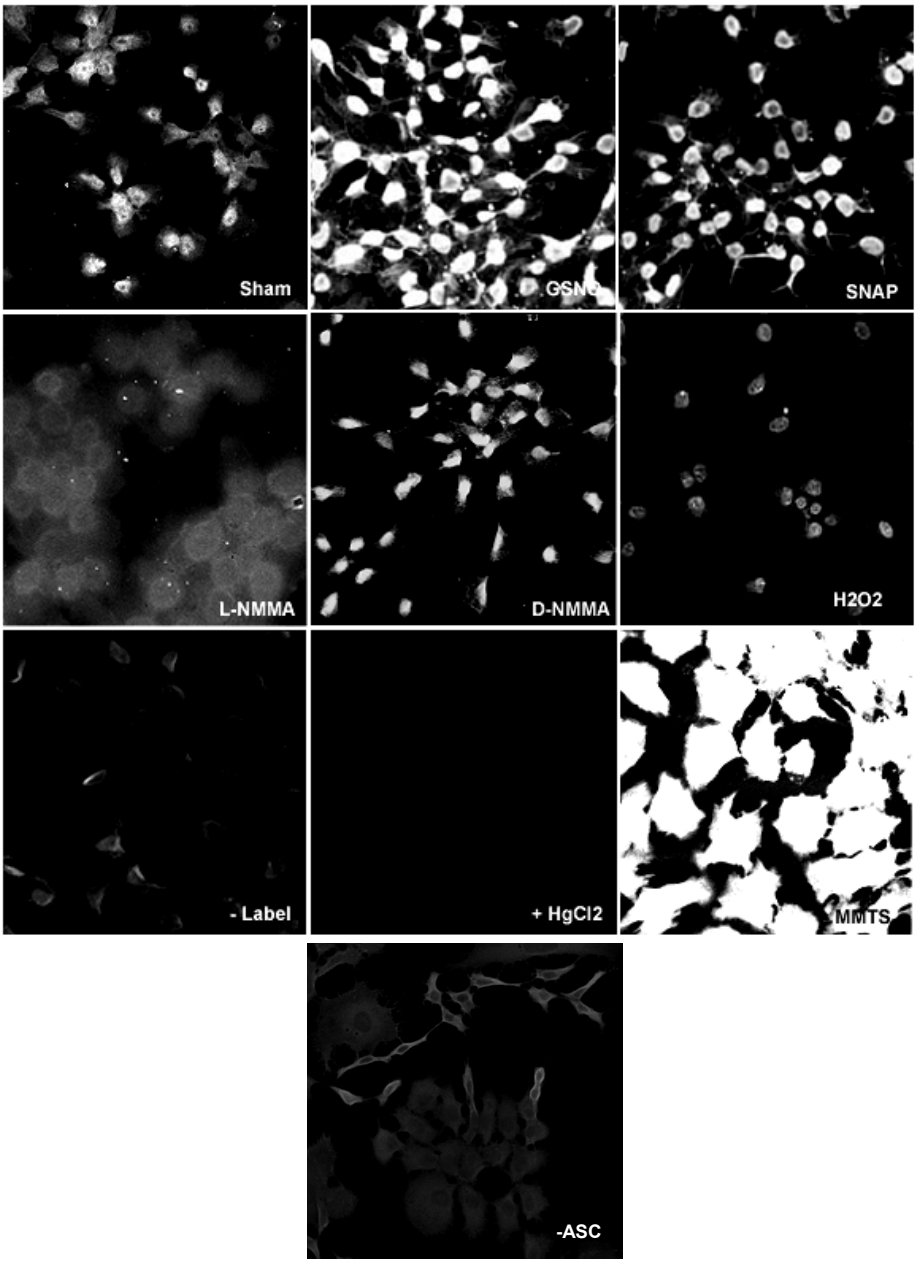


Figure 3 Detection of S-nitrosylated proteins in intact cells. C10 cells were exposed to 1 mM SNAP or 1 mM GSNO for 1 h, or to L-NMMA or D-NMMA for 16 h, fixed, and subjected to biotin derivatization, incubation with streptavidin-FITC, and confocal microscopy. As reagent controls, the biotin-HPDP label (-Label), or ascorbate (- ASC) were omitted, or $HgCl_2$ was added before blocking, to decompose S-nitrosthiols. Omission of MMTS was carried out to visualize all reduced thiols. As an additional control, cells were treated with 500 μM H_2O_2 for 15 min.

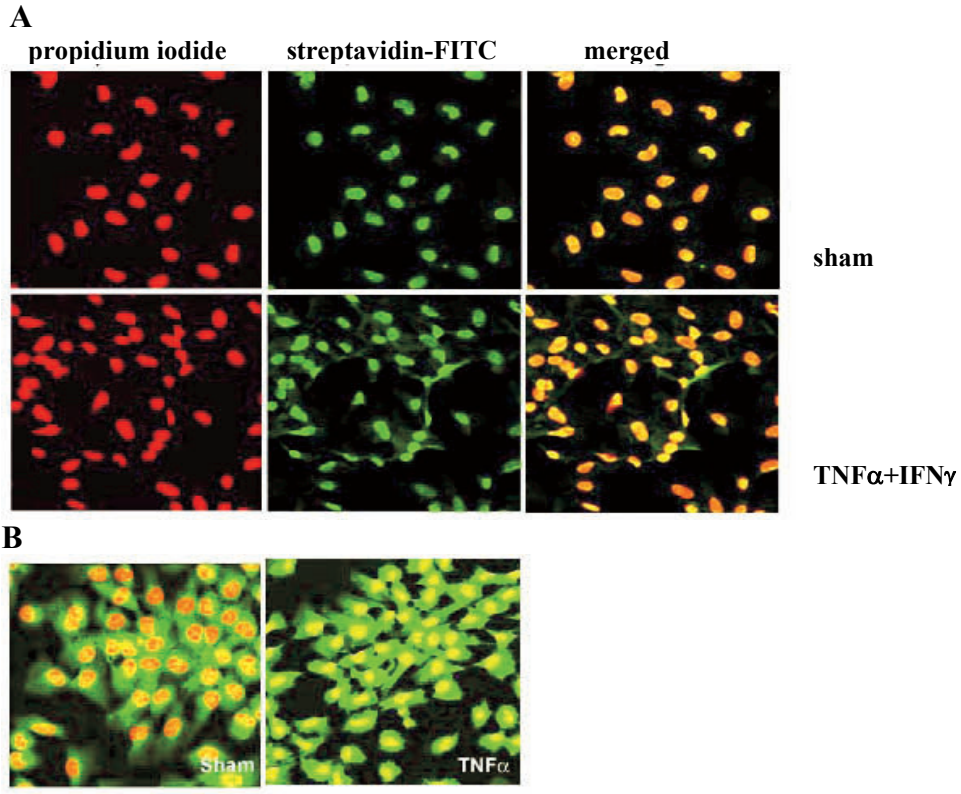


Figure 4 A: Detection of S-nitrosylated proteins in cells following induction of NOS2. Cells were stimulated with TNFα + IFNγ for 24 h, fixed and subjected to biotin derivatization and detection streptavidin-FITC via confocal laser scanning analysis as described in Figure 3. B: Assessment of presence of cytoplasmic protein. C10 cells were mock-treated (sham) or stimulated with TNFα for 30 min, and submitted to the biotinylation protocol described in Figure 1. RelA (p65) was detected using a specific antibody (Santa Cruz). Note the marked cytoplasmic presence of RelA (green) in sham cells and the enhanced nuclear presence in cells treated with TNFα, based upon dual staining with PI (red) which results in a yellow color, indicating the presence of RelA within the nucleus.

Studies aimed at the in situ detection of S-nitrosylated proteins in intact tissues are scant. Nonetheless, S-nitrosylated proteins have been detected in lung tissue using an antibody directed against S-nitroscysteine [18]. We therefore investigated if the *in situ* biotin derivatization method could also be applied to frozen tissue. For this purpose, we used lung sections obtained from mice treated intratracheally with GSNO (50 μL of 10 mM) or PBS. As can be seen in the Fig 5, biotin reactivity, reflecting S-nitrosylated proteins, was apparent in lungs from PBS instilled animals

and markedly increased in the lungs from animals treated with GSNO. Similar to our results with C10 cells (Fig. 3), the reactivity decreased when the sections were incubated with $HgCl_2$, to decompose the SNO, or when the biotin label was omitted.

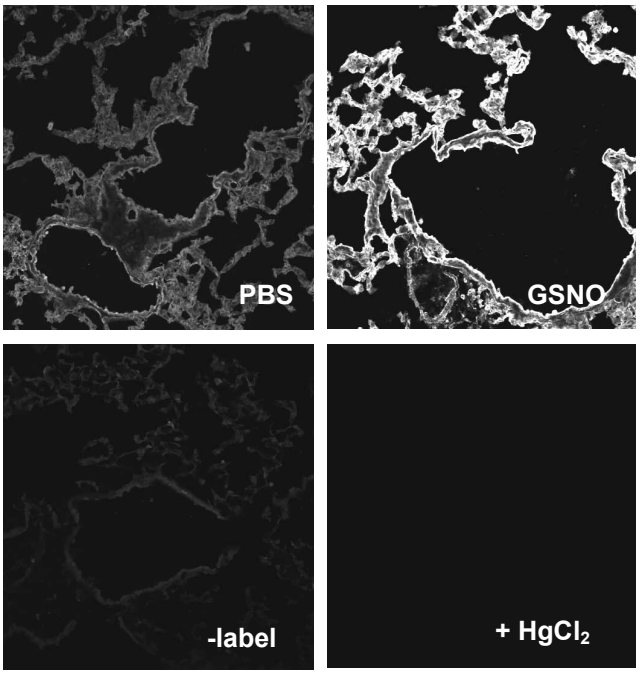


Figure 5 Detection of S-nitrosylated proteins in frozen lung sections. Mice were treated intra-tracheally with 50 µl of 10 mM GSNO or PBS for 2h, euthanized, and the lungs harvested and prepared for biotin derivatization, as described in Material and Methods. The biotinylated proteins were detected with streptavidin-FITC and the sections analyzed via confocal microscopy. As reagent controls, the MPB label was omitted, (-Label) or $HgCl_2$ was used to specifically decompose S-nitrosothiols (+$HgCl_2$).

Detection of S-nitrosylated proteins in the nucleus. Intriguingly, the reactivity of S-nitrosylated proteins detected by confocal microscopy in intact cells (Figs. 3 and 4A) appears to be largely confined, and suggests primarily localization within nuclei. To explore whether S-nitrosylation was indeed localized within nuclei, we used propidium iodide (PI) as a nuclear stain. Merging of images obtained from dual staining with streptavidin-FITC (Fig. 4A, middle panels) and PI (Fig. 4A, left panels), revealed significant co-localization in the nucleus, apparent from the yellow/orange color that was observed (Fig. 4A, right panels). A Z-series taken throughout the cells confirmed that the streptavidin-FITC and PI signals were localized within the same plane of the cells (data not shown). These findings suggest that the nucleus appears to be an important site of localization of S-nitrosylated proteins. One potential artifact in the procedure is the potential loss of soluble cytosolic proteins during the fixation, permeabilization, and biotin derivatization procedures, which would favor detection of S-nitrosylated proteins within nucleus. To ensure

that cytoplasmic targets were still present after this procedure, the presence of RelA was evaluated in sham or TNFα-treated cells. RelA, a subunit of the transcription factor, nuclear factor Kappa B, is normally present in the cytoplasm and is translocated into the nucleus upon exposure to TNFα [19]. Results in Fig. 4B demonstrate that in sham control cells, RelA was readily detected in the cytoplasmic compartment whereas in response to TNFα, increases in the nucleus were apparent, based upon co-localization with PI. These results therefore suggest that the localization of S-nitrosylated proteins in the nucleus is not due to loss of cytoplasmic targets during the fixation and staining procedure. In fact, increased S-nitrosylation of cytoplasmic proteins was observed after induction of NOS2 activity (Fig. 4A).

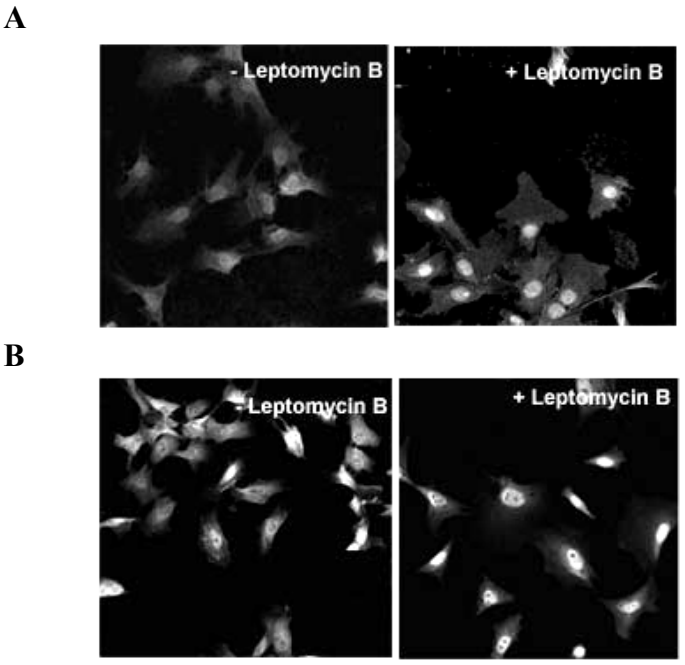


Figure 6 Increases in the content of S-nitrosylated proteins in nucleus after blocking nuclear export. A: Cells were incubated with leptomycin B (5 ng/mL) for 12 h, followed by biotin derivatization, incubation with streptavidin-FITC, and analysis by confocal microscopy. B: Confirmation that nuclear export was blocked by leptomycin B, by immunostaining of RelA (p65).

To show that the nucleus is a site where S-nitrosylated proteins can accumulate, we blocked nuclear export using leptomycin B, which binds SH groups of CRM-1, a protein involved in nuclear export [20], and

assessed the content of S-nitrosylated proteins in the nucleus via biotin derivatization. As demonstrated in Fig. 6A, leptomycin B enhanced biotinylation in the nuclear compartment. As a control, we also demonstrated that the nuclear sequestration of RelA was enhanced by leptomycin B treatment, confirming that nuclear export was blocked (Fig. 6B). These findings support the notion that S-nitrosylation occurs in proteins that are regulated by nuclear trafficking.

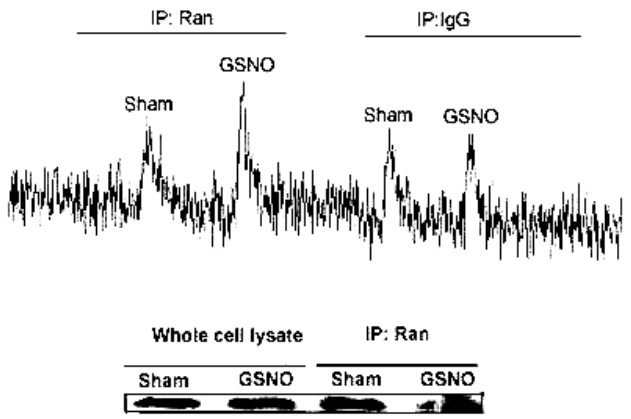
Detection of S-nitrosylation of RanGTPase. After determining that S-nitrosylation is present in the nucleus and enhanced after nuclear export is blocked, we sought to determine a potential target for S-nitrosylation. Ran GTPase is a 25 kDa protein containing three cysteines, that is predominantly found in nuclear compartment in the cells, where it regulates nuclear import through the nuclear pore complex [21,22]. We investigated whether RanGTPase was S-nitrosylated after treatment with GSNO. We first performed chemiluminescence in order to detect the S-nitrosylation of immunoprecipitated RanGTPase. Immunocomplexes from non-treated cells did not produce significant photoelectric signal, when compared to IgG controls. However, after GSNO treatment, an increase in the SNO content of immunopreciptated RanGTPase was apparent, suggesting its S-nitrosylation (Fig 7A). To confirm that Ran GTPase itself was S-nitrosylated, we used the biotin derivatization approach, immunoprecipitated RanGTPase, and detected biotinylated RanGTPase by Western blotting using streptavidin-HRP. Indeed, some basal biotinylation of RanGTPase was observed, which was enhanced after treatment with GSNO. Minimal reactivity occurred in the absence of ascorbate or the biotin label (Fig. 7B, upper panel), consistent with the specificity of the biotinylation approach for S-nitrosylated proteins.

DISCUSSION

S-nitrosylation represents a NO-dependent post-translational modification that plays an important role in signal transduction and cellular function. We have demonstrated here that an adaptation of the biotin-switch method is suitable to detect S-nitrosylated proteins in intact cells and tissues, providing investigators with a method to detect this NO-dependent post-translational modification *in situ*. Using this approach we can detect increases in SNO signal after exogenous administration of S-nitrosothiols, or following induction of NOS2, and decreases in reactivity following inhibition of NOS. We also detected S-nitrosylation in the nuclear

compartment, and demonstrated that Ran is a putative target for S-nitrosylation, suggesting a possible regulatory role for S-nitrosylation in nuclear processes.

A



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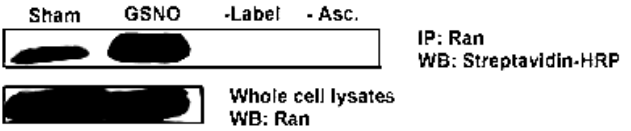

Figure 7 S-Nitrosylation of RanGTPase. (A) Detection of NO released from Ran GTPase by chemi-luminescence. Cells were treated with 1mM GSNO for 1 h, washed, and Ran was immunoprecipitated form the total lysate using a Ran specific antibody, and protein G agarose beads. Immunocomplexes bound to the beads were washed 3 times and directly injected into the purge vessel as described in the "Experimental procedures". As a control, pre-immune IgG was used instead of Ran antibody (upper panel). Western blot analysis confirming the presence of RanGTPase in the immunocomplexes (lower panel). (B) Detection of S-nitrosylation of Ran GTPase using the biotin switch method. Cells were treated with GSNO, lysed and subjected to biotin derivatization. Ran was immunoprecipitated from total cell lysates, and biotinylated RanGTPase was detected by Western blotting and detection by peroxidase-conjugated streptavidin (upper panel). – label; omission of MPB label, -Asc; omission of ascorbate. Lower Panel: As a loading control, Ran was detected in whole cell lysates by Western blotting.

The detection of S-nitrosylated proteins in cells and tissues has involved a multitude of approaches and is the subject of intense debate (See [16]), due to inherent problems with available assays to measure and quantitate

S-nitrosylated proteins. It was stated that more emphasis should perhaps be placed on the qualitative aspect of the data obtained rather than exact quantitation of S-nitrosylated proteins due to those analytical problems [16]. In that context, the *in situ* method described here will allow for the detection of S-nitrosylated proteins in intact cells or tissues and will be informative in the evaluation of altered S-nitrosylation during abnormal physiology, or disease. To verify that S-nitrosylated proteins increase in cells, after administration of exogenous S-nitrosothiols, we first employed chemiluminescence-based detection methods that relied on sulfanilamide quenching or acetone precipitation in presence or absence of administration of $HgCl_2$. Both methods demonstrated increases in SNO signals in cells, in concordance with results from the *in situ* approach. In contrast, whereas we were able to detect S-nitrosylation in control (non SNO-exposed) cells using the *in situ* method, we were not able to detect a SNO signal in these cells via chemiluminescence, suggesting that the SNO content in our cells is below the limit for detection via chemiluminescence. Alternatively, the sample preparation required for chemiluminescence analysis, which requires lysis of cells may have resulted in decomposition of S-nitrosylated proteins, and is not required in the *in situ* detection method, providing a potential explanation for the differences in detection of low levels of S-nitrosylated proteins between these methods.

Technical considerations for utilization of the biotin switch method.

The biotin switch method is based on selective derivatization of protein SNO moieties to a biotin adduct [14], and may suffer from some limitations in specificity and accuracy, due to the reversible nature of S-nitrosylation, the high heterogeneity of protein cysteine residues and variability in thiol redox states that can easily interchange. Hence, the use of this biotin derivatization procedure and its application to *in situ* detection of S-nitrosylated proteins deserves some consideration. The preservation of SNO moieties during sample preparation as well as their specific biotin derivatization is dependent on intricate redox chemistry. The first step is this procedure is the fixation of cells or tissues with paraformaldehyde to preserve the native structure of proteins, prevent unfolding and possible contact with compounds that can destroy SNO moieties. The next step is the blocking of SH groups, which serves to protect reduced SH groups from non specific oxidation and also to prevent further intra and intermolecular transnitrosation reactions. Thus, SNO in the proteins are preserved and reduced by ascorbate and subsequently biotinylated. One major concern in this SH blocking step is its specificity

and efficiency, as incomplete blocking would results in major background in the subsequent biotinylation step and obscure the contribution of SNO to the final signal. Protein SH groups on cysteine are highly heterogeneous, with variable pK_a, and are susceptible to multiple oxidations, such as disulfide interchange, thioether and thioester formation. Cysteine reactivity toward various SH labeling reagents is determined by a number of factors including the buffer composition, ionic strength and pH, which determines the degree of protein unfolding, solvent exposure of SH groups, and the extent of its ionization.

In the original protocol described by Jaffrey and co-workers, MMTS was used as the thiol-blocking agent in a buffer containing SDS, at 55^{o}C. Our results indicated that the use of MMTS in the *in situ* technique was also suitable in the detection of S-nitrosylated proteins, based on our observations that biotinylation subsequent to blocking required the ascorbate reduction step, was dependent on an endogenous or exogenous NO source, and was sensitive to decomposition by $HgCl_2$. However, the relatively harsh blocking conditions using MMTS (which did not allow us to retain frozen tissues sections on slides), and concerns that MMTS form disulfides with protein SH groups that could be reversible, stimulated us to explore the use of NEM as a potentially more suitable thiol-blocking agent. NEM is an alkylating agent with high reactivity for thiolate anion, with a second order rate constant in the order of 10^7 $M^{-1}.min^{-1}$. In contrast, NEM reactivity with protonated thiols is much slower: about 2 x 10^{-4} $M^{-1}.min^{-1}$ [23]. Nevertheless, if steric or other structural considerations are not a factor, average protein SH groups with a pK_a of about 9 will be completely alkylated at neutral pH within 30 min, as indicated by several previous studies [23,24]. Only SH groups with unusually high pK_a, or low reactivity because of other structural factors, may not be sufficiently blocked in this case. To avoid potential issues with specificity, we chose to use a biotinylating agent that relies on the same alkylation chemistry, MPB, to assure that SH groups that are reactive towards MPB would most likely have been alkylated by NEM in the preceding blocking step. In our experimental conditions, the use of NEM at 4^{o}C appeared indeed sufficiently effectively in blocking reduced SH groups and allowed relatively selective *in situ* detection of S-nitrosylated proteins in intact cells (not shown) or in tissue sections (Fig.5).

Another potential limitation of this biotin switch procedure is whether the SNO decomposition by ascorbate is sufficiently specific [25], and there is

reasonable concern that other oxidized forms of protein cysteine residues, such as (mixed) disulfides, sulfenic acids, etc., could also be reduced in the process. We also investigated the ability of ascorbate to significantly reduce GSSG to GSH, under our derivatization conditions. Hence we treated various solutions of GSSG in HENS buffer with either 10 mM or 20 mM ascorbate, and analyzed its potential reduction to GSH by HLPC [26]. As illustrated in Table I, only a minor fraction of GSSG (less than 0.09%) was reduced under the conditions used in the biotin switch method. Thus, we believe that the ascorbate-dependent SH biotinylation in the biotin switch procedure is primarily due to SNO, with only a minor contribution of disulfides.

Table 1. Assessment of reduction of GSSG by 10 or 20 mM ascorbate (asc)

GSSG (μM)	GSH (μM)	GSH (μM) 10 mM asc	GSH (μM) 20 mM asc	%GSSG reduced 10 mM asc	%GSSG reduced 20 mM asc
100	0.10	0.11	0.11	0.01	0.01
500	0.57	0.96	1.29	0.08	0.14
1000	0.60	1.53	2.27	0.09	0.17

Reduction of GSSG to GSH was analyzed by HPLC [21]. Reaction conditions used were identical to those used in the biotin switch procedure for the detection of S-nitrosylated proteins.

Detection of S-nitrosylation in the nucleus. Although previous reports indicate compartimentalization of S-nitrosylated proteins in the mitochondria [27], to our knowledge, the occurrence of nuclear S-nitrosylation has not been previously reported, and is intriguing given the unique local redox environment within the nucleus. Perhaps, the nuclear compartment represents a microenvironment favorable for S-nitrosylation. Protein S-nitrosylation is readily reversible and the microenvironment that surrounds target proteins, such as pH, hydrophobicity, redox status, and the presence of transition metals, are all believed to be important in regulating this post-translational modification [28]. In support of our observations, copper ions associated with chromatin, could catalyze transnitrosylation reactions, in a redox dependent manner [17]. In addition, the perinuclear region, where NOS has been localized [29], is enriched in Golgi membranes, providing a hydrophobic environment which accelerates the reaction between NO and oxygen forming N_2O_3 and consequently facilitates nitrosylation reactions. Thus, it is possible that

perinuclear membranes can enhance the yield of S- nitrosylation [28], providing an example of a spatial environment that may be critical for the specificity of targeting NO reactions [3]. Alternatively, S-nitrosylation of target proteins could have occurred within the cytoplasm prior to their translocation to the nucleus and potentially accumulated within this compartment. It is of interest that RanGTPase is a target for S-nitrosylation. This protein has three cysteines, and is the most abundant member of the Ras superfamily of GTPases, constituting about 0.4% of the total cell protein [30,31]. Nevertheless, it remains to be determined, through site directed mutagenesis approaches, whether S-nitrosylation of RanGTPase has any functional ramifications for nuclear regulatory processes. In support of a role of cysteine oxidation events regulating nuclear trafficking, the yeast Yap-1p protein accumulates in the nucleus under oxidizing conditions [32]. Furthermore, Hic-5 in mammalian cells contains a homologous nuclear export signal (NES) containing two cysteines that contribute to the nuclear retention of this protein under oxidizing conditions [33].

SUMMARY AND CONCLUSIONS

In summary, we have described here a new application of the biotin-switch method enabling the detection of S-nitrosylated proteins in intact cells or tissues. This approach results in biotin labeling of S-nitrosylated proteins following their chemical derivatization. The inclusion of reagent controls, such as $HgCl_2$ to decompose the S-nitrosothiols, and the demonstration that the biotin labeling is dependent on endogenous NOS activity or exogenous S-nitrosothiols, and requires reduction by ascorbate, suggests the specificity for detection of S-nitrosylated proteins. This approach overcomes the necessity to use antibodies directed against the S-nitrosocysteine moiety, which may have problematic specificity, and may be useful to probe altered patterns of S-nitrosylation in pathophysiological conditions. As with most immunolocalization techniques, although this approach will detect changes in patterns of S-nitrosylated proteins in cells or tissues, it will not yield quantitative information, nor elucidate the identity of the S-nitrosylated proteins. Lastly, the intriguing observation of predominant nuclear localization of S-nitrosylated proteins in cultured cells, and the identification of Ran as a target for S-nitrosylation evokes further questions about the role of S-nitrosylation in regulation of nuclear trafficking, gene transcription, or DNA replication.

ACKNOWLEDGEMENTS

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REFERENCES

[1] J.F. Kerwin, Jr., J.R. Lancaster, Jr. ,P.L. Feldman.Nitric oxide: a new paradigm for second messengers, J Med Chem 38(1995) 4343-4362

[2] J.P. Eu, L. Liu, M. Zeng ,J.S. Stamler.An apoptotic model for nitrosative stress, Biochemistry 39(2000) 1040-1047

[3] J.S. Stamler, S. Lamas ,F.C. Fang.Nitrosylation. the prototypic redox-based signaling mechanism, Cell 106(2001) 675-683

[4] D.T. Hess, A. Matsumoto, R. Nudelman ,J.S. Stamler.S-nitrosylation: spectrum and specificity, Nat Cell Biol 3(2001) E46-E49

[5] J.B. Mannick, A. Hausladen, L. Liu, D.T. Hess, M. Zeng, Q.X. Miao, L.S. Kane, A.J. Gow ,J.S. Stamler.Fas-induced caspase denitrosylation, Science 284(1999) 651-654

[6] N.L. Reynaert, K. Ckless, S.H. Korn, N. Vos, A.S. Guala, E.F. Wouters, A. van der Vliet ,Y.M. Janssen-Heininger.Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation, Proc Natl Acad Sci U S A 101(2004) 8945-8950

[7] H.E. Marshall ,J.S. Stamler.Inhibition of NF-kappa B by S-nitrosylation, Biochemistry 40(2001) 1688-1693

[8] J.R. Matthews, C.H. Botting, M. Panico, H.R. Morris ,R.T. Hay.Inhibition of NF-kappaB DNA binding by nitric oxide, Nucleic Acids Res 24(1996) 2236-2242

[9] K.K. Chung, B. Thomas, X. Li, O. Pletnikova, J.C. Troncoso, L. Marsh, V.L. Dawson ,T.M. Dawson.S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function, Science 304(2004) 1328-1331

[10] S.R. Jaffrey, H. Erdjument-Bromage, C.D. Ferris, P. Tempst ,S.H. Snyder.Protein S-nitrosylation: a physiological signal for neuronal nitric oxide, Nat Cell Biol 3(2001) 193-197

[11] S.H. Korn, E.F. Wouters, N. Vos ,Y.M. Janssen-Heininger.Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase, J Biol Chem 276(2001) 35693-35700

[12] Y. Zhang ,N. Hogg.The mechanism of transmembrane S-nitrosothiol transport, Proc Natl Acad Sci U S A 101(2004) 7891-7896

[13] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'Heuil ,M. Kelm.Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo, FASEB J 16(2002) 1775-1785

[14] S.R. Jaffrey ,S.H. Snyder.The biotin switch method for the detection of s-nitrosylated proteins, Sci STKE 2001(2001) L1

[15] S. Li ,A. Richard Whorton.Regulation of protein tyrosine phosphatase 1B in intact cells by S- nitrosothiols, Arch Biochem Biophys 410(2003) 269-279

[16] J.S. Stamler.S-nitrosothiols in the blood: roles, amounts, and methods of analysis, Circ Res 94(2004) 414-417

[17] Y. Li, A. Seacat, P. Kuppusamy, J.L. Zweier, J.D. Yager ,M.A. Trush.Copper redox-dependent activation of 2-tert-butyl(1,4)hydroquinone: formation of reactive oxygen species and induction of oxidative DNA damage in isolated DNA and cultured rat hepatocytes, Mutat Res 518(2002) 123-133

[18] A.J. Gow, Q. Chen, D.T. Hess, B.J. Day, H. Ischiropoulos ,J.S. Stamler.Basal and stimulated protein s-nitrosylation in multiple cell types and tissues, J Biol Chem 277(2002) 9637-9640

[19] M.E. Poynter, C.G. Irvin ,Y.M. Janssen-Heininger.Rapid Activation of Nuclear Factor-kappaB in Airway Epithelium in a Murine Model of Allergic Airway Inflammation, Am J Pathol 160(2002) 1325-1334
[20] N. Kudo, N. Matsumori, H. Taoka, D. Fujiwara, E.P. Schreiner, B. Wolff, M. Yoshida ,S. Horinouchi.Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region, Proc Natl Acad Sci USA 96(1999) 9112-9117
[21] S.A. Richards, K.M. Lounsbury ,I.G. Macara.The C terminus of the nuclear RAN/TC4 GTPase stabilizes the GDP-bound state and mediates interactions with RCC1, RAN-GAP, and HTF9A/RANBP1, J Biol Chem 270(1995) 14405-14411
[22] M.S. Moore.Ran and nuclear transport, J Biol Chem 273(1998) 22857-22860
[23] R.A. Bednar.Reactivity and pH dependence of thiol conjugation to *N*-ethylmaleimide: Detection of a conformational change in chalcone isomerase, Biochemistry 29(1990) 3684-3690
[24] P.J. Britto, L. Knipling ,J. Wolff.The local electrostatic environment determines cysteine reactivity of tubulin, J Biol Chem 277(2002) 29018-29027
[25] M. Xian, X. Chen, Z. Liu, K. Wang ,P.G. Wang.Inhibition of papain by S-nitrosothiols. Formation of mixed disulfides, J Biol Chem 275(2000) 20467-20473
[26] A. van der Vliet, P.A. Hoen, P.S. Wong, A. Bast ,C.E. Cross.Formation of S-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide, J Biol Chem 273(1998) 30255-30262
[27] J.B. Mannick, C. Schonhoff, N. Papeta, P. Ghafourifar, M. Szibor, K. Fang ,B. Gaston.S-Nitrosylation of mitochondrial caspases, J Cell Biol 154(2001) 1111-1116
[28] N. Hogg. The biochemistry and physiology of S-nitrosothiols, Annu Rev Pharmacol Toxicol 42(2002) 585-600
[29] G. Garcia-Cardena, P. Oh, J. Liu, J.E. Schnitzer ,W.C. Sessa.Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling, Proc Natl Acad Sci USA 93(1996) 6448-6453
[30] F.R. Bischoff, C. Klebe, J. Kretschmer, A. Wittinghofer ,H. Ponstingl.RanGAP1 induces GTPase activity of nuclear Ras-related Ran, Proc Natl Acad Sci USA 91(1994) 2587-2591
[31] M. Dasso.The Ran GTPase: theme and variations, Curr Biol 12(2002) R502-R508
[32] S. Kuge, M. Arita, A. Murayama, K. Maeta, S. Izawa, Y. Inoue ,A. Nomoto.Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation, Mol Cell Biol 21(2001) 6139-6150
[33] M. Shibanuma, J.R. Kim-Kaneyama, K. Ishino, N. Sakamoto, T. Hishiki, K. Yamaguchi, K. Mori, J. Mashimo ,K. Nose.Hic-5 Communicates between Focal Adhesions and the Nucleus through Oxidant-Sensitive Nuclear Export Signal, Mol Biol Cell 14(2003) 1158-1171

CHAPTER 5

Dynamic redox control of NF-κB through glutaredoxin-regulated S-glutathionylation of inhibitory kappa B kinase beta

ABSTRACT

The transcription factor nuclear factor kappa B (NF-κB), a central regulator of immunity, is subject to regulation by redox changes. We now report that Cysteine 179 (Cys179) of the IKKβ subunit of the IKK signalsome is a central target for oxidative inactivation via S-glutathionylation. S-glutathionylation of IKKβ Cys179 is reversed by glutaredoxin (GRX), which restores kinase activity. Conversely, GRX1 knock-down sensitizes cells to oxidative inactivation of IKKβ, and dampens TNFα-induced IKK and NF-κB activation. Primary tracheal epithelial cells from *Glrx1*-deficient mice display reduced NF-κB-DNA binding, RelA nuclear translocation as well as MIP-2 and KC production in response to LPS. Collectively these findings demonstrate the physiological relevance of the S-glutathionylation-glutaredoxin redox module in controlling the magnitude of activation of the NF-κB pathway.

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INTRODUCTION

In recent years, inflammatory diseases have commonly been associated with oxidative stress. For instance, lung pathologies like asthma are accompanied by elevated amounts of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) that are produced both by infiltrating inflammatory cells and resident cells that express non-phagocytic NAPDH oxidases (1). Although ROS damage macromolecules there is mounting evidence that they can also serve as regulators of cellular processes.

Amongst the most susceptible oxidant-sensitive targets are protein thiol groups which can be reversibly oxidized to sulfenic acid (-SOH) or irreversibly oxidized to sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acid, although sulfiredoxin has been recently found to specifically reduce the sulfinic acid moiety in peroxiredoxins (2-4). Reversible oxidations are believed to protect proteins from irreversible oxidation, but may also modulate protein function. The sulfenic acid moiety is very unstable and readily reacts with other thiols to form intra- or intermolecular disulfides. For example, the reaction with glutathione to form S-glutathionylated proteins is an important post-translational modification that affects the function of proteins, like H-ras (5), actin (6), HIV-1 protease (7). Furthermore, both the transcription factors NF-κB and AP-1 demonstrate reduced DNA binding activity after S-glutathionylation of their respective p50 (8) and c-Jun subunits (9).

S-glutathionylation is regulated by glutaredoxins (GRX) or thioltransferases, members of the thiol-disulfide oxidoreductase family that contain a thioredoxin fold (10). To date, two mammalian GRX enzymes have been characterized. GRX1 is a cytosolic protein, whereas alternative splicing of the primary RNA transcript controls subcellular trafficking of GRX2 to mitochondria and the nucleus (11-13). GRXs catalyze the reversible reduction of protein-glutathionyl-mixed disulfides to free sulfhydryl groups through a monothiol mechanism (14, 15). In this reaction, GRX itself is S-glutathionylated at Cys22 and the reduced state of GRX is restored using GSH coupled to GSSG reductase (15). GRX2 can also be reduced by the thioredoxin/ thioredoxin reductase system (12). In contrast to bacterial GRX, mammalian GRX display substrate specificity toward S-glutathionylated proteins (14, 16) and could therefore play a unique role in redox signaling.

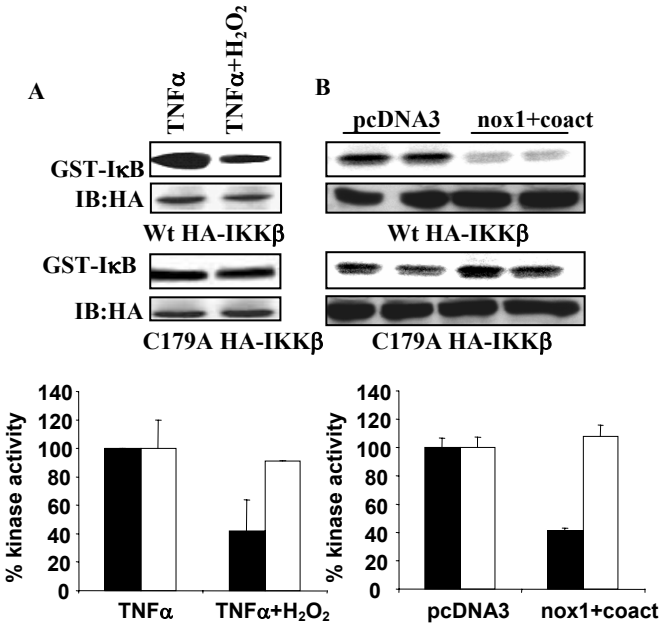
NF-κB is a transcription factor constituted by homo or heterodimers of the Rel protein family with a pivotal role in inflammation, cell survival and proliferation. In unstimulated cells, NF-κB is maintained in a latent form in the cytoplasm via sequestration by IκB inhibitory proteins. NF-κB activating stimuli, like cytokines, viruses and LPS, induce the degradation of IκB's by the proteasome, unmasking the nuclear localization signal of NF-κB, resulting in its nuclear translocation, binding to NF-κB motifs, and gene transcription. The enzyme complex responsible for phosphorylation of IκB's on specific serine residues is I kappa B kinase (IKK), a large, 700-900 kDa complex containing at least 2 catalytic subunits: IKKα and IKKβ (17, 18). Knock-out studies have revealed that IKKβ is responsible for the pro-inflammatory cytokine-induced activation of NF-κB (19). IKKα on the other hand is crucial for B cell maturation and p100 processing, as well as activating NF-κB dependent gene transcription by phosphorylating histone H3 (20, 21). IKKγ is the regulatory subunit (22). The mechanisms that activate the IKK complex are incompletely understood, but it is well defined that activation requires phosphorylation of serine residues 177 and 181 in the activation loop of IKKβ (23).

Although IKK is essential for NF-κB activation is response to most stimuli, IKK is also subject to negative regulation in order to prevent activation of NF-κB. For example, arsenite (24), cyclopentenone prostaglandins (25), S-nitrosothiols (26) and some anti-inflammatory drugs (27, 28) have been reported to inhibit IKKβ via targeting of a critical cysteine residue, resulting in a failure to activate NF-κB. Covalent modification of Cys179 thereby provides a powerful mode by which anti-inflammatory agents repress NF-κB. It has not been established whether the inactivation of IKKβ following oxidation of Cys179 is reversible, permitting rapid regeneration of IKKβ activity and propagation of inflammatory signals.

Previously we established that H_2O_2 inhibits TNF-stimulated IKKβ activity via oxidation of IKKβ (29). The aim of the present study was to investigate the mode of oxidation and identify the critical target that is responsible for the inhibitory effects of H_2O_2 on IKKβ. We also sought to determine whether H_2O_2-induced oxidative inactivation is reversible, to elucidate the redox systems that restore IKK activity, and examine their impact on cytokine-induced NF-κB activation.

RESULTS

Inhibition of IKK activity through reversible oxidation of Cys179 of the IKKβ subunit. Since it has been established that S-nitrosothiols, arsenite and cyclopentenone prostaglandins inhibit IKK activity through oxidation of Cys179 of the β subunit, we first investigated if inhibition by H_2O_2 also occurs via oxidation of this residue. H_2O_2 added exogenously (Fig.1*A*), as well as produced endogenously by expression of nox1 plus its co-activators, p41 and p51 (Fig. 1*B*), markedly inhibited TNFα (Calbiochem, La Jolla, CA)-induced IKKβ activity in cells expressing wt HA-IKKβ. In contrast, a construct containing a Cys179Ala mutant version of HA-IKKβ was largely refractory to inhibition by H_2O_2, indicating that H_2O_2 indeed inhibits IKKβ through oxidation of Cys179.



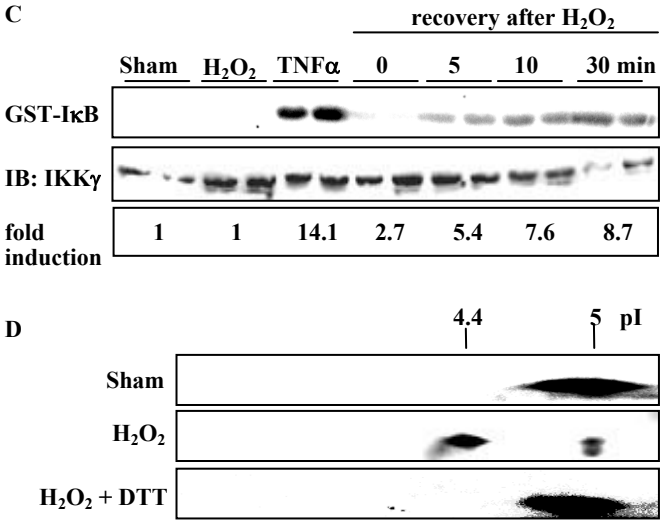
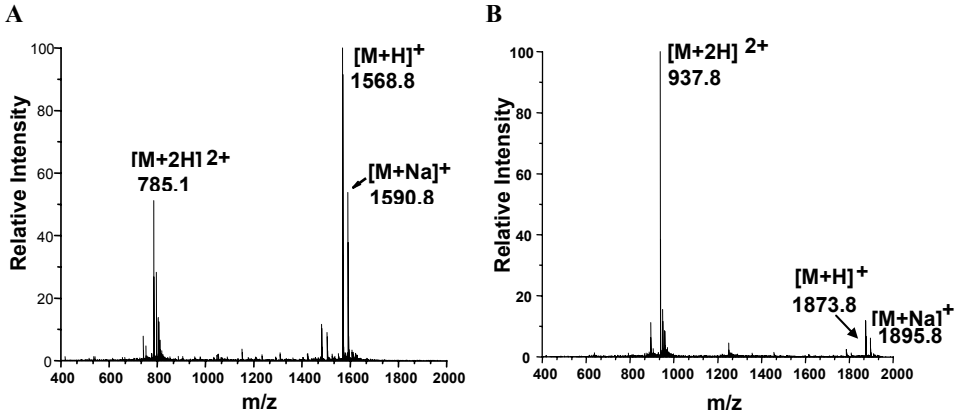
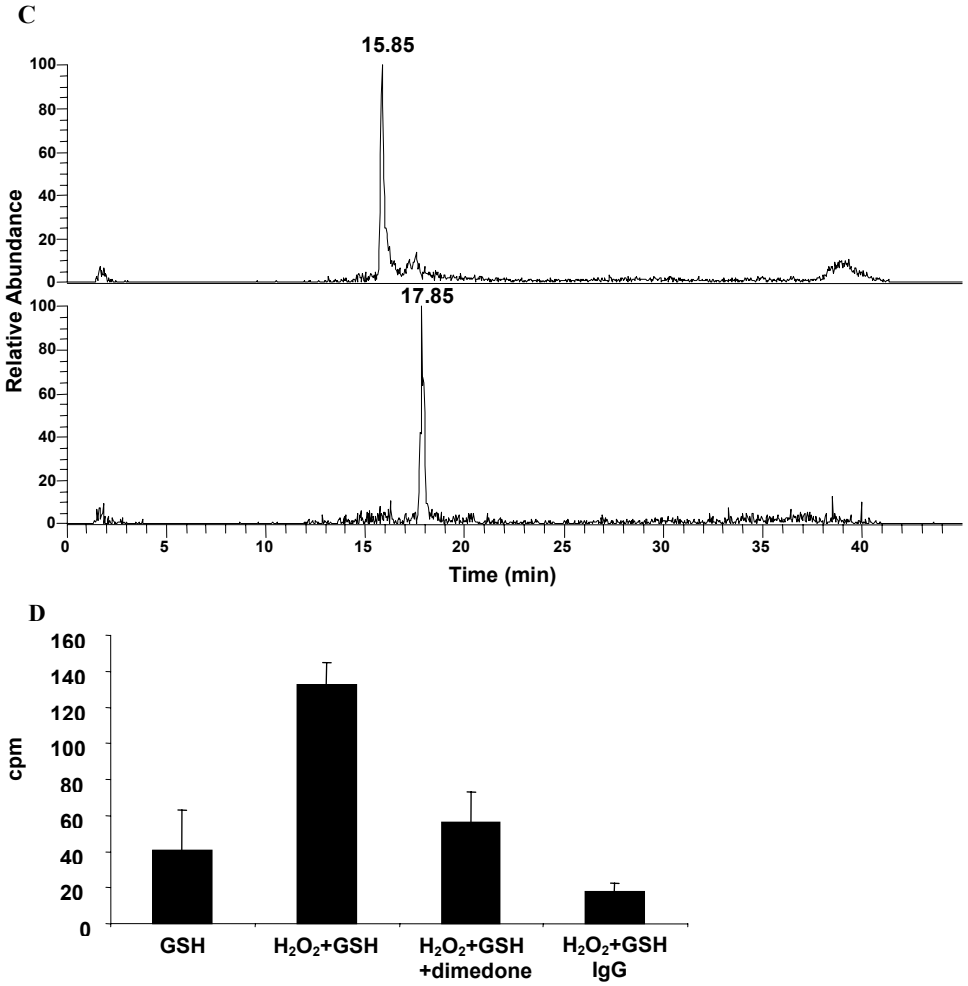


Figure 1 Inhibition of IKK activity through reversible oxidation of Cys179 of IKKβ. *(A)* C10 cells transfected with Wt (▪) or Cys179Ala (C179A) (▫) HA-IKKβ expression vectors were exposed to 10 ng/ml TNFα alone or in combination with 200 μM H_2O_2 for 5 min and IKK activity was assessed. *(B)* C10 cells transfected with Wt (▪) or C179A (▫) HA-IKKβ expression vectors in combination with pcDNA3 or expression vectors of Nox1 plus its co-activators p41 and p51 were stimulated with 10 ng/ml TNFα for 5 min, prior to assessment of IKKβ activity. IB:HA represent Western blots for HA in *(A)* and *(B)*. Lower panels represent quantification using PhosphoImage analysis in *(A)* and *(B)*. *(C)* C10 cells were treated with 200 μM H_2O_2 for 5 min, washed and supplied with fresh media. At different time points thereafter, cells were stimulated with 10 ng/ml TNFα for 5 min and assayed for IKKβ activity (top). Middle panel: Western blot for IKKγ; lower panel: quantification by PhosphoImage analysis. *(D)* C10 cells were transfected with Wt HA-IKKβ, exposed to 200 μM H_2O_2 for 5 min and oxidation of IKKβ was assessed by 2D gel-electrophoresis. To determine the reversibility of the oxidation, cells were incubated with 10 mM DTT for 30 min after treatment with H_2O_2.

To determine whether inhibition of IKKβ by H_2O_2 is reversible, cells were treated with H_2O_2 for 5 min, washed, and at different times thereafter stimulated with TNFα. The ability of TNFα to activate IKKβ started to recover as soon as 5 min after the removal of H_2O_2, with an approximate 61% recovery of maximal TNFα-stimulated activity (Fig. 1*C*). Two dimensional gel-electrophoresis demonstrated that H_2O_2 induced a shift in the apparent pI of wt HA-IKKβ from 5 to 4.4, which was fully reversed by the reducing agent DTT (Fig. 1*D*), consistent with reversible oxidation of IKKβ.

S-glutathionylation of IKKβ 173-187 peptide via the formation of a sulfenic acid intermediate. To investigate how Cys179 of IKKβ is modified under oxidizing conditions, we used a synthetic 15 amino acid peptide corresponding to the primary sequence of mouse IKKβ containing Cys179 (IKKβ 173-187, Online Fig. 1*A*). Analysis by ESI-LCMS revealed S-glutathionylation of the peptide in the presence of H_2O_2 and GSH, indicated by a mass increase of 305 Da, which could be fully reversed by incubation with DTT (Online Table 1) and prevented by pretreatment with the sulfhydryl-specific alkylating agent, *N*-ethylmaleimide (data not shown). These findings indicate that GSH incorporation occurred at Cys179, which was confirmed by MS/MS (data not shown). Incubation of the IKKβ173-187 peptide with GSSG similarly resulted in S-glutathionylation (Online Fig. 1*B*, Online Table 1). Despite not being able to directly detect a sulfenic acid in IKKβ 173-187 after exposure to H_2O_2 alone, presumably because it further reacted with a second peptide to form a disulfide, H_2O_2-mediated S-glutathionylation of IKKβ 173-187 (Online Table 1, Online Fig. 1*C)*, or immunoprecipitated wt HA-IKKβ (Online Fig. 1*D*), was prevented in the presence of the sulfenic acid trapping agent, dimedone. Quantitative assessment of cellular levels of GSH, GSSG or S-glutathionylated proteins (PSSG) in response to 200 μM H_2O_2 (Online Table 2) revealed transient decreases in GSH which were accompanied by 3-fold increases in PSSG. In contrast, cellular levels of GSSG increased only modestly, suggesting that protein S-glutathionylation occurred likely by protein sulfenic acid formation rather than by intermediate formation of GSSG.





Online Figure 1 S-glutathionylation of IKKβ 173-187 peptide via the formation of a sulfenic acid intermediate. All measurements of the synthetic 15-residue peptide of the primary 173-187 residue sequence (including Cys179) of mouse IKKβ (Invitrogen) were made by electrospray ionization (ESI) liquid chromatography-mass spectrometry (LCMS). Immediately at the completion of each experiment conducted with the peptide, a 10-µL aliquot (5 µg of peptide) was injected onto the HPLC of the LCMS (Thermo-Finnigan DECA-XPplus LCQ). HPLC conditions were a C18 1-mm x 150-mm column with a flow rate of 50 µL/min and a program starting with an initial composition of 85% water/15% methanol ramped to 35% water/65% methanol by 10 min, then held for 10 min, followed by a final ramp to 100% methanol at 32 min. To improve ionization, 0.1% formic acid was added to the HPLC solvents. ESI conditions were 4.5 kV on the needle with a small nitrogen sheath-gas flow added. The mass spectrometer ion trap was continuously scanned over a mass range of m/z = 400 – 2000 and spectra collected throughout each HPLC run. In some experiments, data-dependent tandem mass spectrometry was also performed on eluting peaks to obtain MS/MS spectra to provide structural information. *(A)* Electrospray ionization mass spectrum of the IKKβ 173-187

synthetic peptide eluting from the liquid chromatograph. Based on the monoisotopic molecular weight of the peptide (1567.8 Da), the protonated molecular ($[M+H]^+$), sodium adduct ($[M+Na]^+$) and doubly charged molecular ($[M+2H]^{2+}$) ions appear at the expected masses, as shown in the figure. *(B)* Electrospray ionization mass spectrum of the IKKβ 173-187 synthetic peptide after treatment with 0.5 mM GSSG for 15 min. Formation of a thiol-thiol bond between the cysteine of a glutathione and the peptide cysteine would increase the monoisotopic molecular weight to 1872.8 Da. The addition of the glutathione to the peptide is demonstrated by the protonated molecular ($[M+H]^+$), sodium adduct ($[M+Na]^+$) and doubly charged molecular ($[M+2H]^{2+}$) ions appearing at the expected masses. *(C)* Extracted ion chromatogram of the $[M+H]^+$ ion range m/z = 1567-1570 (around the monoisotopic mass of m/z = 1568.8), showing the elution of untreated peptide at 15.85 min (upper panel). The extracted ion chromatogram of the $[M+H]^+$ ion range m/z = 1707-1709 (around the monoisotopic mass of m/z = 1706.8) of the peptide treated for 15 min with 200 μM H_2O_2 plus 1 mM dimedone (5,5-dimethyl-1,3-cyclohexane-dione) eluting at 17.85 min (lower panel). There is an increase in mass of 138 Da going from untreated to H_2O_2 plus dimedone-treated peptide reflecting the covalent attachment of a dimedone (140.2 Da) with the loss of 2 hydrogens. *(D)* WT HA-IKKβ was immunoprecipitated from C10 cells using an antibody directed against HA and reacted for 30 min at RT with 200 μM H_2O_2, 250 μM ^{3}H-GSH (660 cpm/pmol specific activity diluted in cold GSH) and 500 μM dimedone in deoxygenized HBSS supplemented with 4 mM EDTA and 0.4 mM neocuproine. After washing the immunoprecipitates four times with HBSS, cpm were registered and corrected for background. A control immunoprecipitation with a normal mouse IgG was performed.

TREATMENT	% S-glutathionylated
Sham	**0**
GSH 0.5 mM, 15 min RT	**0**
GSH 0.5 mM + H_2O_2 200 μM, 15 min RT	**55**
GSH 0.5 mM + H_2O_2 200 μM, 15 min RT → DTT 15 min, 55 ºC	**0**
GSH 0.5 mM + H_2O_2 200 μM + 1 mM dimedone, 15 min RT	**0**
GSSG 0.5 mM, 15 min RT	**94.6**

Online Table 1 S-glutathionylation of IKKβ 173-187 peptide, MS summary. IKKβ 173-187 peptide was incubated with various agents and analyzed by LC-MS. Results are expressed as % S-glutathionylated compared to unmodified peptide.

S-glutathionylation of Cys 179 of IKKβ corresponds to inactivation by H_2O_2. To assess whether S-glutathionylation of Cys179 is responsible for the observed reversible inhibition of IKK in H_2O_2-exposed cells, we employed two methods to demonstrate S-glutathionylation of IKKβ. First, S-glutathionylated proteins were precipitated from extracts of cells expressing either wt or Cys179Ala HA-IKKβ using an antibody directed against GSH (Virogen, Watertown, MA). As is demonstrated in Fig 2*A*, H_2O_2 caused an increase in S-glutathionylation of wt IKKβ, but not the

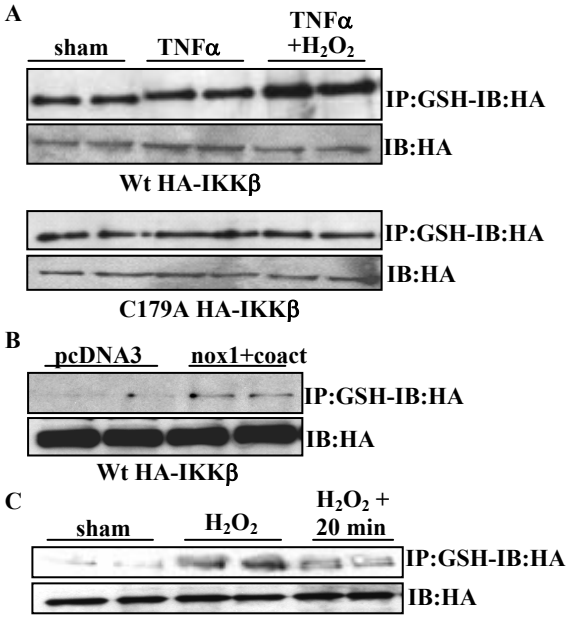
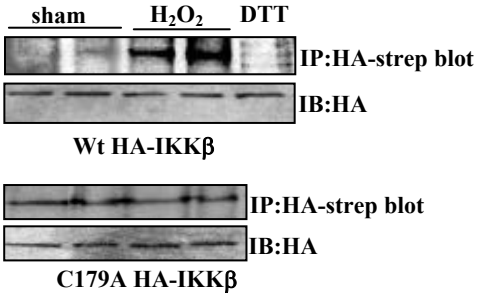


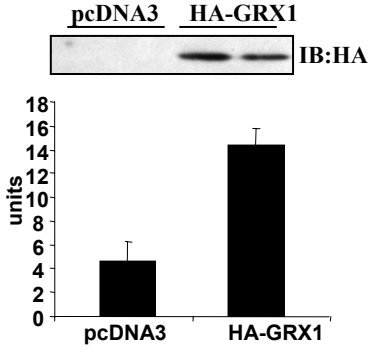
Figure 2 S-glutathionylation of Cys179 of IKKβ corresponds to inactivation by H_2O_2. (*A*) C10 cells transfected with Wt or C179A HA-IKKβ expression vectors were exposed to 10 ng/ml TNFα with or without 200 μM H_2O_2 for 5 min. S-glutathionylated proteins were immunoprecipitated using an antibody directed against GSH, followed by detection of HA-IKKβ by Western blotting. *(B)* C10 cells were transfected with Wt HA-IKKβ plus Nox1, p41, and p51 expression vectors and S-glutathionylation was investigated as in *(A)*. *(C)* C10 cells transfected with Wt HA-IKKβ expression vector were treated with 200 μM H_2O_2 for 5 min. The cells were washed, supplied with fresh media and harvested after 20 min. S-Glutathionylation of IKKβ was investigated as in *(A)*. IB:HA represents control Western blot for HA.

Cys179Ala mutant, whereas TNFα itself had no effect on S-glutathionylation of IKKβ, nor did it influence S-glutathionylation of IKKβ by H_2O_2. Alternatively, cells were preloaded Bio-GEE prior to H_2O_2 treatment. Immunoprecipitation of HA tagged IKKβ followed by blotting with streptavidin–HRP demonstrated enhanced binding of Bio-GEE to wt IKKβ, but not the Cys179Ala mutant in response to H_2O_2 treatment (Online Fig. 2). As a control, a H_2O_2 treated sample was resolved under reducing conditions (DTT) which resulted in a loss of biotin reactivity (Online Fig. 2), consistent with the reversibility of S-glutathionylation (Online Table 1). Furthermore, endogenous generation of H_2O_2 by overexpression of nox1 plus its co-activators also induced S-glutathionylation of IKKβ (Fig. 2*B*), corresponding to the repression of IKK activity seen in Fig.1*B*. Lastly, in agreement with the partial recovery of the kinase activity of IKKβ 20 minutes after removal of H_2O_2 (Fig. 1*C*), S-glutathionylation of IKKβ was markedly diminished by that time (Fig 2*C*). Collectively, these data demonstrate that S-glutathionylation of Cys179 of IKKβ is associated with the reversible inhibition of kinase activity by H_2O_2.

GRX1 modulates the inhibitory effects of H_2O_2 on IKKβ and NF-κB. Our findings demonstrate that cells recover IKKβ activity rapidly upon removal of H_2O_2 (Fig. 1*C*), corresponding to the reversal of S-glutathionylation (Fig. 2*D*). S-glutathionylation may therefore constitute an important regulatory switch allowing rapid regeneration of IKK enzymatic activity through GRX-dependent catalysis. To directly test this hypothesis, we overexpressed cytosolic GRX1 (Online Fig. 3), which abolished the increase in H_2O_2-induced S-glutathionylation of IKKβ (Fig. 3*A*), and allowed activation of IKK enzymatic activity by TNFα in the presence of H_2O_2 (Fig. 3*B*). Consequently, overexpression of GRX1 resulted in marked TNFα–induced degradation of IκBα (Fig 3*C*), and recovery of NF-κB transcriptional activity (Fig. 3*D*) in the presence of H_2O_2. These data demonstrate that S-glutathionylation of Cys179 of IKKβ by H_2O_2 is indeed responsible for inhibition of IKKβ activity, and that inhibitory effects of H_2O_2 can be overcome by GRX1 overexpression, allowing activation of IKKβ and NF-κB in the presence of H_2O_2.



Online Figure 2 S-glutathionylation of Cys179 of IKKβ. C10 cells transfected with WT or C179A HA-IKKβ were loaded with 1.5 mM Bio-GEE for 1 h before treatment with 200 μM H_2O_2 for 5 min. HA-IKKβ was immunoprecipitated using an antibody directed against HA and biotinylation of IKKβ investigated by blotting with streptavidin-HRP (strep). As a control, a H_2O_2 treated sample was resolved under reducing conditions (+DTT).



Online Figure 3 Overexpression of HA-GRX1 in C10 cells. Top panel: Western blot for HA. Lower panel: GRX1 activity assayed using the β-Hydroxyethyl disulfide (HED) assay as previously described (30) with minor modifications. Briefly, cells were lysed in buffer containing 137 mM Tris-HCl (pH 8.0), 130 mM NaCl, 1% NP-40, cleared by centrifugation and equalized for protein content. GRX1 was immunopurified from lysates using a GRX1 antibody (Labfrontier) incubated with reaction buffer containing 137 mM Tris-HCl (pH 8.0), 0.5 mM GSH, 1.2 units GSSG reductase (Roche), 0.35 mM NADPH, 1.5 mM EDTA (pH 8.0) and 0.7 mM HED. The reaction was allowed to proceed at 30˚C and NAPDH consumption was followed spectrophotometrically at 340 nm. The specific enzymatic reaction rate was obtained by subtracting the enzymatic rate omitting the substrate HED from the enzymatic rate including the substrate in the reaction mixture. Data are expressed as units where 1 unit equals 1 μM NADPH/min/mg protein.

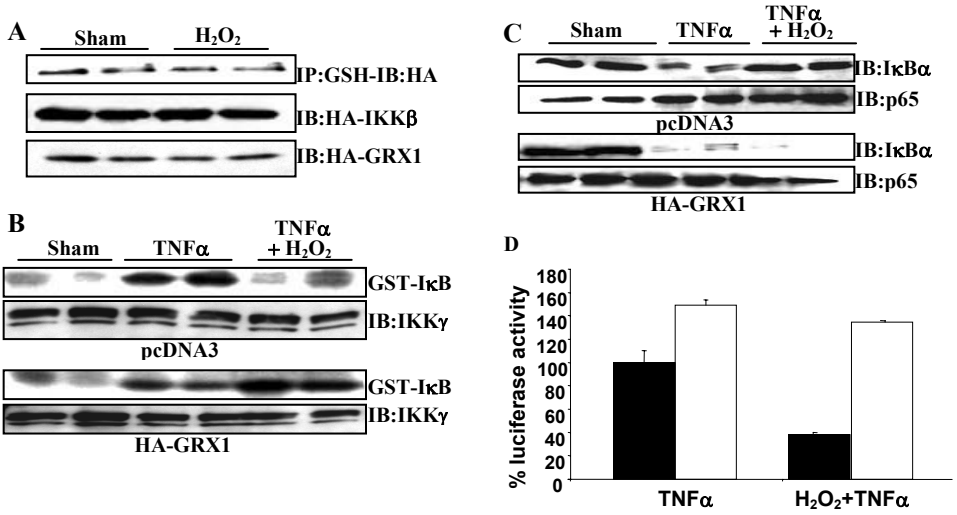
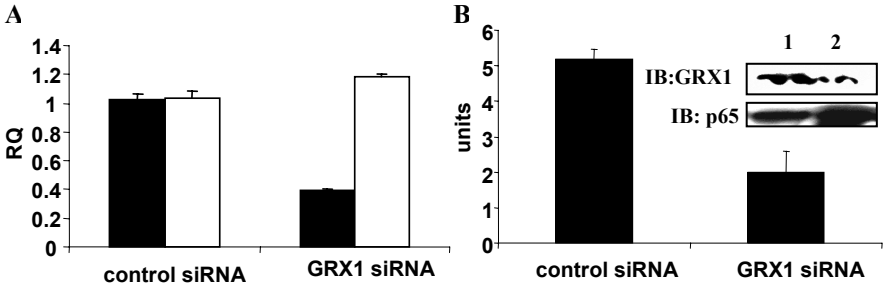


Figure 3 GRX1 modulates the inhibitory effects of H_2O_2 on IKKβ and NF-κB. *(A)* Cells were treated with 200 μM H_2O_2 for 5 min and S-glutathionylation of IKKβ was assessed as described in Fig. 2*B,* top panel. Middle and lower panels represent control Western blots for HA-IKKβ, and HA-GRX1, respectively. *(B,C)* C10 cells overexpressing HA-GRX1 were exposed to agents as before and evaluated after 5 min for IKK activity *(B)*, or after 15 min for IκBα levels *(C)*. The level of RelA (p65) was measured as a loading control. *(D)* Cells were co-transfected with 6 κB-tk-luc reporter vector and pcDNA3 (▪) or GRX1 (▫) expression vectors and exposed to 200 μM H_2O_2 for 5 min before treatment with 10 ng/ml TNFα for 6 h. Luciferase units were corrected for total amount of protein and expressed as percentage of pcDNA3 transfected, TNFα-stimulated luciferase activity.

Based on our observations that GRX1 activity is present in C10 cells (Online Fig. 3), we employed siRNA to knock down expression of GRX1. Results in Online Fig. 4*A* demonstrate that GRX1 siRNA resulted in the specific knock down of GRX1 mRNA by 60%, whereas GRX2 mRNA expression was unaffected. GRX1 protein levels and activity were inhibited to the same extent by siRNA (Online Fig. 4*B*). Importantly, siRNA knock down of GRX1 greatly sensitized cells for inhibition of IKKβ by H_2O_2 (Fig. 4*A*), leading to enhanced repression of NF-κB transcriptional activity (Fig. 4*B*). Moreover, the ability of TNFα to activate IKKβ and NF-κB transcriptional activity was also markedly attenuated following knock down of GRX1. Concomitantly, knock down of GRX1 increased the basal level of S-glutathionylation of IKKβ and enhanced the sensitivity of IKKβ to H_2O_2-induced S-glutathionylation (Fig.4*C*). Together these results indicate that GRX1 expression levels play an important role in controlling the magnitude of activation of the NF-κB pathway through regulation of S-glutathionylation of IKKβ.



Online Figure 4 Knock down of GRX1 using siRNA. C10 cells were transfected with control or GRX1 siRNA. *(A)* GRX1 (filled bars) and GRX2 (open bars) mRNA expression was assessed relative to HPRT by semi-quantitative TaqMan PCR using Assay On Demand for GRX1 and GRX2 from Applied Biosystems. Values were normalized to HPRT. *(B)* GRX1 activity and protein expression (Western blot insert) evaluated as described before.

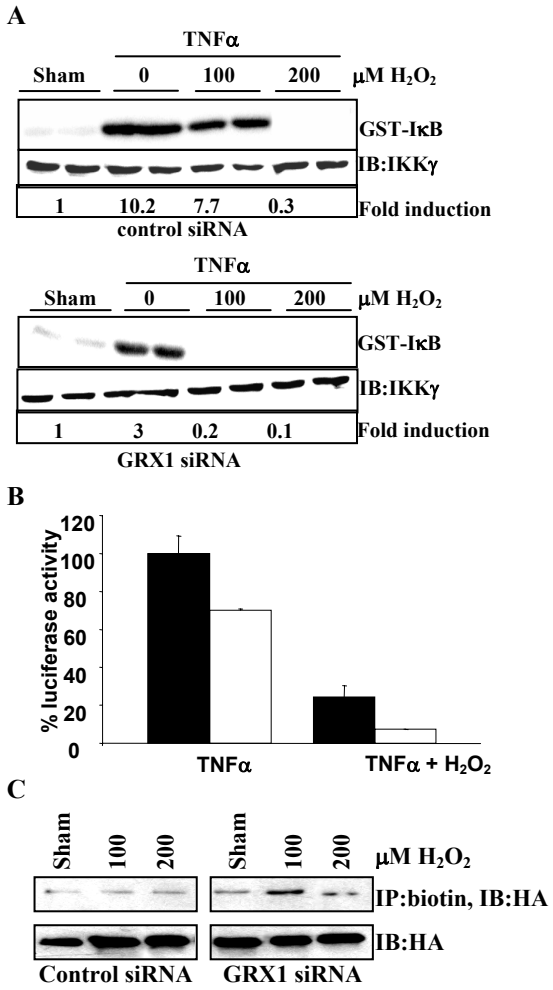


Figure 4 GRX1 knock-down dampens IKKβ and NF-κB activation. *(A)* C10 cells were transfected with control or GRX1 siRNA and treated with TNFα in presence or absence of H_2O_2 and as described before. Top panels represent IKK activity, and lower panels are IKKγ Western blots. Fold induction represent fold increases in IKK activity over sham controls, based upon PhosphoImage analysis. *(B)* C10 cells stably transfected with 6 κB-tk-luc were trans-fected with control siRNA (▪) or siRNA for GRX1 (▫), and treated with 200 μM H_2O_2 and 10 ng/ml TNFα for 6h. Luciferase units were corrected for amount of protein and expressed as percentage of control siRNA TNFα-stimu-lated luciferase activity. (*C*) C10 cells were transfected with HA-IKKβ and control or GRX1 siRNA. Cells were loaded with Bio-GEE and treated with H_2O_2. Biotinylated proteins were immunoprecipitated using a biotin antibody, followed by detection of IKKβ by Western blotting for HA. IB:HA represents control Western blot for HA.

Airway epithelial cells from *Glrx1*-deficient mice display attenuated activation of NF-κB and MIP-2 production in response to lipopolysaccharide (LPS). To further address the physiological relevance of GRX1-controlled IKK and NF-κB activation, we cultured primary tracheal epithelial cells (MTE) from WT or *Glrx1*$^{-/-}$ mice, and assessed the magnitude of activation of NF-κB by LPS. Compared to WT cells, *Glrx1*$^{-/-}$ cells have elevated levels of S-glutathionylated proteins (Fig. 5*A*). Importantly, MTE cells derived from *Glrx1*$^{-/-}$ mice stimulated with LPS displayed no apparent nuclear translocation of RelA, whereas marked RelA nuclear presence was observed in WT cells exposed to LPS (Fig. 5*B*). Similarly, NF-κB-DNA binding activity in response to LPS was also absent in *Glrx-/-* MTE cells in contrast to WT cells which show the expected increases in DNA binding activity in response to LPS (Fig. 5*C*). Lastly, *Glrx1*-deficient MTE cells produced markedly reduced levels of the chemokines, MIP-2 and KC after treatment with LPS when compared to MTE cells isolated from WT mice (Fig. 5*D*), illustrating the importance of GRX1 in the regulation of expression of pro-inflammatory mediators in association with its control over the activation of the NF-κB pathway.

DISCUSSION

Oxidation of Cys179 as a repressive mechanism for IKK activation. We have demonstrated in this study that Cys179 of the β subunit of the IKK complex is a direct target for reversible S-glutathionylation and that this is responsible for the repression of kinase activity by H_2O_2. Cys179 has previously been demonstrated to be redox sensitive and to be oxidized by arsenite (24) and S-nitrosothiols (26), amongst others. Cys179 is strategically located in the activation loop between Ser 177 and 181 that are required to be phosphorylated for IKK enzymatic activity. It is conceivable that oxidation of Cys179 could interfere with phosphorylation of the neighboring Ser residues, but evidence in the previous studies argues against this hypothesis (24, 26). Alternatively, oxidation of Cys179 could prevent the binding of substrate or accessory proteins to the complex or promote dephosphorylation of the neighboring Ser residues. Although additional investigations are needed to unravel the exact mechanism of oxidant inhibition of IKK activity, multiple studies have now underscored the importance of Cys179 in the redox regulation of the IKK complex and consequent NF-κB activation.

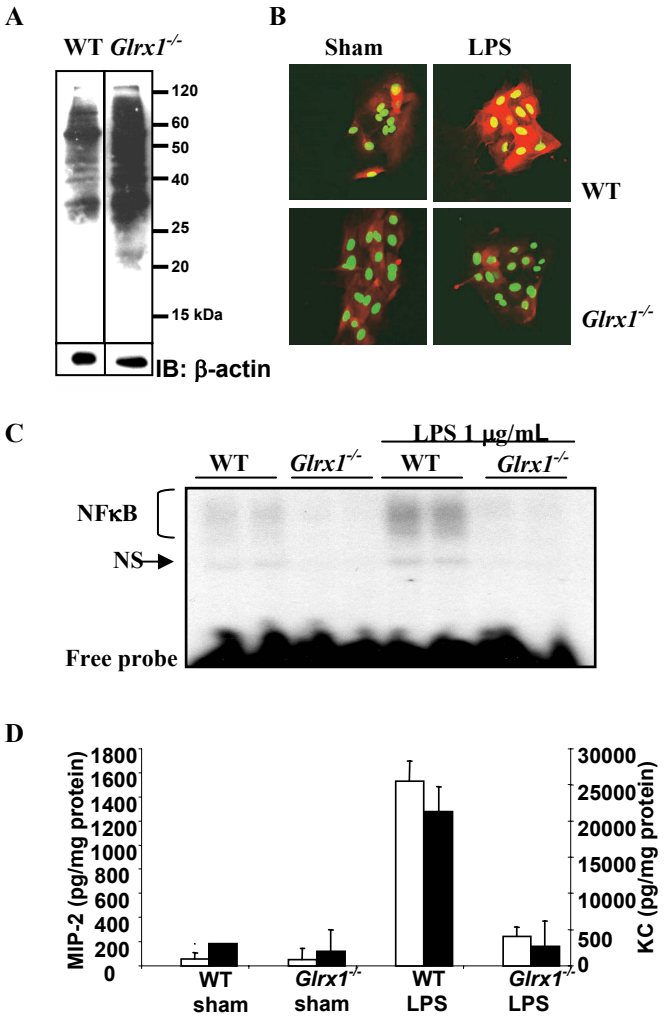


Figure 5 Attenuation of NF-κB activation and chemokine production by primary tracheal epithelial cells from *Glrx1*$^{-/-}$ mice in response to LPS. GRX activity in WT cells was 37.4 Units and undetectable in *Glrx1*$^{-/-}$ cells. (A) Primary tracheal epithelial cells (MTE) were loaded with 1.5 mM Bio-GEE for 1h, lysates resolved by non-reducing SDS-PAGE and blots reacted with Streptavidin-HRP. IB: β-actin, represents a loading control. (B) WT or *Glrx1*$^{-/-}$ MTE cells were treated with 1 μg/ml LPS for 4h for evaluation of RelA nuclear translocation. Red: RelA immunoreactivity, Green: nuclear Sytox Green counterstain. (C) WT or *Glrx1*$^{-/-}$ MTE cells were treated with 1 μg/ml LPS for 6h and NF-κB-DNA binding was assessed by EMSA. NS indicates non-specific binding. (D) WT and *Glrx1*$^{-/-}$ MTE cells were treated with 1 μg/ml LPS for 24h and KC (▪) and MIP-2 (▫) assessed by ELISA on culture media and corrected for protein content.

Redox regulation of the NF-κB activation pathway at multiple levels. It has been well established that NF-κB is a redox sensitive transcription factor. For example, S-glutathionylation of Cys62 of the p50 subunit is known to prevent binding of the transcription factor to κB sites in the promoter regions of genes (8, 31). Recent work from our laboratory has clearly demonstrated that the prerequisite NF-κB activating enzyme IKKβ is also regulated by oxidants (29), providing a second mode of redox control that occurs proximal to DNA binding. The observation that IKK activity remains partially repressed despite reversibility of its proper oxidation (Fig. 1*C* and *D*) suggests that H_2O_2 may also interfere with the activation pathway upstream of the kinase complex. In support of this possibility, our laboratory has demonstrated that H_2O_2 alters the recruitment of adapter proteins to TNF receptor 1, leading to repressed IKK and increased JNK activation in response to TNFα (32).

TREATMENT	GSH	GSSG	PSSG
Control	19.5 ± 0.4	0.39 ± 0.05	0.34 ± 0.07
H_2O_2 200 μM, 5 min	14.9 ± 1.9	0.48 ± 0.06	1.03 ± 0.16

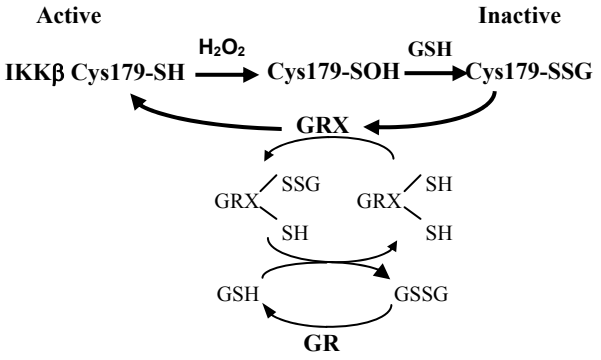
Online Table 2 Cellular levels of GSH, GSSG and S-glutathionylated proteins (PSSG), before and after exposure of C10 cells to 200 μM H2O2 for 5 min. For analysis of PSSG, cell proteins were precipitated with 5% TCA and reduced with 1 mM DTT, after which liberated GSH was determined as described by Jones et al. (*Clin.Chim.Acta*, 1998: 275). Data are expressed in nmol/mg protein (mean ± S.D. from 3 determinations)

S-glutathionylation of IKKβ via a sulfenic acid intermediate. The biochemical mechanisms by which proteins are S-glutathionylated involve either intermediate formation of protein sulfenic acid moieties that subsequently react with GSH, or accumulation of GSSG which then reacts with susceptible protein Cys residues via a thiol exchange mechanism. Studies with the IKKβ peptide and the intact IKK complex (Online Fig.1 and Online Table 1) indicate that H_2O_2-mediated S-glutathionylation of IKKβ occurs though a sulfenic acid intermediate, since it was prevented by the sulfenic acid reactive compound, dimedone. Although GSSG is capable of glutathionylating the IKKβ peptide IKK (Online Fig. 1*B*), *in vitro* kinase assays indicate that the observed levels of GSSG found in H_2O_2-treated cells (Online Table 2) are insufficient to inactivate IKK (data not shown). Indeed, the redox potential of most Cys residues is such that the ratio of GSSG versus GSH in cells would need to change

approximately 100-fold in order to induce S-glutathionylation through a thiol-exchange mechanism (10), which we did not observe, perhaps in part due to the buffering capacity of glutathione reductase. These considerations suggest that thiol-exchange is probably not a significant mechanism for S-glutathionylation of IKKβ *in vivo*, and favor direct intermediate oxidation of Cys179 to a sulfenic acid.

GRX1-controlled NF-κB activation. S-glutathionylation is considered a redox-dependent posttranslational modification with potential relevance to signal transduction. The existence of antioxidant enzymes that serve the unique role of specifically reducing glutathione mixed disulfides emphasizes the importance of S-glutathionylation in modulating protein function. Our findings demonstrate that modulation of GRX1 levels, through both overexpression and knock down by RNA interference, influences S-glutathionylation and consequently impinges upon IKK and NF-κB activation following H_2O_2 or TNFα exposure. The strongly attenuated responses of primary airway epithelial cells from *Glrx1*$^{-/-}$ mice to LPS with regard to NF-κB-DNA binding, RelA nuclear translocation, and chemokine production (Fig. 5) further support a role for GRX1 in regulating inflammation by controlling the magnitude of activation of the NF-κB pathway.

Collectively, the present data demonstrate that S-glutathionylation is a physiologically relevant mechanism for controlling the magnitude of activation of the NF-κB pathway. We propose that GRX1-dependent reversal of S-glutathionylation of IKKβ constitutes a protective mechanism that modulates the extent and timing of activation of NF-κB in response to redox changes by protecting IKKβ from irreversible inactivation (33), and allowing for rapid regeneration of enzymatic activity (Online Fig. 5). As emerging studies document the relevance of H_2O_2 as a second messenger (34) and unravel the intricacies of redox control of biological processes, our findings suggest broad implications in diverse (patho)physiological conditions, many of which have been causally linked to the activation of NF-κB and oxidative stress.



Online Figure 5 Proposed model for the IKKβ redox cycle. Treatment of cells with H_2O_2 results in the S-glutathionylation of IKKβ at Cys179 (-SSG). GRX1-catalyzed reversal of Cys179-SGG restores the reduced cysteine (-SH), thereby rapidly regenerating kinase activity. GRX will become S-glutathionylated itself in this reaction and its reduced state is restored by GSH/glutathione reductase (GR).

EXPERIMENTAL PROCEDURES

Cell culture and reagents. A line of spontaneously transformed mouse alveolar type II epithelial cells (C10) was propagated in CRML-1066 medium containing 50 U/ml penicilline-50μg/ml streptomycin (P/S), 2mM L-glutamine and 10% fetal bovine serum (FBS), all from GIBCO-BRL. One h before adding the test agents, cells were switched to phenol red-free DMEM/F12 containing P/S and 0.5% FBS. Primary tracheal epithelial cells were isolated from homozygous GRX1 knock-out (*Glrx1*$^{-/-}$) mice in C57BL/6 and 129SV hybrid background and genetically matched wild-type mice according to Wu et al. (35) with minor modifications (36). The knock-out mice were generated by deleting exons 1 and 2 of the *Glrx1* gene, resulting in abolishment of GRX1 expression in all tissues (Ho et al., unpublished observations). For experiments, primary cells were plated on Collagen I coated culture dishes or glass slides. The Institutional Animal Care and Use Committee granted approval for all procedures. Reagents were purchased from Sigma (St. Louis, MO), unless stated otherwise. Experiments were repeated at least three times.

Kinase assays. C10 cells were exposed to test agents, transferred to ice, washed twice with PBS, and lysed in buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM Na_3VO_4, 1 mM PMSF, 0.1 % NP40, 10 μg/ml leupeptin, 1 % aprotenin, 250 μM DTT, 100 μM NaF. The IKK complex was immunoprecipitated from 200 μg

protein with an IKKγ (Santa Cruz Biotechnology) or HA antibody (Upstate) using protein G agarose beads. Precipitates were washed once with lyses buffer and twice with kinase buffer (20 mM HEPES/ 20 mM β-glycerolphosphate/ 1 mM $MnCl_2$, 5 mM $MgCl_2$/ 2 mM NaF/ 250 μM DTT). Kinase reactions were performed with 1 μg GST-IκBα as a substrate, provided by Dr. Rosa Ten (Mayo Clinic, Rochester, MN), and 5 μCi [γ^{32}P]adenosine triphosphate at 30°C for 30 min. Reactions were stopped by addition of 2x Laemmli sample buffer. Samples were boiled and separated on 15% polyacrylamide gel, gels were dried and examined by autoradiography. Results were quantitated by phospho-imaging.

Western blotting. Cell lysates were resolved on polyacrylamide gels, transferred to nitrocellulose and levels of IκBα, IKKγ, IKKβ, p65 and HA were detected according to the following immunoblotting protocol: membranes blocked in TBS/5% milk were washed two times in TBS containing 0.05% Tween-20 (TBS-Tween) and incubated with primary antibodies for 1h at RT. Membranes were washed three times in TBS-Tween, and incubated with a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1h at RT. After three washes with TBS-Tween, conjugated peroxidase was detected by chemiluminescence according to the manufacturer's instructions (Amersham, Biosciences, Piscataway, NJ).

Two-dimensional gel electrophoresis. At the indicated times, cells were washed twice with 10 mM Tris, 25 mM sorbitol pH 7.0 and lysed in sample buffer containing 9 M urea, 2% CHAPS, 1 mM EDTA, 4 mM AEBSF, 0.8% ampholytes (Amersham Biosciences), 0.007% bromophenol blue and 10mM iodoacetamide. Lysates were cleared by centrifugation and applied to an immobilized pH gradient (pH 3-10, 13cm, Amersham Biosciences). Strips were rehydrated with sample for 10 h at 50V and iso-electric focusing was performed for a total of 13299 Vh at a maximum of 8000 volts. Each strip was equilibrated two times 15 min in buffer containing 6 M urea, 30% glycerol, 50 mM Tris-HCl pH 6.8, 0.007% bromophenol blue, 2% SDS and 65 mM DTT. The second dimension was carried out by 10% SDS-PAGE, proteins were transferred to nitrocellulose and Western blot was performed as described above.

Transfections. The expression vectors of HA-tagged WT and Cys179Ala (C179A) mutant IKKβ (HA-IKKβ) were a gift of Dr. Karin, University of California, San Diego, La Jolla, CA. The expression vectors of nox1, p41

nox and p51 nox were a gift of Dr. Lambeth, Emory University, Atlanta, GA and the HA-tagged mouse GRX1 expression plasmid was made as previously described (36). Plasmids were transfected into cells using Lipofectamine plus (Invitrogen) according to the manufacturer's directions. Two h after transfection with nox1 and 24 h after transfection with other plasmids, test reagents were added. Control and GRX1 siRNA were purchased from Ambion and transfected into cells at a concentration of 20 nM using siPORTamine and test agents were added 48h later.

Biotinylated glutathione ethyl ester (Bio-GEE) preparation and assessment of S-glutathionylation. Bio-GEE was prepared as described in (37). Cells were loaded with 1.5mM Bio-GEE for 1h prior to the addition of test agents and lysed in buffer containing 25 mM Hepes pH 7.7, 0.1 mM EDTA, 0.01 mM neocuproine, 0.5% CHAPS and 20 mM *N*-ethylmaleimide. After resolution by SDS-PAGE electrophoresis, proteins were transferred to nitrocellulose and membranes were blocked in TBS containing 5% BSA at RT. After two washes in TBS-Tween membranes were incubated with streptavidin-peroxidase for 4h at RT to detect biotinylated proteins. Alternatively, biotinylated proteins were immunoprecipitated using an antibody directed against biotin, followed by detection of HA-IKKβ by immunoblotting.

NF-κB luciferase reporter assay. A stable C10 cell line harboring a 6x κB-tk-luc reporter gene (29) was transfected with plasmids or siRNA. Luciferase units were corrected for total amount of protein.

Electrophoretic Mobility Shift Assay. To assay DNA binding activity of NF-kB complexes, binding to a radiolabeled double-stranded oligonucleotide containing a NF-kB consensus sequence was analyzed (Promega, Madison, WI). Nuclear extracts and gel shift assays were prepared as described previously (38).

RelA immunostaining. Cells were fixed with 4% PFA for 30 min at RT and permeabilized for 20 min using 0.2% Triton X-100. After blocking with 1% BSA for 30 min, cells were incubated with an antibody directed against RelA (Santa Cruz) for 1h followed by a 1h incubation with an Alexa-Fluor568 conjugated secondary antibody. Nuclei were counterstained with Sytox Green (Molecular Probes). Sections were scanned using an Olympus BX50 upright microscope configured to a Bio-Rad (Hercules, CA) MRX 1000 confocal scanning laser microscope system.

ELISA. MIP-2 and KC concentrations were determined in cell culture media using a DuoSet ELISA kit (R&D Systems). Values were normalized to a standard curve, corrected for protein content, and expressed as pg/mg protein.

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REFERENCES

1. Lambeth, J. D. (2004) *Nat Rev Immunol* **4,** 181-9.
2. Budanov, A. V., Sablina, A. A., Feinstein, E., Koonin, E. V. & Chumakov, P. M. (2004) *Science* **304,** 596-600.
3. Woo, H. A., Jeong, W., Chang, T. S., Park, K. J., Park, S. J., Yang, J. S. & Rhee, S. G. (2005) *J Biol Chem* **280,** 3125-8.
4. Chang, T. S., Jeong, W., Woo, H. A., Lee, S. M., Park, S. & Rhee, S. G. (2004) *J Biol Chem* **279,** 50994-1001.
5. Mallis, R. J., Buss, J. E. & Thomas, J. A. (2001) *Biochem J* **355,** 145-53.
6. Wang, J., Boja, E. S., Tan, W., Tekle, E., Fales, H. M., English, S., Mieyal, J. J. & Chock, P. B. (2001) *J Biol Chem* **276,** 47763-6.
7. Davis, D. A., Dorsey, K., Wingfield, P. T., Stahl, S. J., Kaufman, J., Fales, H. M. & Levine, R. L. (1996) *Biochemistry* **35,** 2482-8.
8. Pineda-Molina, E., Klatt, P., Vazquez, J., Marina, A., Garcia de Lacoba, M., Perez-Sala, D. & Lamas, S. (2001) *Biochemistry* **40,** 14134-42.
9. Klatt, P., Molina, E. P. & Lamas, S. (1999) *J Biol Chem* **274,** 15857-64.
10. Shelton, M. D., Chock, P. B. & Mieyal, J. J. (2005) *Antioxid Redox Signal* **7,** 348-66.
11. Su, D. & Gladyshev, V. N. (2004) *Biochemistry* **43,** 12177-88.
12. Gladyshev, V. N., Liu, A., Novoselov, S. V., Krysan, K., Sun, Q. A., Kryukov, V. M., Kryukov, G. V. & Lou, M. F. (2001) *J Biol Chem* **276,** 30374-80.
13. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M. & Holmgren, A. (2001) *J Biol Chem* **276,** 26269-75.
14. Gravina, S. A. & Mieyal, J. J. (1993) *Biochemistry* **32,** 3368-76.
15. Yang, Y., Jao, S., Nanduri, S., Starke, D. W., Mieyal, J. J. & Qin, J. (1998) *Biochemistry* **37,** 17145-56.
16. Fernandes, A. P. & Holmgren, A. (2004) *Antioxid Redox Signal* **6,** 63-74.
17. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. & Karin, M. (1997) *Nature* **388,** 548-54.
18. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A. & Rao, A. (1997) *Science* **278,** 860-6.
19. May, M. J. & Ghosh, S. (1999) *Science* **284,** 271-3.
20. Anest, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D. & Baldwin, A. S. (2003) *Nature* **423,** 659-63.
21. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T. & Gaynor, R. B. (2003) *Nature* **423,** 655-9.
22. Rothwarf, D. M., Zandi, E., Natoli, G. & Karin, M. (1998) *Nature* **395,** 297-300.
23. Delhase, M., Hayakawa, M., Chen, Y. & Karin, M. (1999) *Science* **284,** 309-13.
24. Kapahi, P., Takahashi, T., Natoli, G., Adams, S. R., Chen, Y., Tsien, R. Y. & Karin, M. (2000) *J Biol Chem* **275,** 36062-6.
25. Ji, C., Kozak, K. R. & Marnett, L. J. (2001) *J Biol Chem* **276,** 18223-8.
26. Reynaert, N. L., Ckless, K., Korn, S. H., Vos, N., Guala, A. S., Wouters, E. F., van der Vliet, A. & Janssen-Heininger, Y. M. (2004) *Proc Natl Acad Sci U S A* **101,** 8945-50.
27. Jeon, K. I., Byun, M. S. & Jue, D. M. (2003) *Exp Mol Med* **35,** 61-6.
28. Kwok, B. H., Koh, B., Ndubuisi, M. I., Elofsson, M. & Crews, C. M. (2001) *Chem Biol* **8,** 759-66.

29. Korn, S. H., Wouters, E. F., Vos, N. & Janssen-Heininger, Y. M. (2001) *J Biol Chem* **276,** 35693-700.
30. Luthman, M. & Holmgren, A. (1982) *J Biol Chem* **257,** 6686-90.
31. Matthews, J. R., Botting, C. H., Panico, M., Morris, H. R. & Hay, R. T. (1996) *Nucleic Acids Res* **24,** 2236-42.
32. Pantano, C., Shrivastava, P., McElhinney, B. & Janssen-Heininger, Y. (2003) *J Biol Chem* **278,** 44091-6.
33. Adachi, T., Weisbrod, R. M., Pimentel, D. R., Ying, J., Sharov, V. S., Schoneich, C. & Cohen, R. A. (2004) *Nat Med* **10,** 1200-7.
34. Finkel, T. (2000) *FEBS Lett* **476,** 52-4.
35. Wu, R. & Smith, D. (1982) *In Vitro* **18,** 800-12.
36. Reynaert, N. L., Ckless, K., Guala, A. S., Wouters, E. F., van der Vliet, A. & Janssen-Heininger, Y. M. (2006) *Biochim Biophys Acta* **1760,** 180-187.
37. Demasi, M., Silva, G. M. & Netto, L. E. (2003) *J Biol Chem* **278,** 679-85.
38. Janssen, Y. M., Driscoll, K. E., Howard, B., Quinlan, T. R., Treadwell, M., Barchowsky, A. & Mossman, B. T. (1997) *Am J Pathol* **151,** 389-401.

CHAPTER 6

In situ detection of S-glutathionylated proteins following glutaredoxin-1 catalyzed cysteine derivatization

ABSTRACT

S-glutathionylation is rapidly emerging as an important post-translational modification, responsible for transducing oxidant signals. However, few approaches are available that allow visualization of glutathione mixed disulfides in intact cells. We describe here a glutaredoxin 1-dependent cysteine derivatization and labeling approach, in order to visualize S-glutathionylation patterns *in situ*. Using this new method, marked S-glutathionylation was observed in epithelial cells, which was predominant at membrane ruffles. As expected, the labeling intensity was further enhanced in response to bolus oxidant treatments, or in cells overexpressing Nox1 plus its coactivators. In addition, manipulation of endogenous levels of glutaredoxin 1 via RNAi, or overexpression resulted in altered sensitivity to H_2O_2 induced formation of glutathione mixed disulfides. Overall, the derivatization approach described here preferentially detects S-glutathionylation and provides an important means to visualize this post-translational modification in sub-cellular compartments, and to investigate its relation to normal physiology as well as pathology.

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INTRODUCTION

The tripeptide glutathione is considered one of the major anti-oxidants of the human body, with cellular concentrations in the millimolar range. A number of enzyme systems exist that are dedicated to maintaining glutathione homeostasis, including the rate-limiting enzyme for its synthesis, γ-glutamylcysteine synthetase, and glutathione reductase, which reduces GSSG, using NADPH as a cofactor. Glutathione serves a major role in maintaining the reduced state of cellular protein thiol groups. It accomplishes this role mainly through the function of glutathione peroxidases, which utilize GSH to reduce hydroperoxides [1]. In addition, upon oxidative stress, glutathione will form mixed disulfides with protein thiol groups, causing reversible S-glutathionylation [2]. The commonly held notion is that S-glutathionylation of thiols confers protection against their irreversible oxidation, like for instance the formation of sulphonic acid moieties. If the targeted cysteine is a functionally critical amino acid, S-glutathionylation will however also modify protein function. For instance S-glutathionylation of the p50 subunit of NF-κB [3] as well as of the c-Jun subunit of AP-1 [4] have been linked to repression of DNA binding activity of these transcription factors. The activities of protein kinase C [5], glyceraldehyde-3-phosphate dehydrogenase [6] and HIV-1 protease [7] have been also demonstrated to be adversely affected by S-glutathionylation. On the other hand, S-glutathionylation of peroxiredoxin 6, mediated by πGST, appears to be required for its activity [8].

Mammalian glutaredoxins (GRX), or thioltransferases, are members of the thiol-disulfide oxidoreductase family that contain the typical thioredoxin fold [9]. Two mammalian GRX enzymes have been characterized to date. GRX1 is a cytosolic protein with a Cys-Pro-Tyr-Cys active site, whereas GRX2 contains a Cys-Pro-Phe-Cys active site and is directed to the mitochondria by a mitochondrial leader sequence and can also occur in the nucleus following alternative splicing [10-12]. GRX catalyze the reversible reduction of protein-glutathionyl-mixed disulfides to free sulfhydryl groups through a monothiol mechanism that only depends on the N-terminal Cys22 that displays an unusual low pK_a (3.5) [13, 14]. Cys22 will become S-glutathionylated itself in this reaction and the reduced state of GRX will subsequently be restored using GSH coupled to GSSG reductase [14]. GRX2 in addition can be reduced by the thioredoxin/ thioredoxin reductase system [11]. In addition to the monothiol reduction of glutathionyl disulfides, bacterial GRX have been demonstrated to reduce low molecular weight disulfides as well as

disulfides in ribonucleotide reductase through a dithiol mechanism [15]. For mammalian GRX, substrate specificity towards S-glutathionylated proteins has been demonstrated [13] and GRXs could therefore play a unique role in redox signaling.

The detection and identification of S-glutathionylated proteins has mainly been limited to cell lysates, and purified proteins, and has relied on the use of HPLC based methods [16], ^{35}S-Cys-radiolabeled GSH [17, 18], biotinylated glutathione [19, 20] and more recently, an antibody directed against GSH [21]. In addition, the anti-glutathione antibody has been employed to visualize S-glutathionylated proteins in intact cells. Since this antibody can also recognize GSSG, the specificity of this method depends on the complete elimination of GSSG [22]. More recently, a glutathione-S-transferase overlay approach was applied to detect S-glutathionylated proteins in lysates and tissue, but this method would also detect free GSH [23].

Given the limitations associated with the current methods for detection of S-gutathionylated proteins, we utilized the catalytic activity of GRX1 to specifically derivatize protein-glutathionyl-mixed disulfides in intact cells. Through the comprehensive implementation of diverse reagent controls, mouse GRX1 overexpression and knock-down approaches we demonstrate the specificity of detection of S-gutathionylated proteins in intact cells, and reveal their unique cellular localization in response to oxidative stress.

MATERIALS AND METHODS

Cell culture and reagents. A line of spontaneously transformed mouse alveolar type II epithelial cells (C10) [24] was propagated in cell culture media-1066 containing 50 units/ml penicillin and 50 mg/ml streptomycin (P/S), 2 mM L-glutamine and 10% FBS, all from GIBCO/BRL. For experiments involving microscopic analysis, cells were grown on glass coverslips. One h before exposure to test agents the cells were switched to phenol red free DMEM/F12, containing 0.5% FBS and P/S.
Primary epithelial cells were isolated from C57BL/6 according to Wu and Smith [25] with minor modifications. Briefly, trachea were cannulated, filled with MEM media containing 0.1% Protease 14, tied-off and removed from the mouse. After overnight incubation at 4°C in MEM, cells

were dislodged by opening the ends of the trachea and flushing through 5ml of MEM containing 10% FBS. Cells were pelleted and plated on collagen gel coated tissue culture flasks in DMEM/F12 media containing 20ng/ml Cholera toxin, 4μg/ml Insulin, 5μg/ml Transferrin, 5 μg/ml Bovine Pituitary Extract, 10 ng/ml EGF, 100 nM dexamethasone, 2mM L-Glutamine and P/S. For experiment, cells were plated on Collagen I coated glass slides. The Institutional Animal Care and Use Committee granted approval for all procedures.
All reagents were purchased from Sigma unless otherwise stated.

Vector construction and transfection. Full length mouse glutaredoxin (GRX)1 was amplified from mouse lung cDNA using PCR with 5'-CATGGCTCAGGAGTTTGTGA-3' as the 5'-primer and 5'-GCCACCCCTTT TATAACTGC-3' as 3'-primer and inserted into TA cloning vector. GRX1 was amplified from this vector using 5'-primer 5'-CCGGATCCATGTACCCATACACGTCCCAGACTACGCTGCTCAGG AGTTTTGTGAACTG-3' that introduced a BamHI site, a start codon and HA sequence and as the 3'-primer, 5'-GCCACCCCTTTTA TAACTGCGAATTCCGG-3', inserting an EcoRI site and a stop codon. The amplified fragment was digested using BamHI and EcoRI and cloned into pcDNA3 expression vector.
Plasmids for Nox1, p41 Nox and p51 Nox were gifts of Dr. David Lambeth, Emory University, Atlanta, Ga. C10 cells were transfected with 1 μg HA-GRX1 or pcDNA3 or 0.5 μg of Nox1 plus 0.5 μg of p41 Nox plus 0.5 μg of p51 Nox according to the manufacturer's directions (Lipofectamine Plus, Invitrogen) and 24 h after transfection, test agents were added.
Control and GRX1 siRNA (cat no. 4611 and 85588, Ambion) were transfected into C10 cells at a concentration of 20 nM using siPORTamine in 2 ml plain CRML according to the manufacturer's directions. After 4 hours, 2 ml of complete CRML was added and 48 h later test agents were added and experiments performed.

Immunocytochemistry for GRX1. Cells were exposed to test agents, washed twice with PBS and fixed with 4% PFA for 10 min at RT. After three washes with PBS, cells were permeabilized and blocked simultaneously with PBS containing 0.5 % triton and 2% BSA for 10 min at RT. Next, cells were incubated with rabbit anti-human GRX1 antibody (Labfrontier), diluted 1:100 in blocking buffer, for 1 h at RT. After three washes with PBS the cells were incubated for 1 h with goat anti-rabbit Cy-3 in blocking buffer. Nuclei were counterstained with Sytox Green

(Molecular Probes) for 5 min at RT, coverslips were mounted and cells analyzed by confocal microscopy using an Olympus BX50 microscope coupled to a Bio-Rad MRC 1024 confocal scanning laser microscope system.

Assessment of GRX1-catalyzed cysteine derivatization to visualize protein S-glutathionylation in intact cells. Cells were exposed to test agents, washed twice with PBS and fixed with 4% PFA for 10 min at RT. After three washes with PBS, cells were permeabilized and free sulfhydryl groups blocked with buffer containing 25 mM Hepes, pH 7.7, 0.1 mM EDTA, 0.01 mM neocuproine, 20 mM *N*-ethylmaleimide and 1% Triton X-100 for 30 min at 4°C. After three washes with PBS, S-glutathionyl mixed disulfides were reduced by incubation with 27 μg/ml *E.coli* or human GRX1 (American Diagnostica), 4 U/ml GSSG reductase (Roche), 1 mM GSH, 1 mM NADPH and 1 mM EDTA in 50 mM Tris, pH 7.5, for 15 min at 37°C [19]. Next, cells were washed three times with PBS and newly reduced sulfhydryl groups were labeled with 1mM *N*-(3-maleimidylpropionyl) biocytin (MPB, Molecular Probes) for 1 h at RT. After removal of excess MPB by three washes with PBS, cells were incubated with 10 μg/ml streptavidin-FITC for 1 h at RT and nuclei counter stained with 10 μg/ml propidium iodide for 30 min at RT. Coverslips were mounted and cells analyzed by confocal microscopy using an Olympus BX50 microscope coupled to a Bio-Rad MRC 1024 confocal scanning laser microscope system. As a negative control, GRX1 alone, or GRX1, GSSG reductase, GSH and NADPH were omitted in the reduction step. Furthermore, MPB was omitted in some coverslips to assess the contribution of endogenous biotin.

Preparation of cysteinylated and S-glutathionylated BSA. A 5 mg/ml solution of BSA in 50 mM Tris (pH 7.5)/1 mM EDTA was reduced by incubation with 1 mM DTT for 1 h at 37°C. DTT was removed using Micro Bio-Spin 6 chromatography columns (Bio-Rad) and BSA was subsequently reacted with 5 mg L-cysteine or GSSG for 30 min at 37°C. The GRX reduction mixture contained a final concentration of 2.5 mg/ml BSA.

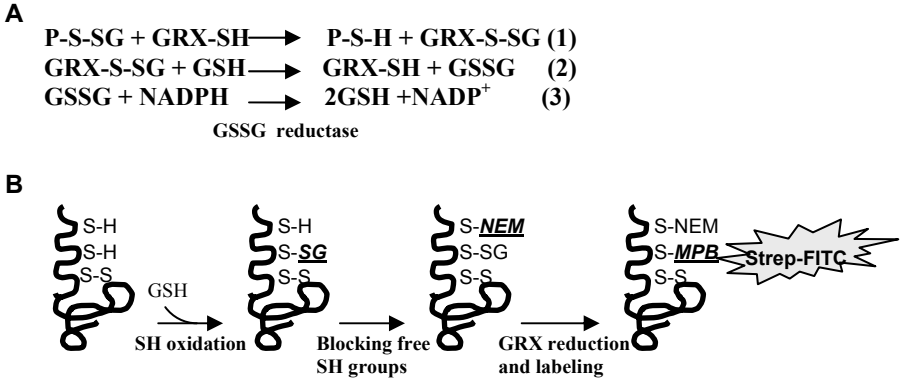

Figure 1 *(A)* Reactions involved in GRX mediated deglutathionylation. In reaction 1, the S-glutathionyl moiety is transferred to GRX. The GRX-S-SG intermediate is reduced by GSH in reaction 2 and GSSG reductase reduces the resulting GSSG using NAPDH in reaction 3. *(B)* Schematic representation of the staining method for GRX reversible cysteine oxidation. In the first step, free protein thiols are blocked with NEM. In the second step S-glutathionyl moieties are reduced using GRX1, and next labeled using MPB. Newly biotinylated proteins are then visualized with streptavidin-FITC.

Western blotting. Cells were lysed in buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM Na_3VO_4, 1 mM PMSF, 0.1 % NP40, 10 μg/ml leupeptin, 1 % aprotenin, 250 μM DTT DTT, 100 μM NaF, equalized for protein content and an equal volume of 2X Laemmli sample buffer was added. After boiling the samples for 5 min, proteins were separated on 15% polyacrylamide gels and transferred to nitrocellulose. Following blocking of the membranes overnight in TBS containing 0.05% Tween-20 (TBST) and 5% milk at 4°C, primary antibodies against HA (Upstate) or GRX1 (Labfrontier) were incubated for 4 h at RT. After three 20 min washes with TBST, membranes were incubated with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at RT. Conjugated peroxidase was detected by chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

GRX-catalyzed cysteine derivatization to visualize protein S-glutathionylation in intact cells. We first evaluated whether GRX1-catalyzed reversal of proteins S-glutathionylation could be observed in control cells, according to the protocol depicted in Fig. 1. Indeed results in Fig. 2*A* demonstrate marked MPB-FITC labeling in control cells, which depends on the presence of GRX in the reaction mixture. Furthermore, omission of MBP resulted in minimal staining, demonstrating that endogenous biotin does not contribute to the observed signal. Furthermore, reduction of disulfides with DTT prior to blocking with NEM abolished all labeling.

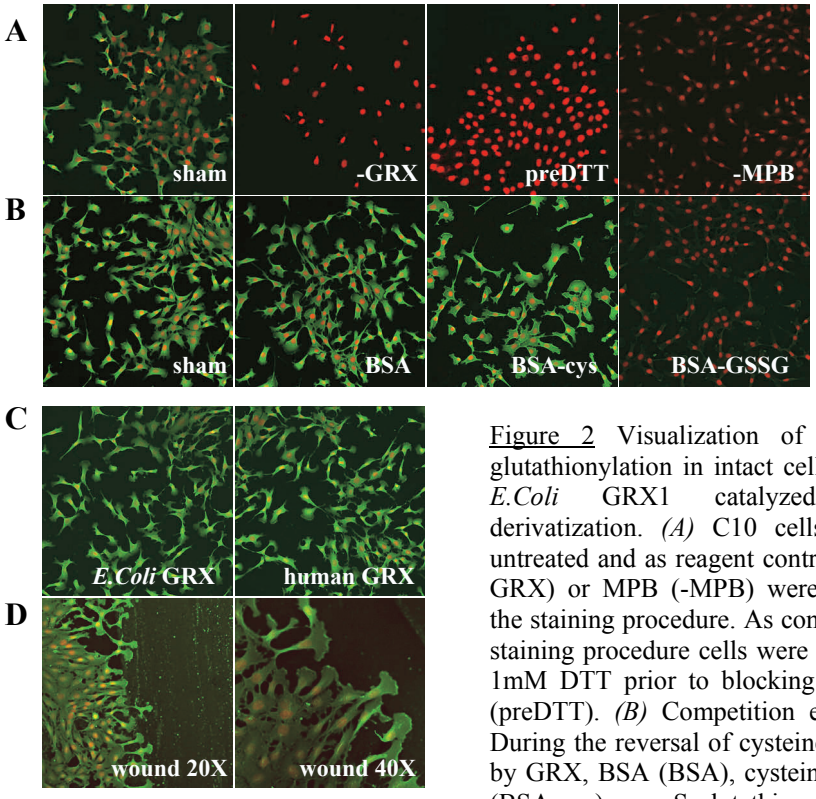

Figure 2 Visualization of protein S-glutathionylation in intact cells following *E.Coli* GRX1 catalyzed cysteine derivatization. *(A)* C10 cells were left untreated and as reagent controls, GRX (-GRX) or MPB (-MPB) were omitted in the staining procedure. As controls for the staining procedure cells were treated with 1mM DTT prior to blocking with NEM (preDTT). *(B)* Competition experiments. During the reversal of cysteine oxidations by GRX, BSA (BSA), cysteinylated BSA (BSA-cys) or S-glutathionylated BSA (BSA-GSSG) were co-incubated in the reaction mixture to evaluate competition with endogenous substrates for GRX1-catalyzed reduction. *(C) E.Coli* or human GRX1 were used in the staining procedure (20X objective). *(D)* A wound was created using a 1 ml pipet tip on a coverslip of confluent cells and cells were left to recover for 4h (lower panels). *E.Coli* GRX1-reversible cysteine oxidation staining was performed (green) and nuclei were counter stained with propidium iodide (red).

Since *E.Coli* GRX1 could be reducing both glutathionyl disulfides, and low molecular weight disulfides, the concern exists that utilization of *E.Coli* GRX1 lacks specificity for S-glutathionylated proteins. Therefore, we conducted a series of competition experiments that included either cysteinylated or S-glutathionylated BSA in the reaction mixture. As is apparent from Fig. 2*B*, cysteinylated BSA did not attenuate the staining of *E.coli* GRX1 catalyzed-reversible cysteine oxidations, whereas as expected, S-glutathionylated BSA abolished all staining. Additionally, human GRX1 has been demonstrated to specifically reduce glutathione mixed disulfides [13]. Staining intensities as well as staining patterns were identical when *E.Coli* or human GRX1 were used in the labeling procedure (Fig.2*C*). Collectively, these controls demonstrate that the labeling method used indeed preferentially detects S-glutathionylated protein oxidations, and illustrates that basal protein S-glutathionylation occurs in control cells. It is of interest to note that GRX1-catalyzed MPB-FITC labeling is predominant in the cell periphery in association with membrane ruffles, which was particularly noticeable in cells at the leading edge of a wound (Fig. 2*D*).

Increased GRX reversible cysteine oxidation in cells exposed to oxidants. Following these observations, we exposed cells to oxidants that are known to cause the formation of protein glutathione mixed disulfides and again visualized GRX1-reversible cysteine oxidations. According to our expectations, glucose oxidase (GOX), the thiol oxidizing agent, diamide, or the nitrosothiol, GSNO, all caused a marked increase in *E.Coli* GRX1 catalyzed FITC-MBP labeling (Fig. 3*A*). It is of interest to note that the pattern of protein S-glutathionylation after diamide exposure appeared to be highly punctate in nature, whereas GOX or GSNO caused uniform increases in labeling throughout the cells. Staining intensities and patterns observed after exposure to these oxidants were identical when human GRX1 was substituted for *E.Coli* GRX1 (data not shown), indicating the preferential detection of S-glutathionylated proteins using this in situ method.

Primary epithelial cells isolated from C57BL/6 mice also demonstrated a basal level of glutathione mixed disulfides, which was enhanced after treatment with H_2O_2 (Fig. 3*B*), similar to the C10 cell line.

In order to assess the formation of glutathione mixed disulfides in cells that endogenously produce an elevated flux of H_2O_2, cells were transfected with Nox 1 plus its co-activators. Indeed, Nox1 dependent

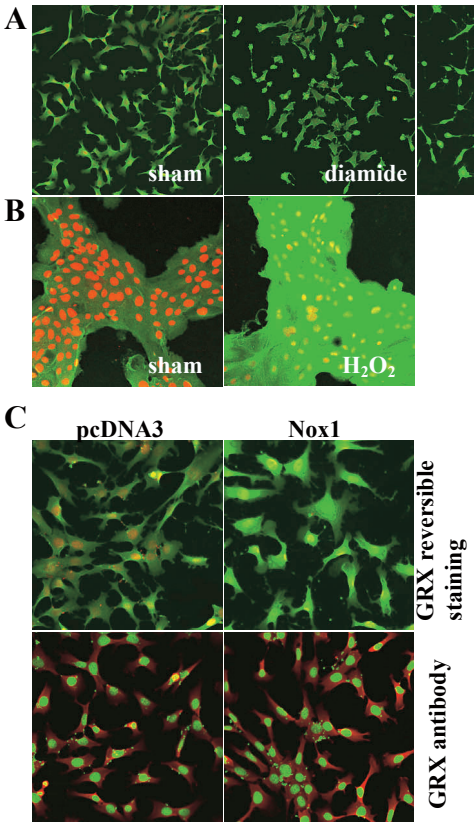

Figure 3 Increased *E.Coli* GRX1 reversible cysteine oxidation in cells exposed to oxidants. *(A)* C10 cells were left untreated or treated with 400 µM diamide for 15 min, 5 U/ml GOX for 1h or 1mM GSNO for 1h. GRX reversible cysteine oxidation was performed by incubation with *E.Coli* GRX1 (green). Nuclei were counterstained with propidium iodide (red, 20X objective). *(B)* Primary tracheal epithelial cells from C57BL/6 mice were left untreated or were exposed to 200 µM H_2O_2 for 15 min. *E.Coli* GRX1 reversible cysteine oxidation staining was performed (green) and nuclei were counter stained with propidium iodide (red, 20X objective). *(C)* C10 cells were transfected with pcDNA3 or Nox1 plus p41 Nox and p51 Nox and stained for *E.Coli* GRX1 reversible cysteine oxidation as in *(A)*. As a control, immunocytochemistry for GRX1 was performed (red, bottom panels). Nuclei were counter stained with Sytox Green (green, 40X objective).

generation of H_2O_2 also resulted in markedly enhanced formation of glutathione mixed disulfides (Fig. 3*C*). We also assessed GRX1 expression in cells that overexpress Nox1 plus its co-activators, because differences in GRX1 expression could affect the levels of protein-S-glutathionylation (further addressed below). Results in Fig. 3*B* suggest

that GRX-1 immunoreactivity was not different between pcDNA3 and Nox1 overexpressing cells, illustrating that the differences in S-gluathionylation in Nox1 overexpressing cells are not due to intrinsic differences in GRX1 content.

Depletion of glutathione enhances GRX reversible cysteine oxidation. While γ-glutamylcysteine synthetase inhibitor, DL-buthionine-[S,R]-sulfoximine (BSO) is well known to deplete the cellular glutathione pool, this agent also has been demonstrated to cause increases in levels of glutathione mixed disulfides [26]. In agreement with those previous observations, results in Fig. 4 demonstrate marked increases in GRX1-dependent MPB-FITC labeling in cell treated with BSO, which was most prominent in membrane ruffles, and was further enhanced in cells exposed to H_2O_2.

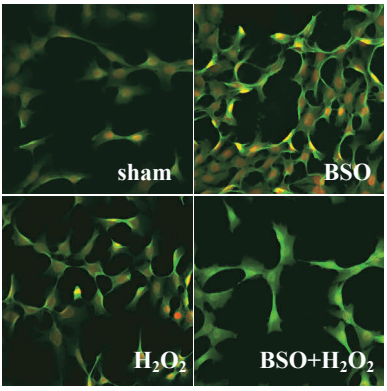

Figure 4 Depletion of glutathione enhances *E.Coli* GRX1 reversible cysteine oxidation. C10 cells were treated with 0.1 mM BSO for 16 h to deplete glutathione, followed by 200 μM H_2O_2 for 15 min. *E.Coli* GRX1 reversible cysteine oxidation was stained according to the protocol (green) and nuclei were counter stained with propidium iodide (red, 40X objective).

Manipulation of cellular GRX1 affects levels of S-glutathionylated proteins detected in situ. Since mammalian GRX1 specifically reverses protein-glutathione mixed disulfides, we next manipulated the GRX1 expression in cells, in order to augment or attenuate S-glutathionylation, to confirm that the labeling approach used so far indeed detects S-glutathionylated proteins. We first transfected C10 cells with mouse HA-GRX1, and enhanced expression was confirmed by Western blot for HA (Fig. 5*A*, left). Whereas overexpression of GRX1 in C10 cells did not appear to attenuate the basal level of cellular glutathione mixed disulfides (Fig. 5*B*, left), GRX1 overexpression completely prevented the increased formation of S-glutathionylated proteins in response to H_2O_2, seen in pcDNA3 transfected cells. Lastly, RNA interference to selectively inhibit

the expression of GRX1 resulted in significantly decreases in protein expression of GRX1 (Fig. 5*A*, right). Importantly, knock-down of GRX1 was sufficient to enhance basal cellular S-glutathionylation, and substantially increased the formation of S-glutathionylated proteins in response to H_2O_2 (Fig. 5*B*, right). Collectively these findings demonstrate that the patterns of FITC-MBP labeling observed in the presence of catalytically active GRX1 are indeed due to protein-S-glutathionylation, and that the staining patterns change substantially in a cell under conditions of oxidative stress or following manipulation of endogenous GRX1.

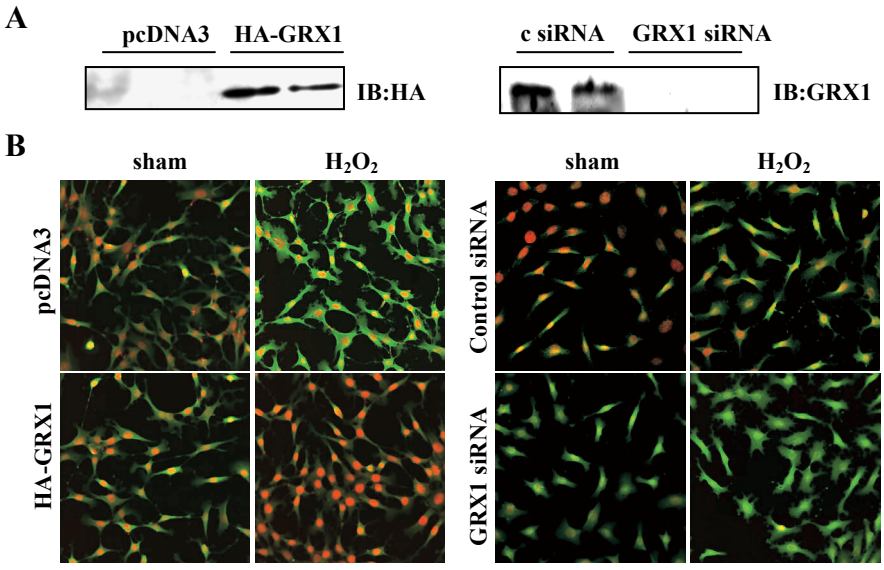

Figure 5 Manipulation of cellular GRX1 affects levels of S-glutathionylated proteins detected in situ. C10 cells were transfected with pcDNA3, mouse HA-GRX1 *(left panels)*, control siRNA (c siRNA) or GRX1 siRNA *(right panels)*. *(A)* Western blotting for HA or GRX1 was performed. *(B)* Cells were left untreated or treated with 200 μM H_2O_2 for 15 min and stained for *E.Coli* GRX1 reversible cysteine oxidation (green). Nuclei were counter stained with propidium iodide (red, 40X objective).

DISCUSSION

Protein S-glutathionylation is emerging as a post-translational modification that transduces oxidative signals by altering the function of effecter proteins. Analysis of this post-translational modification in situ

has been hampered by the lack of specific reagents that unequivocally detect this event with sufficient specificity. Here, we have described a new method to visualize S-glutathionylated proteins in intact cells, taking advantage of the unique catalytic activity of GRX which catalyzes the reduction of S-glutathionylated proteins, followed by specific labeling of newly reduced cysteines. Using the appropriate reagent controls that include direct manipulation of intracellular levels of GRX1 to change steady state levels of S-glutathionylated proteins, we demonstrated the preferential specificity of this new application towards the detection of S-glutathionylated proteins in situ.

E.Coli GRX catalyze both the reduction of S-glutathionylated proteins as well as low molecular weight disulfides [15]. Using cysteinylated BSA as a competitive substrate in the reduction mixture (Fig.2*B*), we did not observe any attenuation of staining intensity or staining patterns, demonstrating that *E.Coli* GRX1-catalyzed cysteine derivatization preferentially detects S-glutathionylated proteins using the current method. Additionally, using human GRX1, which in *in vitro* studies has been demonstrated to be specifically reactive towards S-glutathionylated substrates [13], we observed similar staining intensities and patterns as when *E.Coli* GRX1 was used (Fig.2*C*). It appears that GRX1 from both species can be used for the preferential detection of S-glutathionylated proteins in situ, although human GRX1 may still yield a higher specificity. Furthermore, as is the case for many, if not all approaches to detect redox-dependent posttranslational modifications, this *in situ* method will preferentially detect S-glutathionylation of highly abundant proteins.

Two mammalian GRX enzymes have been identified and partially characterized to date. GRX1 and 2 essentially contain the same catalytic core, but differ in their intracellular localization. GRX1 is a cytoplasmic variant, whereas GRX2 contains a mitochondrial leader sequence and can also reside in the nucleus due to alternative splicing of the gene [10-12]. In addition, evidence is emerging that they display discrete sensitivity to regulation by oxidants [11, 27]. However, both isozymes recognize only the GSH-moiety of protein mixed disulfides [15] and therefore target different subsets of proteins due to their alternative localization. Since cells are sufficiently permeabilized to access both nuclear and mitochondrial compartments [28] targets for both enzymes are likely to be accessible for labeling using GRX-dependent catalysis.

Quite surprisingly, in resting conditions, or in response to some oxidants, marked staining was revealed at the periphery of cells, consistent with another study, using alternative approaches to visualize protein-S-gluathionylation [22]. Moreover, cells at the leading edge of a wound display a greater extent of glutathione mixed disulfides when compared to cells in confluent unwounded areas, which is consistent with enhanced patterns of DCF oxidation at these sites [29]. It is of interest to note that the cell membrane is where the H_2O_2 generating enzymes Nox and Duox are localized [30, 31], providing a direct source of oxidants in order to produce S-glutathionylated proteins locally. As the cytoskeleton, and in particular its actin component are involved in the formation of membrane ruffles, as well as in migration and cellular plasticity, it is conceivable that actin may represent one of the targets for S-glutathionylation. S-glutathionylation of actin has been shown to inhibit its ability to undergo polymerization and form F-actin [32] and additionally, GRX, has been implicated in actively mediating actin depolymerization [33]. It has been speculated that the dynamic control of actin polymerization/ depolymerizaton could represent a key feature in the response of cells to growth factors and other mediators, through its role in the formation of signal transduction scaffolds [32]. The present findings suggest a potential role for protein S-glutathionylation in these processes, although experiments to formally test this possibility are awaiting.

As expected, various oxidants, including bolus H_2O_2, diamide, GSNO, glucose oxidase, and H_2O_2 production through overexpression of Nox1 all led to enhanced staining of S-glutathionylated proteins. However, the staining patterns that these various oxidants and oxidant generating systems inflicted displayed marked differences. Despite previous reports demonstrating the capability of H_2O_2 to stimulate GRX expression [34], Nox1 overexpression appeared not to have altered GRX1 protein levels. The observation that S-glutathionylation is enhanced in cells under glutathione depleted conditions might be surprising at first. However, GSH is an essential cofactor for GRX-catalyzed deglutathionylation, and therefore depletion of GSH could limit the extent of GRX activity, resulting in enhanced S-glutathionylation. On the other hand, if S-glutathionylation represents a mechanism that protects protein thiols from irreversible oxidation, one could speculate that available GSH would become conjugated to protein thiols in a pro-oxidative environment of low GSH levels.

In conclusion, we have described here a GRX1 catalyzed derivatization and labeling approach in order to preferentially visualize glutathione mixed disulfides in intact cells or tissues. This provides a powerful means to investigate patterns of S-glutathionylation *in situ* in relation to normal physiology as well as pathology.

ACKNOWLEDGMENTS

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REFERENCES

[1] F. Ursini, M. Maiorino, R. Brigelius-Flohe, K.D. Aumann, A. Roveri, D. Schomburg and L. Flohe. Diversity of glutathione peroxidases. Methods Enzymol 252 (1995) 38-53.

[2] D. Giustarini, R. Rossi, A. Milzani, R. Colombo and I. Dalle-Donne. S-Glutathionylation: from redox regulation of protein functions to human diseases. J Cell Mol Med 8 (2004) 201-12.

[3] E. Pineda-Molina, P. Klatt, J. Vazquez, A. Marina, M. Garcia de Lacoba, D. Perez-Sala and S. Lamas. Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. Biochemistry 40 (2001) 14134-42.

[4] P. Klatt, E.P. Molina, M.G. De Lacoba, C.A. Padilla, E. Martinez-Galesteo, J.A. Barcena and S. Lamas. Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. Faseb J 13 (1999) 1481-90.

[5] N.E. Ward, F. Chu and C.A. O'Brian. Regulation of protein kinase C isozyme activity by S-glutathiolation. Methods Enzymol 353 (2002) 89-100.

[6] S. Mohr, J.S. Stamler and B. Brune. Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by S-nitrosylation and subsequent NADH attachment. J Biol Chem 271 (1996) 4209-14.

[7] D.A. Davis, F.M. Newcomb, D.W. Starke, D.E. Ott, J.J. Mieyal and R. Yarchoan. Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. J Biol Chem 272 (1997) 25935-40.

[8] Y. Manevich, S.I. Feinstein and A.B. Fisher. Activation of the antioxidant enzyme 1-CYS peroxiredoxin requires glutathionylation mediated by heterodimerization with pi GST. Proc Natl Acad Sci U S A 101 (2004) 3780-5.

[9] M.D. Shelton, P.B. Chock and J.J. Mieyal. Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. Antioxid Redox Signal 7 (2005) 348-66.

[10] D. Su and V.N. Gladyshev. Alternative splicing involving the thioredoxin reductase module in mammals: a glutaredoxin-containing thioredoxin reductase 1. Biochemistry 43 (2004) 12177-88.

[11] V.N. Gladyshev, A. Liu, S.V. Novoselov, K. Krysan, Q.A. Sun, V.M. Kryukov, G.V. Kryukov and M.F. Lou. Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2. J Biol Chem 276 (2001) 30374-80.

[12] M. Lundberg, C. Johansson, J. Chandra, M. Enoksson, G. Jacobsson, J. Ljung, M. Johansson and A. Holmgren. Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. J Biol Chem 276 (2001) 26269-75.

[13] S.A. Gravina and J.J. Mieyal. Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. Biochemistry 32 (1993) 3368-76.

[14] Y. Yang, S. Jao, S. Nanduri, D.W. Starke, J.J. Mieyal and J. Qin. Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity. Biochemistry 37 (1998) 17145-56.

[15] A.P. Fernandes and A. Holmgren. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal 6 (2004) 63-74.

[16] T. Dandrea, E. Bajak, L. Warngard and I.A. Cotgreave. Protein S-glutathionylation correlates to selective stress gene expression and cytoprotection. Arch Biochem Biophys 406 (2002) 241-52.
[17] S. Casagrande, V. Bonetto, M. Fratelli, E. Gianazza, I. Eberini, T. Massignan, M. Salmona, G. Chang, A. Holmgren and P. Ghezzi. Glutathionylation of human thioredoxin: a possible crosstalk between the glutathione and thioredoxin systems. Proc Natl Acad Sci U S A 99 (2002) 9745-9.
[18] M. Fratelli, H. Demol, M. Puype, S. Casagrande, I. Eberini, M. Salmona, V. Bonetto, M. Mengozzi, F. Duffieux, E. Miclet, A. Bachi, J. Vandekerckhove, E. Gianazza and P. Ghezzi. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proc Natl Acad Sci U S A 99 (2002) 3505-10.
[19] C. Lind, R. Gerdes, Y. Hamnell, I. Schuppe-Koistinen, H.B. von Lowenhielm, A. Holmgren and I.A. Cotgreave. Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. Arch Biochem Biophys 406 (2002) 229-40.
[20] P. Eaton, N. Wright, D.J. Hearse and M.J. Shattock. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. J Mol Cell Cardiol 34 (2002) 1549-60.
[21] S. Mawatari and K. Murakami. Different types of glutathionylation of hemoglobin can exist in intact erythrocytes. Arch Biochem Biophys 421 (2004) 108-14.
[22] T. Soderdahl, M. Enoksson, M. Lundberg, A. Holmgren, O.P. Ottersen, S. Orrenius, G. Bolcsfoldi and I.A. Cotgreave. Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells. Faseb J 17 (2003) 124-6.
[23] G. Cheng, Y. Ikeda, Y. Iuchi and J. Fujii. Detection of S-glutathionylated proteins by glutathione S-transferase overlay. Arch Biochem Biophys 435 (2005) 42-9.
[24] A.M. Malkinson, L.D. Dwyer-Nield, P.L. Rice and D. Dinsdale. Mouse lung epithelial cell lines--tools for the study of differentiation and the neoplastic phenotype. Toxicology 123 (1997) 53-100.
[25] R. Wu and D. Smith. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. In Vitro 18 (1982) 800-12.
[26] P. Ghezzi, B. Romines, M. Fratelli, I. Eberini, E. Gianazza, S. Casagrande, T. Laragione, M. Mengozzi and L.A. Herzenberg. Protein glutathionylation: coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection. Mol Immunol 38 (2002) 773-80.
[27] C.H. Lillig, C. Berndt, O. Vergnolle, M.E. Lonn, C. Hudemann, E. Bill and A. Holmgren. Characterization of human glutaredoxin 2 as iron-sulfur protein: A possible role as redox sensor. Proc Natl Acad Sci U S A 102 (2005) 8168-73.
[28] H. Fung, Y.W. Kow, B. Van Houten, D.J. Taatjes, Z. Hatahet, Y.M. Janssen, P. Vacek, S.P. Faux and B.T. Mossman. Asbestos increases mammalian AP-endonuclease gene expression, protein levels, and enzyme activity in mesothelial cells. Cancer Res 58 (1998) 189-94.
[29] L. Moldovan, N.I. Moldovan, R.H. Sohn, S.A. Parikh and P.J. Goldschmidt-Clermont. Redox changes of cultured endothelial cells and actin dynamics. Circ Res 86 (2000) 549-57.
[30] G. Cheng and J.D. Lambeth. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. J Biol Chem 279 (2004) 4737-42.

[31] D. Wang, X. De Deken, M. Milenkovic, Y. Song, I. Pirson, J.E. Dumont and F. Miot. Identification of a novel partner of duox: EFP1, a thioredoxin-related protein. J Biol Chem 280 (2005) 3096-103.
[32] J. Wang, E.S. Boja, W. Tan, E. Tekle, H.M. Fales, S. English, J.J. Mieyal and P.B. Chock. Reversible glutathionylation regulates actin polymerization in A431 cells. J Biol Chem 276 (2001) 47763-6.
[33] J. Wang, E. Tekle, H. Oubrahim, J.J. Mieyal, E.R. Stadtman and P.B. Chock. Stable and controllable RNA interference: Investigating the physiological function of glutathionylated actin. Proc Natl Acad Sci U S A 100 (2003) 5103-6.
[34] M. Okuda, N. Inoue, H. Azumi, T. Seno, Y. Sumi, K. Hirata, S. Kawashima, Y. Hayashi, H. Itoh, J. Yodoi and M. Yokoyama. Expression of glutaredoxin in human coronary arteries: its potential role in antioxidant protection against atherosclerosis. Arterioscler Thromb Vasc Biol 21 (2001) 1483-7.

CHAPTER 7

Modulation of glutaredoxin expression in a mouse model of allergic airway disease

ABSTRACT

Glutaredoxins (GRX) are antioxidant enzymes that preferentially catalyze the reduction of protein-glutathione mixed disulfides. The formation of mixed disulfides with GSH is known as S-glutathionylation, a posttranslational modification that is emerging as an important mode of redox signaling. Since asthma is a disease that is associated with increased oxidative stress and altered antioxidant defenses, we investigated the expression of GRX in a murine model of allergic airway disease. Sensitization and challenge of C57BL/6 mice with ovalbumin resulted in increased expression of GRX1 mRNA, as well as increased amounts of GRX1 protein and total GRX activity in the lung. Because GRX-1 expression is prominent in bronchial epithelium, we isolated primary epithelial cells from mouse trachea to investigate the presence of GRX. Primary tracheal epithelial cells were found to express both GRX1 and 2 mRNA, and detectable GRX activity. Treatment with IFNγ increased the expression of GRX1 and overall GRX activity, resulting in attenuation of protein S-glutathionylation. In contrast, TGFβ caused decreased GRX1 expression and overall GRX activity, leading to markedly enhanced protein S-glutathionylation. GRX1 joins the cadre of antioxidant defenses known to be modulated during allergic airway inflammation. The positive regulation of GRX1 by IFNγ and its negative regulation by TGFβ suggest a possible role for GRX1 in tissue destruction and/or repair.

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INTRODUCTION

Chronic inflammatory diseases of the lung, such as asthma are accompanied by oxidative stress. The bulk of oxidant production has been attributed to inflammatory cells, which mainly consist of eosinophils in asthma. In addition, during normal cellular respiration and aerobic metabolism as well as through activation of non-phagocytic-NADPH oxidases (Nox (1)), resident epithelial cells can also generate oxidants. Lastly, environmental exposures that cause asthma exacerbations increase the oxidative burden in the lungs.

Because oxidants can cause damage to macromolecules, lung tissue has evolved with a battery of antioxidant enzymes to protect against these oxidative insults and glutathione plays a major role herein. The importance of glutathione in protecting airspace epithelium against oxidant-mediated injury is underscored by the fact that its levels in the epithelial lining fluid are approximately one hundred fold higher than in serum (2). As evidence of oxidative stress, oxidized glutathione levels (GSSG) were reported to be increased in BAL fluid, or induced sputum samples of patients with asthma compared to healthy controls(3, 4). Glutathione levels are regulated by a system of antioxidant enzymes that include glutathione peroxidase (GPx), glutathione reductase (GR), γ–glutamyl-cysteinyl synthase (γGCS), glutathione-S-transferase (GST) and glutaredoxins (GRX). Changes in some of these enzymes have been demonstrated in asthma, locally in lung tissue, as well as systemically. For instance, erythrocytes of patients with asthma display reduced GPx activity compared to control individuals (5), whereas levels of extracellular GPx were found to be increased in the lungs of asthmatics compared to controls (6). Polymorphisms in the GST-M1, GST-T1 and GST-P1 genes have also been associated with asthma, for review see (7). Decreases in activities of superoxide dismutases (8, 9) and catalase (10) in patients with asthma further contribute to enhanced oxidative stress.

As part of the antioxidant properties of glutathione, the tripeptide can incorporate into protein amino acid sulfhydryls through its proper thiol group in order to protect them directly against irreversible oxidations. The formation of protein mixed disulfides is also known as S-glutathionylation, which has been demonstrated to occur in a number of proteins, like the transcription factors NF-κB (11) and AP-1 (12), under baseline conditions and is increased after oxidative stress. Since S-glutathionylation has been established to alter the function of these

proteins, it is considered a posttranslational modification through which oxidants can transduce signals, and serve as second messenger molecules. Mammalian GRX enzymes (thioltransferases) belong to the thioredoxin superfamily of enzymes and have been demonstrated to regulate this posttranslational modification (13). GRXs are oxidoreductases that catalyze the preferential reduction of protein-glutathione mixed disulfides or deglutathionylation to restore the reduced sulfhydryl group through a monothiol mechanism (14). In this reaction, GRX itself is S-glutathionylated and the reduced state of GRX is restored by GSH coupled to GSSG reductase (15). Two mammalian GRX proteins have been identified to date. GRX1 is a cytosolic protein, whereas GRX2 contains a mitochondrial leader sequence but can also occur in the nucleus following alternative splicing (16, 17).

Studies on GRX enzymes in the lung are scant. To date GRX1 was found to be decreased in alveolar macrophages of patients with sarcoidosis and allergic alveolitis, but no difference was observed by immunohistochemistry between patients with interstitial pneumonia and controls (18). In addition, hyperoxia was found not to affect GRX expression (19). Given the importance of glutathione in maintaining the redox status of the lung and the presence of oxidative stress in asthma, the goal of the current study was to investigate the expression of GRX in a mouse model of allergic airway disease. Furthermore, the modulation of GRX by cytokines that are relevant to allergic airway disease was examined in primary mouse tracheal epithelial cells.

MATERIALS AND METHODS

Animals. Six to 8 week-old female C57BL/6 mice (Jackson Laboratories) were housed in the University of Vermont Animal Facility. Mice were subjected to Ovalbumin (OVA) sensitization and challenge as described elsewhere (20). The Institutional Animal Care and Use Committee granted approval for all studies.

Primary cell culture. Primary epithelial cells were isolated from C57BL/6 mice according to Wu et al. (21) with minor modifications (22). For experiment, cells were plated on Collagen I coated culture dishes or glass slides and switched to phenol red free DMEM/F12 media containing 2mM L-Glutamine and P/S 24h before initiation of experiments.

Semi-quantitative PCR. Total RNA was isolated from lung using an RNeasy Mini Kit (Qiagen, Valencia, CA), DNase treated and reverse transcribed into cDNA. Semi-quantitative TaqMan PCR for GRX1 and GRX2 were performed (Applied Biosystems, Framingham, MA) and values were normalized to HPRT.

Immunohistochemistry. Following euthanasia, lungs were instilled with 50% Tissue-Tek OCT Compound (Sakura Finetek Inc. Torrance, CA) in PBS and frozen in liquid nitrogen-chilled isopentane for the preparation of 10-μm frozen sections. Slides or cells were fixed for 10 min with 4% paraformaldehyde (PFA), washed and permeabilized with 1% Triton X-100 in PBS for 20 min. After blocking with 1% BSA in PBS (PBS/1% BSA) slides were incubated with rabbit anti-human GRX1 antibody (10 μg/ml, Labfrontier) overnight at 4°C. Following three washes in PBS/1% BSA, slides were incubated with goat anti-rabbit Cy3 secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and counterstained with Sytox Green (Molecular Probes) to label DNA. Slides were washed and coverslipped and sections were scanned using an Olympus BX50 upright microscope configured to a Bio-Rad MRC 1024 confocal scanning laser microscope system using a 20X objective.

Western Blotting. Lung lysates were mixed with 2x Laemmli sample buffer, boiled and loaded on polyacrylamide gels. Proteins were transferred to nitrocellulose and Western blotting for GRX1 was performed using a GRX1 antibody (Labfrontier) as previously described (22).

GRX activity assay. GRX activity was assayed as previously described (23). Briefly, cells or lungs were lysed in buffer containing 137 mM Tris-HCl (pH 8.0), 130 mM NaCl, 1% NP-40, cleared by centrifugation and equalized for protein content. For analysis of whole lung GRX activity, excess NADPH was removed from lung lysates through a Micro Bio-Spin 6 chromatography column (Bio-Rad) prior to analysis. The lysates were incubated with reaction buffer containing 137 mM Tris-HCl (pH 8.0), 0.5 mM GSH, 1.2 units GSSG reductase (Roche), 0.35 mM NADPH, 1.5 mM EDTA (pH 8.0) and 2.5 mM Cys-SO_3. The reaction was allowed to proceed at 30°C and NAPDH consumption was followed spectrophotometrically at 340 nm. Data are expressed as units, where 1 unit equals the oxidation of 1 μM NADPH/min/mg.

Assessment of GRX1-catalyzed cysteine derivatization to visualize S-glutathionylation. Cells were grown on Collagen I coated glass slides, exposed to test agents and S-glutathionylation was assessed using GRX1-catalyzed cysteine derivatization. This newly developed assay utilizes GRX1-dependent reduction of protein-glutathione mixed disulfides coupled to biotin labeling in order to visualize S-glutathionylation patterns in situ (22). Briefly, S-glutathionylated proteins are stained in red, using streptavidin-Alexa Fluor 568 and nuclei are counterstained with Sytox Green. Slides were analyzed by confocal microscopy using 20X objective.

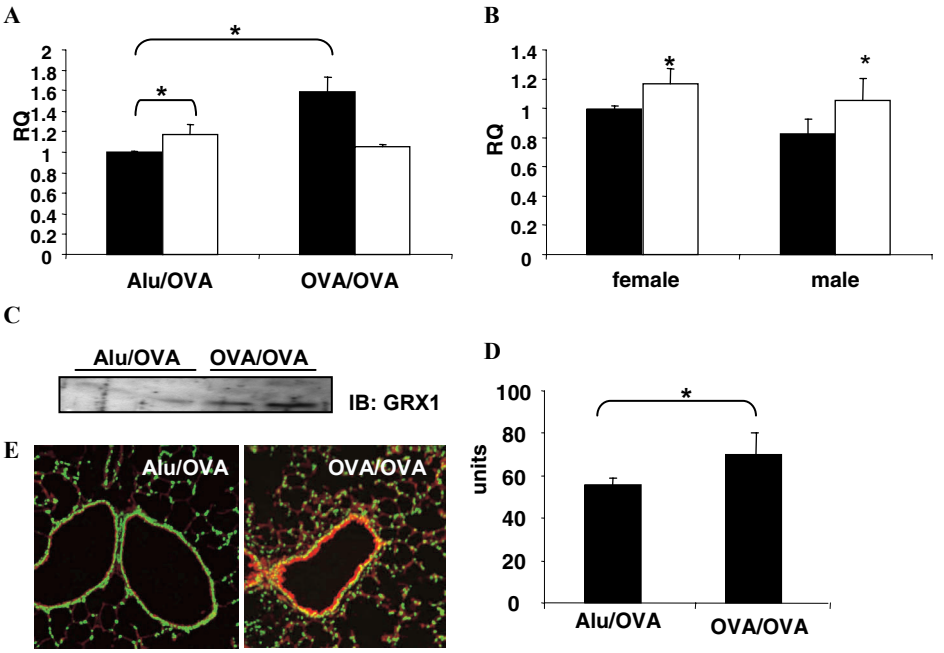

Figure 1 GRX is increased in allergic airway disease. (A) RNA was collected from lungs 48h after the last challenge, reverse-transcribed and analyzed for GRX1 (■) and 2 (□) expression relative to HPRT by TaqMan PCR. Data are expressed as mean RQ from five mice per group (± SEM). * represents $p<0.05$. (B) RNA was collected from lungs of mock-sensitized male and female mice 48h after the last challenge and analyzed for GRX1 (■) and 2 (□) expression relative to HPRT by TaqMan PCR. Data are expressed as mean RQ from five mice per group (± SEM). * represents $p<0.05$ between GRX1 and GRX2. (C) Western blotting for GRX1 was performed on lung lysates. (D) GRX activity was assessed in lung lysates as described in Materials and Methods. Result is representative of three experiments and values are mean from three mice per group (± SEM). (E) Frozen sections were stained with an antibody directed against GRX1, followed by incubation with a Cy3 conjugated secondary antibody (red). Sytox Green was used as a nuclear counter stain and sections were scanned by confocal microscopy. Images are representative of results from five to six mice per group.

Statistical analysis. Data were expressed as mean ± SEM and compared by analysis of variance (ANOVA). Differences were considered significant when $p<0.05$. All experiments were repeated at least two times.

RESULTS

GRX is increased in allergic airway disease. Immunization and challenge with Ova caused eosinophilic inflammation (24) and marked increases in levels of amongst others Interleukins 4, 5, 6, KC and MCP1 in BALF (manuscript submitted). Total lung homogenates from mock-sensitized (Alu/OVA) mice expressed both GRX1 and GRX2 mRNA, in agreement with previous reports (18, 25). Basal expression of GRX2 mRNA in lung tissue was significantly higher then that of GRX1, which was also reported previously (25). Allergen sensitization and challenge (OVA/OVA) increased mRNA levels of GRX1, while not affecting GRX2 mRNA levels (Fig. 1A). Since GRX1 mRNA expression was reported to be higher in the brain of female compared to male mice, and this was associated with protection against the induction of experimental Parkinson's disease (26), we investigated the differential expression of GRX in the lungs of both sexes. Fig. 1B demonstrates that in contrast to the brain, lungs of control male and female mice displayed no significant differences in the levels of either GRX1 or GRX2 mRNA, nor differences in GRX activity (data not shown). To corroborate increases in GRX1 mRNA expression in response to OVA, we performed Western blot analysis for GRX1. Results in Fig.1C demonstrate elevated levels of GRX1 protein in whole lung homogenates after antigen sensitization and challenge when compared to mock-sensitized controls, which corresponds to increases in GRX activity (Fig. 1D). Immunofluorescence analysis of lung sections revealed that in naïve mice GRX1 was localized to both airways and parenchyma, and increased predominantly in the airway epithelium in this experimental model of allergic airway disease (Fig. 1E). Collectively, these data indicate that lung GRX1, but not GRX2 expression increases in allergic airway disease and that this corresponds to elevated levels of GRX activity.

Regulation of GRX1 and S-glutathionylation by IFNγ and TGFβ *in vitro*. Since little information exists on the regulation of GRX expression and activity, we exposed primary mouse tracheal epithelial cells to cytokines relevant to asthma. We focused on tracheal epithelial cells since these cells demonstrated high levels of GRX expression (Fig.1E) and are

known to play an important role in the defense against oxidants and the regulation of innate and adaptive immune responses (20). While no significant effects on GRX activity were observed in response to TNFα on GRX activity, LPS, CpG DNA, IL-4 or TGFβ1 decreased GRX enzymatic activity (Table 1). In contrast, IL-13 and IFNγ were the only mediators tested that caused an increase in GRX activity (Table 1). Since TGFβ1 and IFNγ caused the strongest modulation of GRX activity, we further evaluated GRX expression profiles and protein S-glutathionylation in response to these cytokines. Consistent with decreased GRX activity, TGFβ1 caused attenuated expression of GRX1 mRNA, while not affecting GRX2 mRNA levels (Fig.2A). Although we could not detect clear decreases in GRX1 protein levels in response to TGFβ1 compared to controls via immunofluorescence (Fig.2C, upper panels), possibly due to the low level of staining present in control cells, TGFβ1 did result in marked increases in protein S-glutathionylation (Fig.2C, lower panels). Consistent with the increases in GRX activity in cells exposed to IFNγ (Table 1), exposure to IFNγ resulted in increased expression of GRX1 mRNA, whereas GRX2 mRNA levels were not affected (Fig.2B). Concomitant increases in GRX1 protein levels (Fig.2C, upper panels), and decreases in protein S-glutathionylation (Fig.2C, lower panels) were also detected by immunofluorescence in cells exposed to IFNγ.

	Fold change	*p*-value
Sham	1	
TNFα	0.76±0.07	0.19
LPS	0.89±0.07	0.03
CpG DNA	0.71±0.003	0.03
IL-13	1.42±0.27	0.01
IL-4	0.73±0.07	0.03
TGFβ	0.60±0.02	0.01
IFNγ	1.93±0.05	0.002

Table 1: GRX activity in primary airway epithelial cells. Cells were treated for 48 h with each agent at the following concentrations: 20 ng/ml TNFα, 5 μg/ml LPS, 1 μg/ml CpG DNA, 10 pg/ml IL-13, 20 ng/ml IL-4, 10 ng/ml TGFβ or 20 pg/ml IFNγ and GRX activity was determined.

DISCUSSION

In this study we identified GRX1 as part of the antioxidant defense system of the lungs that is upregulated during allergic airway disease. Although basal expression levels of GRX2 mRNA were higher then GRX1, only increases in GRX1 mRNA were detected in lung tissue of mice with allergic airway disease (Fig.1). The two isoforms of GRX do not only differ in their intracellular localization (16, 17), but evidence is emerging that they might be differentially regulated as well.

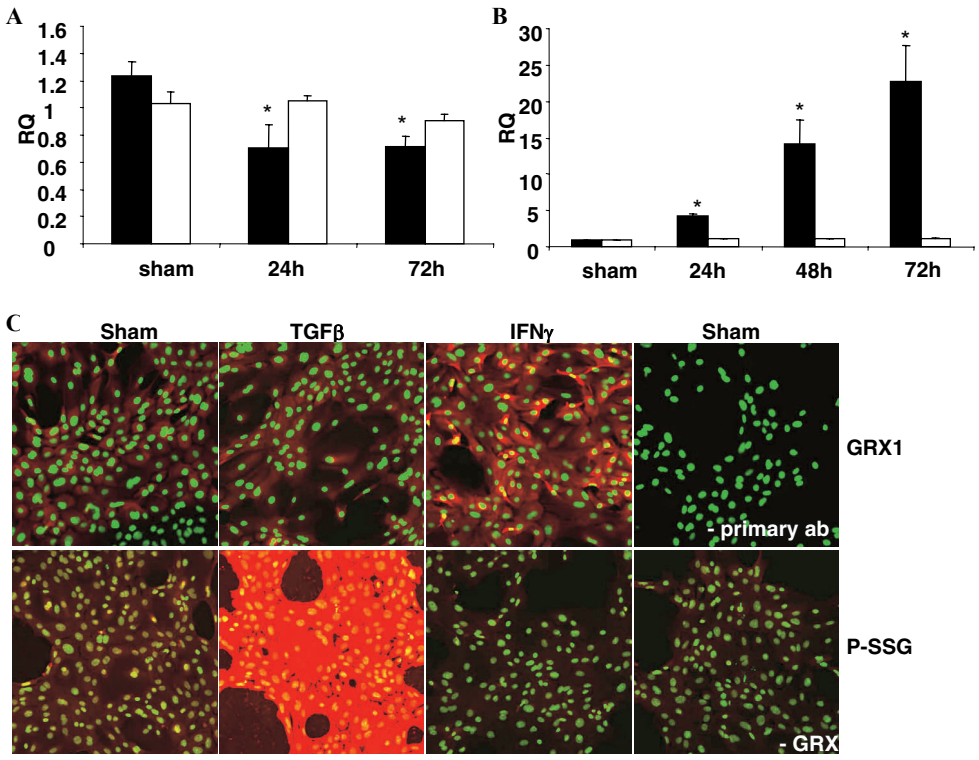

Figure 2: Regulation of GRX1 and S-glutathionylation by IFNγ and TGFβ *in vitro*. Primary tracheal epithelial cells were treated with (A) 5 ng/ml TGFβ1 or (B) 20 pg/ml IFNγ for the indicated time frames, mRNA was collected, reverse-transcribed and analyzed for GRX1 (■) and 2 (□) expression relative to HPRT by TaqMan PCR. Data are expressed as mean (±SD) and * represents $p<0.05$. (C) Primary airway epithelial cells were treated with 5 ng/ml TGFβ1 or 20 pg/ml IFNγ for 48h. Upper panels: Immunohistochemistry for GRX1 was performed (red) and nuclei were counterstained with Sytox Green (green). Lower panels: GRX1-reversible cysteine oxidation was performed (red) and nuclei were counterstained with Sytox Green (green). As reagent controls, either primary antibody (top) or GRX (-GRX, bottom) were omitted from the reaction mix.

Recent studies have indicated that GRX2 is a redox sensor that is inactive as a dimer through the formation of an 2Fe-2S cluster. Upon oxidative stress, GRX2 is activated through oxidation of the 2Fe-2S cluster and its subsequent monomerization (27). Our present work demonstrates that GRX1 mRNA levels are increased after treatment with IFNγ while conversely TGFβ treatment led to repressed expression of GRX1 mRNA (Fig.2A&B, respectively). These findings also suggest that GRX1 is regulated at the transcriptional level, whereas GRX2 may be regulated posttranslationally. Such diverse regulatory mechanisms would allow for quick activation of GRX2 upon oxidative stress to confer rapid restoration of sulfhydryl groups, whereas GRX1 would provide delayed but sustained protection.

In the current study we report that IFNγ enhanced GRX1 mRNA levels in primary tracheal epithelial cells. The antioxidant enzymes thioredoxin reductase (28) and MnSOD (29) have also been demonstrated to be positively regulated by IFNγ. The promoter region of the human GRX1 gene however does not contain GAS elements (30) and IFNγ is therefore likely to act in concert with other transcription factors to induce GRX1. The repression of GRX1 expression by TGFβ has been observed previously (18). Surprisingly, a repressive effect of TGFβ has also been reported towards multiple other antioxidant enzymes, like for instance catalase, MnSOD, Cu,ZnSOD (31) and γ-glutamylcysteinyl synthetase (32). Although the mechanism and functional outcome of repression of antioxidant defenses by TGFβ remain elusive, several potential interactions between TGFβ and oxidants/antioxidants in the lung have been described, and it is thought that oxidants and TGFβ may co-operate to enhance fibrotic responses, for review (33).

Asthma or allergic airway disease is characterized by a Th-2 bias. GRX1 expression is elevated during allergic airway disease, but GRX1 appears to be negatively regulated by at least two Th-2 cytokines, TGFβ and IL-4, whereas the Th-1 cytokine IFNγ positively impacted on GRX1 expression. While at first glance these results seem contradictory, Th-1 co-development has been demonstrated to occur during allergic airway inflammation (34) and IFNγ in particular has been found to be elevated in mouse models that use similar protocols to the one used in this current study (35). The increase in GRX1 and total GRX activity during allergic airway disease could be part of the protective antioxidant defense of the lung. Through its deglutathiolating activity, GRX1 could be responsible

for liberating GSH from proteins in order to increase GSH levels to combat the oxidant stress associated with the inflammatory response. Additionally, attenuation of protein S-glutathionylation following GRX1 activation in response to IFNγ (Fig.2C), could play an important role in the disease process by affecting cell signaling cascades. For instance S-glutathionylation of the p50 and c-jun subunit of the transcription factors NF-κB and AP-1 have been demonstrated to prevent DNA-binding and consequent inflammatory gene transcription (11, 12). However, additional experiments aimed at unraveling changes in S-glutathionylation of critical protein targets will need to be implemented to formally test these scenarios.

In vivo GRX1 expression appears to be mainly localized to the airway epithelium (Fig.1E). Airway epithelium represents an important barrier of the lungs and confers antioxidant protection. It is therefore not surprising that GRX1 expression is prominently localized here under basal conditions, in addition to being upregulated during allergic airway inflammation (Fig.1E). However, a previous study reported that human GRX1 was mainly localized in alveolar macrophages, and only weakly to bronchial epithelium (18), which might reflect differences in GRX expression among different species.

In summary, the present study provides the first evidence that expression of GRX is altered in mice with allergic airway inflammation. Given the emerging significance of GRX catalytic activity both in antioxidant defenses and cell signaling (13), it is plausible that this newly recognized enzyme could play a critical role in the etiology of allergic airway inflammation.

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REFERENCES

1. Forteza, R., M. Salathe, F. Miot, R. Forteza, and G. E. Conner. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2005; 32(5):462-469.
2. Cantin, A. M., S. L. North, R. C. Hubbard, and R. G. Crystal. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol* 1987; 63(1):152-157.
3. Kelly, F. J., I. Mudway, A. Blomberg, A. Frew, and T. Sandstrom. Altered lung antioxidant status in patients with mild asthma. *Lancet* 1999; 354(9177):482-483.
4. Dauletbaev, N., J. Rickmann, K. Viel, R. Buhl, T. O. Wagner, and J. Bargon. Glutathione in induced sputum of healthy individuals and patients with asthma. *Thorax* 2001; 56(1):13-18.
5. Mak, J. C., H. C. Leung, S. P. Ho, B. K. Law, W. K. Lam, K. W. Tsang, M. S. Ip, and M. Chan-Yeung. Systemic oxidative and antioxidative status in Chinese patients with asthma. *J Allergy Clin Immunol* 2004; 114(2):260-264.
6. Comhair, S. A., P. R. Bhathena, C. Farver, F. B. Thunnissen, and S. C. Erzurum. Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells. *Faseb J* 2001; 15(1):70-78.
7. Malerba, G., and P. F. Pignatti. A review of asthma genetics: gene expression studies and recent candidates. *J Appl Genet* 2005; 46(1):93-104.
8. Comhair, S. A., W. Xu, S. Ghosh, F. B. Thunnissen, A. Almasan, W. J. Calhoun, A. J. Janocha, L. Zheng, S. L. Hazen, and S. C. Erzurum. Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. *Am J Pathol* 2005; 166(3):663-674.
9. De Raeve, H. R., F. B. Thunnissen, F. T. Kaneko, F. H. Guo, M. Lewis, M. S. Kavuru, M. Secic, M. J. Thomassen, and S. C. Erzurum. Decreased Cu,Zn-SOD activity in asthmatic airway epithelium: correction by inhaled corticosteroid in vivo. *Am J Physiol* 1997; 272(1 Pt 1):L148-154.
10. Ghosh, S., A. J. Janocha, M. A. Aronica, S. Swaidani, S. A. Comhair, W. Xu, L. Zheng, S. Kaveti, M. Kinter, S. L. Hazen, and S. C. Erzurum. Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. *J Immunol* 2006; 176(9):5587-5597.
11. Pineda-Molina, E., P. Klatt, J. Vazquez, A. Marina, M. Garcia de Lacoba, D. Perez-Sala, and S. Lamas. Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 2001; 40(47):14134-14142.
12. Klatt, P., E. P. Molina, and S. Lamas. Nitric oxide inhibits c-Jun DNA binding by specifically targeted S- glutathionylation. *J Biol Chem* 1999; 274(22):15857-15864.
13. Shelton, M. D., P. B. Chock, and J. J. Mieyal. Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxid Redox Signal* 2005; 7(3-4):348-366.
14. Gravina, S. A., and J. J. Mieyal. Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. *Biochemistry* 1993; 32(13):3368-3376.
15. Yang, Y., S. Jao, S. Nanduri, D. W. Starke, J. J. Mieyal, and J. Qin. Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity. *Biochemistry* 1998; 37(49):17145-17156.

16. Gladyshev, V. N., A. Liu, S. V. Novoselov, K. Krysan, Q. A. Sun, V. M. Kryukov, G. V. Kryukov, and M. F. Lou. Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2. *J Biol Chem* 2001; 276(32):30374-30380.
17. Lundberg, M., C. Johansson, J. Chandra, M. Enoksson, G. Jacobsson, J. Ljung, M. Johansson, and A. Holmgren. Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *J Biol Chem* 2001; 276(28):26269-26275.
18. Peltoniemi, M., R. Kaarteenaho-Wiik, M. Saily, R. Sormunen, P. Paakko, A. Holmgren, Y. Soini, and V. L. Kinnula. Expression of glutaredoxin is highly cell specific in human lung and is decreased by transforming growth factor-beta in vitro and in interstitial lung diseases in vivo. *Hum Pathol* 2004; 35(8):1000-1007.
19. Barrios, R., Z. Z. Shi, S. V. Kala, A. L. Wiseman, S. E. Welty, G. Kala, A. A. Bahler, C. N. Ou, and M. W. Lieberman. Oxygen-induced pulmonary injury in gamma-glutamyl transpeptidase-deficient mice. *Lung* 2001; 179(5):319-330.
20. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol* 2002; 160(4):1325-1334.
21. Wu, R., and D. Smith. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. *In Vitro* 1982; 18(9):800-812.
22. Reynaert, N. L., K. Ckless, A. S. Guala, E. F. Wouters, A. van der Vliet, and Y. M. Janssen-Heininger. In situ detection of S-glutathionylated proteins following glutaredoxin-1 catalyzed cysteine derivatization. *Biochim Biophys Acta* 2006; 1760(3):180-187.
23. Gan, Z. R., and W. W. Wells. Purification and properties of thioltransferase. *J Biol Chem* 1986; 261(3):996-1001.
24. Takeda, K., A. Haczku, J. J. Lee, C. G. Irvin, and E. W. Gelfand. Strain dependence of airway hyperresponsiveness reflects differences in eosinophil localization in the lung. *Am J Physiol Lung Cell Mol Physiol* 2001; 281(2):L394-402.
25. Jurado, J., M. J. Prieto-Alamo, J. Madrid-Risquez, and C. Pueyo. Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse. *J Biol Chem* 2003; 278(46):45546-45554.
26. Kenchappa, R. S., L. Diwakar, J. Annepu, and V. Ravindranath. Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. *Faseb J* 2004; 18(10):1102-1104.
27. Lillig, C. H., C. Berndt, O. Vergnolle, M. E. Lonn, C. Hudemann, E. Bill, and A. Holmgren. Characterization of human glutaredoxin 2 as iron-sulfur protein: A possible role as redox sensor. *Proc Natl Acad Sci U S A* 2005; 102(23):8168-8173.
28. Soderberg, A., B. Sahaf, and A. Rosen. Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res* 2000; 60(8):2281-2289.
29. Harris, C. A., K. S. Derbin, B. Hunte-McDonough, M. R. Krauss, K. T. Chen, D. M. Smith, and L. B. Epstein. Manganese superoxide dismutase is induced by IFN-gamma in multiple cell types. Synergistic induction by IFN-gamma and tumor necrosis factor or IL-1. *J Immunol* 1991; 147(1):149-154.
30. Park, J. B., and M. Levine. The human glutaredoxin gene: determination of its organization, transcription start point, and promoter analysis. *Gene* 1997; 197(1-2):189-193.

31. Kayanoki, Y., J. Fujii, K. Suzuki, S. Kawata, Y. Matsuzawa, and N. Taniguchi. Suppression of antioxidative enzyme expression by transforming growth factor-beta 1 in rat hepatocytes. *J Biol Chem* 1994; 269(22):15488-15492.
32. Arsalane, K., C. M. Dubois, T. Muanza, R. Begin, F. Boudreau, C. Asselin, and A. M. Cantin. Transforming growth factor-beta1 is a potent inhibitor of glutathione synthesis in the lung epithelial cell line A549: transcriptional effect on the GSH rate-limiting enzyme gamma-glutamylcysteine synthetase. *Am J Respir Cell Mol Biol* 1997; 17(5):599-607.
33. Kinnula, V. L., C. L. Fattman, R. J. Tan, and T. D. Oury. Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. *Am J Respir Crit Care Med* 2005; 172(4):417-422.
34. Randolph, D. A., R. Stephens, C. J. Carruthers, and D. D. Chaplin. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* 1999; 104(8):1021-1029.
35. Poynter, M. E., R. Cloots, T. van Woerkom, K. J. Butnor, P. Vacek, D. J. Taatjes, C. G. Irvin, and Y. M. Janssen-Heininger. NF-kappa B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol* 2004; 173(11):7003-7009.

CHAPTER 8

Catalase overexpression does not attenuate inflammation and increases airway hyperresponsiveness in a murine model of allergic airway disease

ABSTRACT

Oxidative stress is a hallmark of asthma, and increased levels of oxidants are considered markers of the inflammatory process. Most studies to date addressing the role of oxidants in the etiology of asthma were based on the therapeutic administration of low molecular weight antioxidants or antioxidant mimetic compounds. To directly address the function of endogenous hydrogen peroxide in the pathophysiology of allergic airway disease, we comparatively evaluated mice systemically overexpressing catalase, a major antioxidant enzyme that detoxifies hydrogen peroxide, and C57BL/6 strain matched controls in the Ovalbumin (OVA) model of allergic airways disease. Despite 8-fold increases in catalase activity in lung tissue of catalase transgenic mice compared to C57BL/6 controls, both strains showed similar increases in OVA-specific IgE, IgG1, and IgG2a levels, comparable airway and tissue inflammation, and identical increases in pro-collagen 1 mRNA expression, following sensitization and challenge with ovalbumin. Unexpectedly, mRNA expression of Muc5ac and CLCA3 genes were enhanced in catalase transgenic mice, compared to C57BL/6 mice subjected to antigen. Furthermore, when compared to control mice, catalase overexpression increased airway hyper-responsiveness to methacholine both in naïve mice as well as in response to antigen. In contrast to the prevailing notion that hydrogen peroxide is positively associated with the etiology of allergic airways disease, the current findings implicate that endogenous hydrogen peroxide serves a role in suppressing both mucus production and airway hyper-responsiveness.

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INTRODUCTION

Oxidative stress is one of the hallmarks of asthma. Inflammatory cells associated with asthma are considered to be the main source of oxidants. In addition, oxidants are continuously produced by resident pulmonary cells, including epithelial cells, during normal cellular respiration and aerobic metabolism as well as through non-phagocytic-NADPH oxidases (Duox (1, 2)). Duox1 and 2 are found at the apical surface of tracheal and airway epithelial cells, and recently Duox1 was found to be inducible in response to the Th2 cytokines IL-4 and IL-13 (1, 3). Lastly, asthma exacerbations are often caused by environmental agents that increase the oxidative burden in the lung, like tobacco smoke (4), air pollution (5), ozone (6) and pollen (7).

Oxidative stress has been reported in patients with exacerbations. Elevated levels of the oxidants, hydrogen peroxide (H_2O_2) (8), and nitric oxide (9) are detected in exhaled breath, and increases in levels of 8-isoprostane (10), malondialdehyde (11), nitrotyrosine (12), and protein carbonyls (13) which reflect lipid or protein oxidation, are also present in these patients. In stable asthmatics on the other hand, variable data have been reported with regard to oxidative stress (11, 14). Since levels of oxidants correlate directly with disease severity, oxidants, or markers of oxidation currently serve as parameters for the assessment of disease severity (15), and as measures of the therapeutic effect of inhaled corticosteroids (15, 16).

Oxidants are believed to play a causal role in pathophysiology of asthma. Several studies have indicated that oxidants may contribute to the development of some of the hallmarks of asthma. For instance, oxidants are known to cause epithelial cell death through DNA damage (17) and the consequent loss of epithelial barrier function can increase airway hyperreactivity (18, 19). Furthermore oxidants have been demonstrated to cause mucus hypersecretion and impair mucociliary clearance (20-22) which can lead to airflow limitation. Lower levels of Cu,ZnSOD (23) and MnSOD activity (24) are also well known to occur in lungs of asthmatics, contributing to the pro-oxidative environment.

A number of strategies to increase the antioxidant capacity of the lung have been evaluated in patients with asthma. These studies mainly investigated dietary supplementation of antioxidants like vitamin C (25) and E (26, 27), as well as cysteine precursors (28, 29), and reported variable success rates in alleviating asthma symptoms.

Additional approaches, aimed at restoring normal levels of antioxidant enzymes encompassed SOD mimetic compounds, which have been shown to improve inflammation and other features of allergic airway disease in animal models (30, 31).

Catalase represents an important component of the endogenous antioxidant defense system of the lung. Catalase is responsible for detoxifying H_2O_2 produced under physiological conditions. Catalase transgenic mice have been shown to exhibit attenuated disease parameters in various models associated with oxidative stress, like hypoxia-reoxygenation (32) and doxorubicin toxicity in the heart (33) as well as oxidant injury to pancreatic β-cells (34). Most recently, mice that overexpress catalase in mitochondria were found to display an extended life span (35).
Since most studies that address the role of oxidants in the pathophysiology of allergic airways disease have relied on the use of antioxidant compounds with diverse reactivities, the causal role of endogenously generated H_2O_2 in the disease process remains unraveled. The goal of the present study therefore was to elucidate the contribution of H_2O_2 in the pathophysiology of allergic airway disease. For this purpose we utilized mice that systemically overexpress catalase and strain matched controls, in the ovalbumin model of allergic airway disease.

MATERIALS AND METHODS

Animals. Homozygous Tg $(CAT)^{+/+}$ mice were a kind gift of Dr. Holly Van Remmen (University of Texas at San Antonio, San Antonio, Tx (36)). Briefly, catalase transgenic mice were generated using a 80 kb genomic DNA fragment containing the human CAT gene and ‘5 and ‘3 flanking regions (37), that led to the integration of a 65 kb fragment. Thus the human CAT gene is controlled by endogenous regulatory elements which leads to systemic gene expression. Mice were backcrossed onto the C57BL/6 background. Six-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used as controls. Mice were administered 20 μg ovalbumin (OVA) with 2.25 mg Imject Alum (OVA-sensitized, OVA/OVA) or 2.25 mg Imject Alum alone (mock-sensitized, Alu/OVA) via intraperitoneal injection on day 0 and 7. All mice were challenged for 30 min with aerosolized 1% OVA in PBS on day 14, 15 and 16, as previously described (38). Grade V OVA was purchased from Sigma-Aldrich (St.Louis, MO) and ImjectAlum from Pierce

Biotechnology (Rockford, IL). Mice were euthanized by a lethal dose of pentobarbital via intraperitoneal injection, 48 h after the last challenge. The Institutional Animal Care and Use Committee granted approval for all studies.

Pulmonary function assessment. Anesthetized mice were tracheotomized and mechanically ventilated for the assessment of pulmonary function using the forced oscillation technique as described previously (39) (flexiVent; SCIREQ Inc., Montreal, Canada). Briefly, mice were ventilated at a rate of 2.5 Hz with a tidal volume of 0.2 ml and 3 cm H_2O positive end-expiratory pressure. Data from prior to methacholine challenge were collected to establish the baseline for each animal. Next, inhaled doses of aerosolized methacholine (Sigma-Aldrich) in saline were administered in successive increasing concentrations (0, 3.125, 12.5 and 50 mg/ml). Multiple linear regression was used to fit impedance spectra derived from measured pressure and volume to the constant phase model of the lung: $Z(f) = R_n + J\omega I + [(G_{ti} + jH_{ti})/\omega^a]$ (40). We determined the following physiological parameters: R_n (a measure of central airways resistance), H_{ti} (elastance) and G_{ti} (a measure of visco-elastic properties and/or airflow heterogeneity, (41)). The peak response for each variable was determined, and the percentage change from baseline, as measured at the beginning of the protocol, was calculated.

Bronchoalveolar lavage (BAL). BAL fluid was collected from euthanized mice, using 1 ml of phosphate buffered saline for the assessment of total and differential cell counts.

Plasma collection and immunoglobulin analysis. Following euthanasia, blood was collected by heart puncture, transferred to plasma separator tubes, centrifuged, and plasma was kept frozen at -80°C. For Ig ELISAs, 96-well plates were coated with 1 μg/ml OVA in PBS overnight at 4°C and washed with PBS containing 0.05% Tween 20 (PBS-T). After blocking with 1% BSA in PBS for 1 h, plates were washed with PBS-T, plasma was applied at dilutions of 1:2 – 1:250 and incubated for 2 h. Plates were washed with PBS-T and incubated with biotinylated secondary antibodies (BD Biosciences Pharmigen, San Diego, CA), followed by incubation with streptavidin/peroxidase (R&D Systems) for 1 h and detection using reagents from R&D Systems. ODs were read at 405 nm with a wavelength correction at 540 nm (Bio-Tek Instruments $PowerWave_x$,Winooski, VT). Data is reported as delta OD values (± SEM) from identical dilutions within the linear range of the readings.

Histopathology and morphometry. Following euthanasia and BAL, the left lung lobe was instilled with 4% paraformaldehyde (PFA) in PBS and placed into 4% PFA at 4°C overnight, before embedding in paraffin. Next, 7-μm sections were cut, affixed to glass microscope slides, deparaffinized with xylene, and rehydrated through a series of ethanols and stained with haematoxylin and eosin, periodic acid Schiff (PAS) stains or Pico Sirius red, coverslipped, and examined by light microscopy (20X objective). Sections with a length:diameter ratio of <2:1 were evaluated for PAS positivity and collagen deposition. The percentage of PAS positive airway epithelial cells was recorded. For the assessment of pulmonary fibrosis, Pico Sirius red stained sections were visualized by differential interference contrast microscopy (42) and scored using a scale of 1 to 3 for airway as well as parenchyma associated collagen deposition by two independent, blinded observers. The cumulative score from each mouse was then averaged according to treatment group.

Catalase activity. Pulverized lung tissue was dissolved in 9 volumes Na-phosphate buffer, and different dilutions were reacted with 30 mM H_2O_2. Decomposition of H_2O_2 was followed spectrophotometrically at 240 nm (43). Recombinant catalase was used to generate a standard curve. Data are expressed in units, where 1 unit equals the amount of enzyme that will decompose 1 μM H_2O_2 per minute at 25°C, and results are normalized to protein content.

Semi-quantitative PCR. Total RNA was DNase treated and reverse transcribed into cDNA. Semi-quantitative TaqMan PCR was performed using TaqMan Gene Expression Assays for MUC5AC, CLCA3 and COL1A1 (Applied Biosystems, Foster City, CA). Values were normalized to the expression levels of HPRT.

Bio-Plex analysis. The Bio-Plex (Bio-Rad) kit used allowed analysis of twenty three different cytokines, and was used according to the manufacturer's instructions. Standard curves were established using a stock of lyophilized multiplex cytokine. The anti-cytokine beads were vortexed and a 25-fold working dilution was prepared in a stock solution of Assay Buffer A. The bead solution was added to the plate and washed twice with Bio-Plex wash buffer A. Standards and samples were added to the plate and incubated for 30 minutes at RT with shaking. Following this incubation, the plate was washed 3 times with Bio-Plex wash buffer A. Detection antibody A was incubated for 30 minutes at RT with shaking, washed 3 times and incubated with streptavadin-phycoerythrin for 10 min

at RT with shaking. After 3 washes, the beads were resuspended in Bio-Plex wash buffer A and the plate was read on the Bio-Plex suspension array reader.

Statistical analysis. All data were expressed as mean ± SEM and compared by analysis of variance (ANOVA). Differences were considered significant when $p<0.05$. Pulmonary function assessment was analyzed using repeated measures ANOVA with dose as the within-animal repeated measure. Mouse and treatment were the grouping factors, and the F-statistic associated with the dose by group interaction was used to test for differences in dose-response patterns between groups.

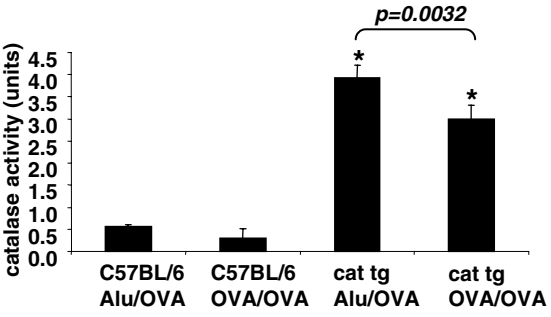

Figure 1 Lung catalase activity in catalase transgenic or C57BL/6 mice. Pulverized lung tissue was homogenized in 9 volumes of Na-phosphate buffer, and reacted with 30 mM H_2O_2. Decomposition of H_2O_2 was recorded spectro-photometrically at 240 nm, and compared to a standard curve generated with catalase of known catalytic activity. Data were normalized to protein, and are expressed as units. * represents $p<0.05$ between Alu/OVA and OVA/OVA. Cat tg: Catalase transgenic mice

RESULTS

Enhanced catalase activity in lungs from catalase transgenic mice. We first determined the basal level of catalase activity in the lungs of control and catalase transgenic mice as well as after OVA sensitization and challenge. Catalase transgenic mice demonstrated an approximate 8-fold increase in lung catalase activity compared to control mice (Fig.1). Sensitization and challenge with OVA did not alter lung catalase activity in C57BL/6 mice, but significantly decreased the activity in transgenic mice.

Catalase overexpression does not alter immunoglobulin production following antigen sensitization and challenge. To ensure that overexpression of catalase did not affect the immunization process, plasma levels of OVA-specific immunoglobulins were measured. Ovalbumin sensitization and challenge increased plasma levels of OVA-specific IgE, IgG1 and IgG2a in C57BL/6 mice (Fig.2). Catalase transgenic mice demonstrated similar increases in levels of these OVA-specific immunoglobulins (Fig.2), indicating that catalase transgenic mice mount an immune response to OVA that is similar to C57BL/6 mice.

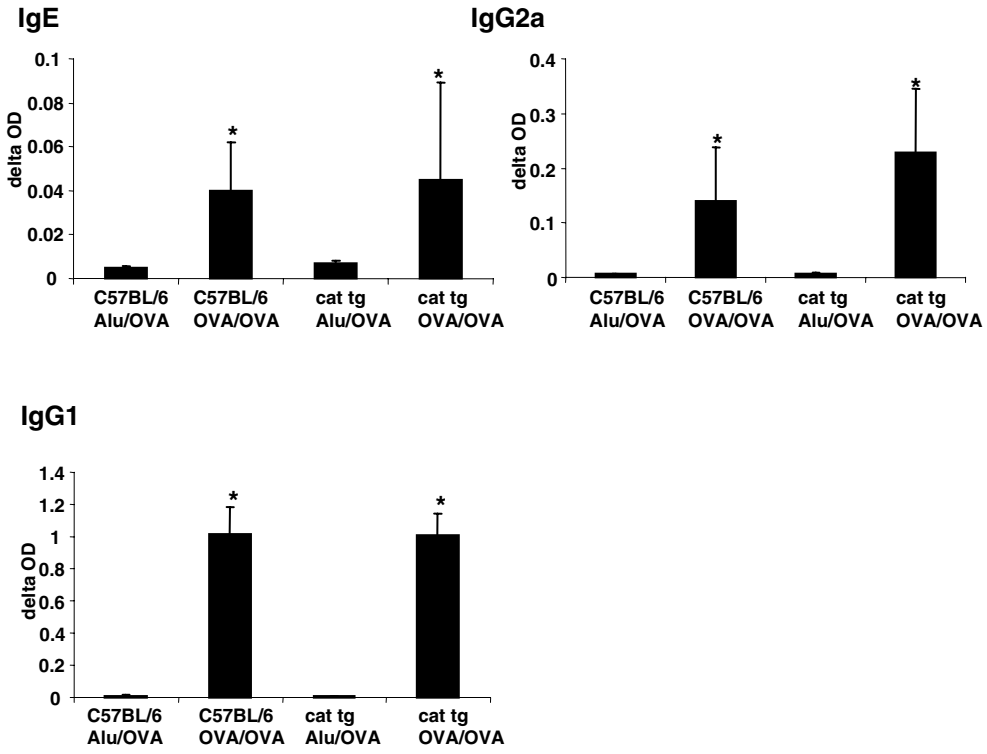

Figure 2 Immunoglobulin production following sensitization and challenge with OVA in catalase transgenic and C57BL/6 mice. Plasma from mock or OVA sensitized mice subjected to Ova challenge (Alu/OVA and OVA/OVA, respectively) was analyzed for OVA-specific IgE, IgG2a and IgG1 by ELISA. Values are corrected mean optical densities (±SEM) from 11-13 mice per group. * represents $p<0.05$ between Alu/OVA and OVA/OVA. Cat tg: Catalase transgenic mice

Catalase overexpression does not confer protection against cellular infiltration following antigen sensitization and challenge. We next evaluated the impact of catalase overexpression on OVA-induced pulmonary inflammation in tissue (Fig.3A), and BAL (Figs 3B and 3C).

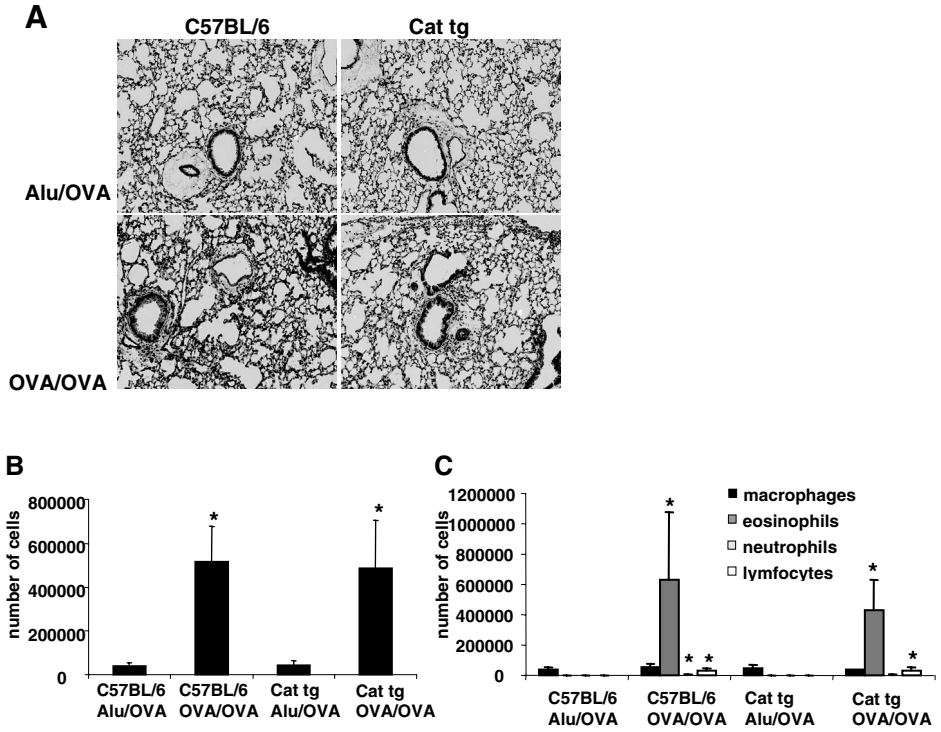

Figure 3 Airway and tissue inflammation following sensitization and challenge with OVA in catalase transgenic and C57BL/6 mice. A. Lung histopathology was evaluated by staining representative sections from paraffin-embedded lungs with haematoxylin and eosin. BAL fluid was collected and total (B) and differential cell counts were performed (C). Values are means (±SEM) from 10-13 mice per group. * represents $p<0.05$ between Alu/OVA and OVA/OVA. Cat tg: Catalase transgenic mice

As expected, sensitization and challenge with OVA caused prominent perivascular and peribronchial cell infiltration in C57BL/6 mice, and marked increases in eosinophils in BAL. Surprisingly, catalase transgenic mice displayed inflammatory responses to antigen (Fig. 3) that were indistinguishable from C57BL/6 mice. Evaluation of BAL cytokines revealed no detectable levels of IL12 (p70), IL13, IL10, IFNγ, TNFα or eotaxin in mock immunized mice, nor detectable expression of GM-CSF, IL2 or IL3 in response to Ova sensitization plus challenge in C57BL/6 or

catalase transgenic mice (data not shown). Similar OVA-dependent increases in IL4, IL5, IL6, MCP1, G-CSF and MIP1β occurred in both C57BL/6 and catalase transgenic mice (Table 1). Although KC and IL12 (p40) increased in an OVA-dependent manner in both mouse strains, these increases were enhanced in C57BL/6 mice compared to catalase transgenic mice. In contrast, IL1α, MIP1α, RANTES and IL17 were found only to be significantly elevated after OVA immunization and challenge in catalase transgenic mice. Lastly, naïve C57BL/6 mice demonstrated higher levels of RANTES and MIP1β compared to catalase transgenic mice (Table 1).

	C57BL/6		cat tg	
cytokine	**Alu/OVA**	**OVA/OVA**	**Alu/OVA**	**OVA/OVA**
IL4	0	10.65±2.54*	0	3.94±1.28*
IL5	0.93±0.62	16.30±4.05*	0.36±0.31	11.17±2.79*
IL6	1.52±0.30	11.95±3.84*	0.10±0.52	12.76±4.76*
MCP 1	12.92±2.87	56.32±13.75*	6.22±6.22	141.10±51.43*
G CSF	1.19±0.45	10.16±3.61*	1.14±0.21	19.17±12.23*
KC	23.73±8.18	137.99±34.76*§	14.05±6.71	80.53±30.14*
IL1α	1.06±0.18	1.20±0.31	1.10±0.28	1.87±0.78*
MIP 1α	3.95±1.35	11.38±7.62	3.04±0.14	6.34±1.42*
MIP 1β	1.98±0.62†	8.94±3.29*	1.49±0	9.06±3.54*
Rantes	6.57±1.89†	9.75±3.17	6.02±0.32	14.04±5.76*
IL17	0.92±0.53	1.19±0.67	0.61±0.60	2.18±1.64*
IL12 (p40)	32.26±10.43	372.07±148.03*§	31.59±21.78	132.34±40.38*

Table 1 Cytokines in BAL fluid. Cytokines were measured in BAL fluid by Bio-Plex analysis. Values are expressed in pg/ml and represent mean of 3 to 7 animals per group ± SEM. * OVA-induced significant difference.
† significant difference between C57BL/6 Alu/OVA and cat tg Alu/OVA.
§ significant difference between C57BL/6 OVA/OVA and cat tg OVA/OVA.

Catalase transgenic mice display enhanced mucin gene expression in response to OVA, compared to C57BL/6 controls. Since oxidants have been reported to stimulate mucus production (22), we next evaluated mucus metaplasia. PAS positive epithelial cells were detected both in C57BL/6 and catalase transgenic mice in response to Ova (Fig. 4A). Although scoring of percent PAS-positive cells in the airways indicated a trend toward enhanced goblet cell metaplasia in catalase transgenic mice (Fig. 4B), this failed to reach statistical significance. Evaluation of mRNA expression of CLCA3 (Fig. 4C) and MUC5AC (Fig. 4D) in lung tissues

revealed marked increases in both strains of mice in response to sensitization and challenged with OVA. However mRNA increases CLCA3 and MUC5AC were more pronounced in catalase transgenic mice compared to C57BL/6 strain matched controls.

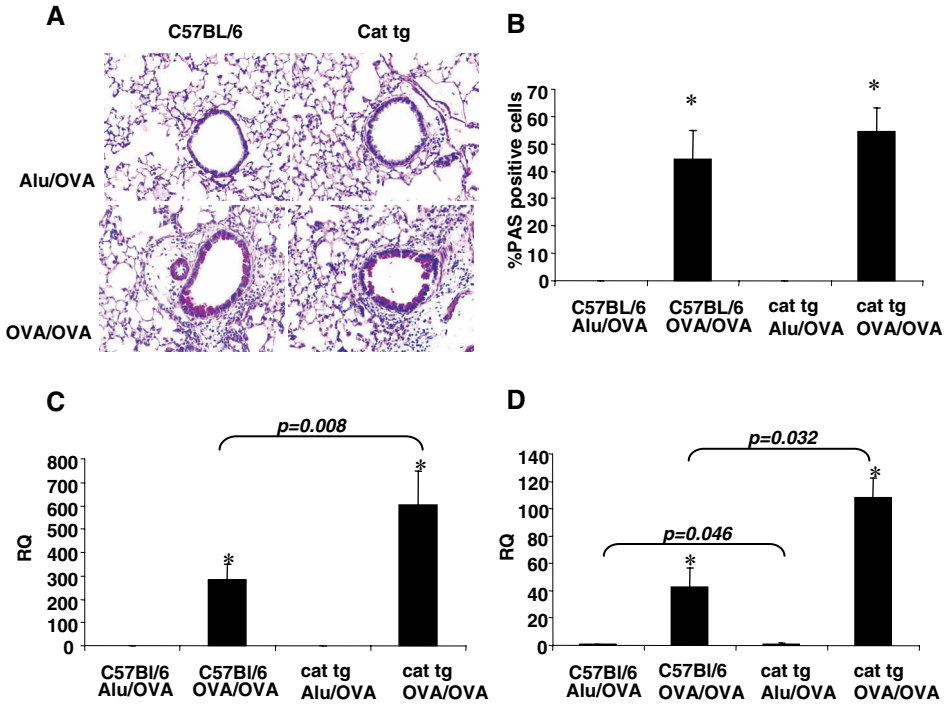

Figure 4 Mucus metaplasia and mucin gene expression following sensitization and challenge with OVA in catalase transgenic and C57BL/6 mice. A. Representative sections from paraffin-embedded lungs, stained using PAS reagent to visualize mucus producing airway cells. B. Airways with a length to diameter ratio of <2:1 were evaluated for PAS positivity and the percentage of PAS positive airway epithelial cells was recorded. Data are expressed as mean of 4 mice per group, using multiple airways per mouse (± SEM). RNA was collected from lungs, reverse-transcribed and analyzed for CLCA3 (C) and MUC5AC (D) expression relative to HPRT by semiquantitative TaqMan PCR. Data are expressed as mean RQ from six mice per group (± SEM). * represents $p<0.05$ between Alu/OVA and OVA/OVA. Cat tg: Catalase transgenic mice

Catalase overexpression does not alter collagen expression or deposition. To address whether H_2O_2 could affect the development of subepithelial fibrosis, lung sections were stained with Pico Sirius red (Fig.

5A) and collagen deposition scored in airways as well as parenchymal regions (Fig. 5B). Although subepithelial fibrosis can be detected in OVA sensitized and challenged mice (44) and C57BL/6 mice are prone to the development of fibrosis (45), the acute exposure regimen used here was not sufficient to enhance collagen deposition (Fig.5A and B), and no differences were observed between C57BL/6 and catalase transgenic mice. However, increases in mRNA expression of COL1A1 were detected in this acute study, and comparable increases in COL1A1 mRNA occurred in both mouse strains (Fig.5C).

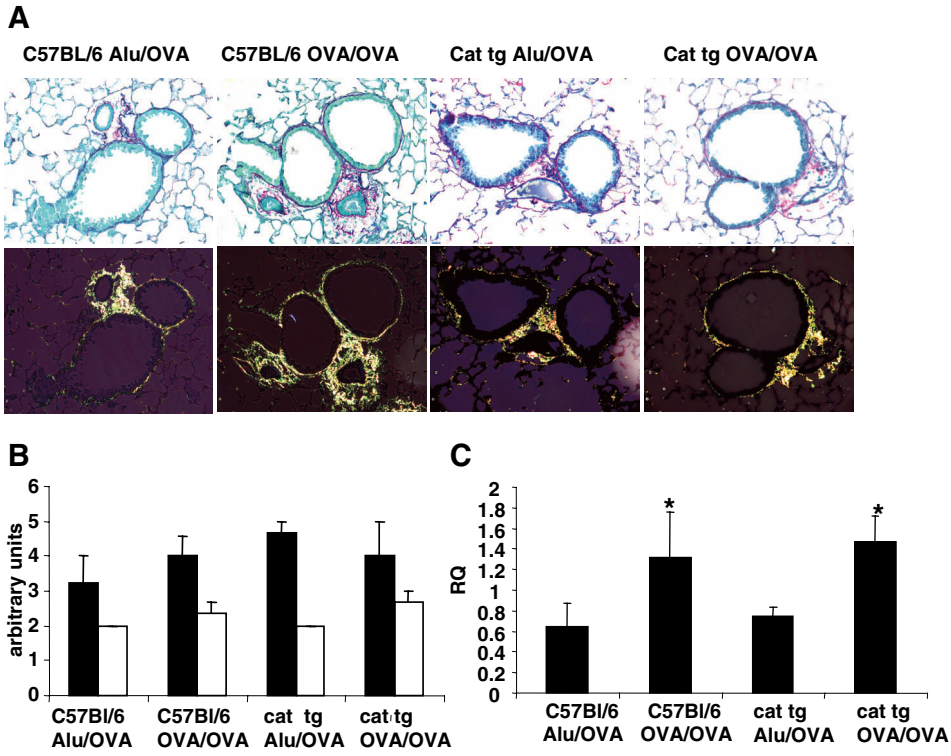

Figure 5 Collagen deposition and COL1A1 mRNA expression in catalase transgenic and C57BL/6 mice after OVA sensitization and challenge. A. Representative sections from paraffin-embedded lungs that were stained using Pico Sirius red, which stains collagen red in bright-field microscopy (upper panels) and bright green when visualized by differential interference contrast microscopy (lower panels). B. Scoring by two blinded observers of collagen deposition in airways (■) or parenchyma (□) using a scale from 1 to 3. The cumulative score for each mouse was averaged according to treatment group. C. RNA was collected from lungs, reverse-transcribed and analyzed for COL1A1 mRNA expression by TaqMan PCR. Data are expressed as mean RQ from six mice per group (± SEM), following normalization to the housekeeping gene, HPRT. * represents $p<0.05$ between Alu/OVA and OVA/OVA.

Enhanced airway hyperresponsiveness following OVA sensitization and challenge in catalase transgenic mice compared to C57BL/6 controls. To assess whether catalase overexpression affected respiratory physiology, pulmonary function was determined using the forced oscillation technique and the constant phase model of the lung (39). OVA-induced increases in central airway resistance (R_n) after challenge with methacholine occurred to similar extents in C57BL/6 and catalase transgenic mouse strains (Fig.6A). Intriguingly, tissue visco-elastic properties and/or airflow heterogeneity (G_{ti},) were elevated in naïve catalase transgenic mice after methacholine challenge compared to naïve C57BL/6 controls, masking increases that are observed in response to OVA challenge in C57BL/6 mice (Fig.6B). Lastly, tissue elastance (H_{ti}) responses to methacholine were similar in naïve animals of the two mouse strains, although Ova-induced increases in elastance were enhanced in mice that overexpress catalase (Fig.6C).

DISCUSSION

The implementation of catalase transgenic mice in various disease models has suggested a damaging role of H_2O_2 in their pathophysiology. In contrast, the present study demonstrating that catalase transgenic mice exhibit worsened airways hyperresponsiveness, and enhanced expression of genes important to mucus production, points to a protective role of H_2O_2 in these manifestations of antigen-induced allergic airway disease. In line with our findings, the same catalase transgenic mice were shown to have increased sensitivity to γ-irradiation induced death (36). Although the results from the present study might be surprising, conflicting reports exist on the role of H_2O_2 in airways hyperresponsiveness. For example, studies using isolated organ preparations have demonstrated that H_2O_2 could increase airway contractility by decreasing epithelial barrier function through damage to epithelia, thereby increasing epithelial permeability to methacholine (18, 19). Furthermore, cyclooxygenase (COX)-2 dependent formation of PGD_2 was shown to be a mediator of H_2O_2-induced bronchoconstriction, whereas conversely, COX-2 dependent formation of PGE_2 has been demonstrated to be involved in H_2O_2-induced repression of airway smooth muscle contractility (46-48). H_2O_2 has furthermore been shown to upregulate the expression of eNOS (49, 50), and the consequent rise in NO production could also account for the bronchoprotective effects of H_2O_2.

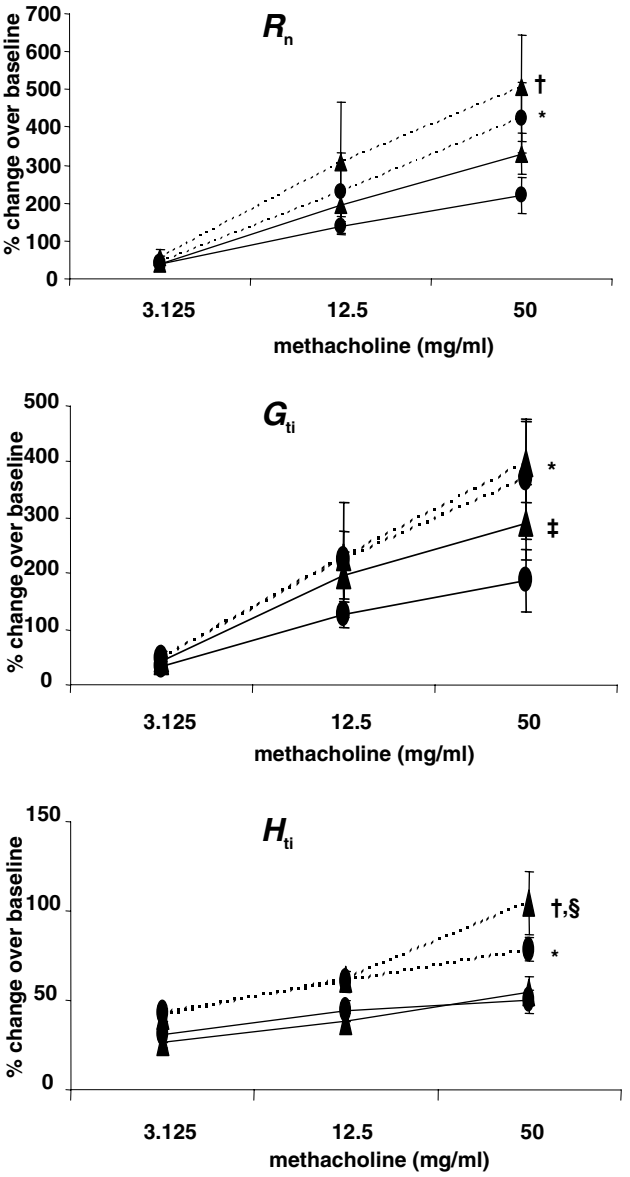

Figure 6 Assessment of airway hyperresponsiveness following OVA sensitization and challenge in catalase transgenic and C57 BL/6 mice. Pulmonary hyperresponsiveness to increasing doses of nebulized methacholine was assessed from forced oscillations and expressed as R_n (central airway resistance, top), G_{ti} (tissue visco-elastic properties and/or heterogeneity, middle) and H_{ti} (elastance, bottom). Solid lines: Alu/OVA; dotted lines: OVA/OVA; ● C57 BL/6; ▲ Cat tg. Data are derived from the constant phase model and are expressed as percentage change from baseline measure-ments (±SEM) and comprised of 7-8 mice per group. * $p<0.05$ between C57BL/6 Alu/OVA and C57 BL/6 OVA/OVA, † $p<0.05$ between cat tg Alu/OVA and cat tg OVA/OVA, ‡ $p<0.05$ between C57BL/6 Alu/OVA and cat tg Alu/OVA, § $p<0.05$ between C57BL/6 OVA/OVA and cat tg OVA/OVA.

Our present findings indicate that catalase overexpression may enhance mucus production, in association with increased expression of MUC5AC and CLCA3 in allergic airway disease. No other studies to date have indicated a direct role for H_2O_2 in the repression of mucus hyperproduction or mucin gene expression. In contrast, oxidants generated through the activity of neutrophil elastase (51), Duox 1 (22) as well as

xanthine/xanthine oxidase (20) have been demonstrated to increase the expression of MUC5AC, consequently leading to increased mucus secretion. However, studies have shown that Interleukin-17 (IL17) is a potent inducer of mucus metaplasia through the induction of MUC5AC (52, 53). The higher levels of IL17 in the BAL fluid of catalase transgenic mice compared to C57BL/6 controls (Table 1) could therefore be responsible for the observed mucus metaplasia. This scenario, which remains to be formally tested, would suggest an indirect role for H_2O_2 in the alterations of mucin gene expression seen in catalase transgenic mice compared to C57BL/6 controls in response to antigen challenge.

Catalase transgenic mice appear to be capable of mounting a normal immune response against OVA, based upon comparable increases in levels of OVA-specific immunoglobulins seen in both strains of mice subjected to antigen sensitization and challenge (Fig.2). Yet recent evidence supports a role for oxidant production, and a second messenger function of oxidants, in cells of the adaptive immune response after receptor activation (for review (54)). In particular, oxidants have been shown to regulate antigen processing and presentation by dendritic cells (55). Some evidence also points to a role of ROS in skewing Th-2 responses (for review (56)). However, levels of the Th-2 cytokines, IL-4 and IL-5, and lung inflammation were similar in catalase transgenic and C57BL/6 mice, suggesting that these observed effects in catalase transgenic mice may not be due to altered regulation of immune cell function.

It is of importance to consider that catalase is not the only enzyme responsible for the detoxification of H_2O_2. Glutathione peroxidase and the more recently discovered members of the peroxiredoxin family of enzymes are also capable of eliminating H_2O_2 (for review see (57)). One of the potential pitfalls of using catalase transgenic mice is that catalase in most cells is primarily localized to peroxisomes (58). This limits investigating the role of H_2O_2 in the extracellular space, like the extracellular lining fluid of the lung where eGPx would be more likely to account for H_2O_2 detoxifying activity. eGPx has furthermore been demonstrated to be elevated in asthmatic patients compared to control individuals (59). Additionally, catalase transgenic mice used in the present study overexpress catalase in all tissues and all compartments of the lung making it impossible to asses the local environment where H_2O_2 affects hyperresponsiveness or mucin gene expression. Tissue specific or inducible transgenic approaches would be required to unravel these questions. It is furthermore critical to consider that most antioxidant

systems are interrelated and interconnected. Compensation in response to changes in the steady state activities of a particular antioxidant module by other functionally related enzyme systems has been recognized (59-62). However, previous studies that addressed antioxidant compensation in catalase transgenic mice failed to detect changes in levels of MnSOD, Cu,ZnSOD, GPx in various tissues, including the lungs, compared to wild type controls (36), suggesting that the enhanced hyperresponsiveness and mucin gene expression are due to changes in endogenous levels of H_2O_2, and not other antioxidant defenses. Although it would appear advantageous to measure endogenous H_2O_2 directly, the currently available assay systems are flawed with non-specific reactivities, and ultimately rely on the use of catalase.

Taken together, the present study indicates a protective role for H_2O_2 in airway hyperresponsiveness and mucus metaplasia and illustrates the importance of H_2O_2 in regulating a diverse spectrum of physiological functions. These findings appear to warrant reconsideration of the damaging role of H_2O_2 in the pathophysiology of allergic airway disease.

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REFERENCES

1. Geiszt, M., J. Witta, J. Baffi, K. Lekstrom, and T. L. Leto. 2003. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *Faseb J.* 17*:*1502-1504.
2. Forteza, R., M. Salathe, F. Miot, R. Forteza, and G. E. Conner. 2005. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol.* 32*:*462-469.
3. Harper, R. W., C. Xu, J. P. Eiserich, Y. Chen, C. Y. Kao, P. Thai, H. Setiadi, and R. Wu. 2005. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett.* 579*:*4911-4917.
4. Eisner, M. D. 2002. Environmental tobacco smoke and adult asthma. *Clin Chest Med.* 23*:*749-761.
5. McConnell, R., K. Berhane, F. Gilliland, J. Molitor, D. Thomas, F. Lurmann, E. Avol, W. J. Gauderman, and J. M. Peters. 2003. Prospective Study of Air Pollution and Bronchitic Symptoms in Children with Asthma. *Am J Respir Crit Care Med.* 168*:*790-797.
6. Vagaggini, B., M. Taccola, S. Cianchetti, S. Carnevali, M. L. Bartoli, E. Bacci, F. L. Dente, A. Di Franco, D. Giannini, and P. L. Paggiaro. 2002. Ozone exposure increases eosinophilic airway response induced by previous allergen challenge. *Am J Respir Crit Care Med.* 166*:*1073-1077.
7. Boldogh, I., A. Bacsi, B. K. Choudhury, N. Dharajiya, R. Alam, T. K. Hazra, S. Mitra, R. M. Goldblum, and S. Sur. 2005. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest.* 115*:*2169-2179.
8. Emelyanov, A., G. Fedoseev, A. Abulimity, K. Rudinski, A. Fedoulov, A. Karabanov, and P. J. Barnes. 2001. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. *Chest.* 120*:*1136-1139.
9. Kharitonov, S. A., D. Yates, R. A. Robbins, R. Logan-Sinclair, E. A. Shinebourne, and P. J. Barnes. 1994. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet.* 343*:*133-135.
10. Montuschi, P., M. Corradi, G. Ciabattoni, J. Nightingale, S. A. Kharitonov, and P. J. Barnes. 1999. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med.* 160*:*216-220.
11. Hanta, I., S. Kuleci, N. Canacankatan, and A. Kocabas. 2003. The oxidant-antioxidant balance in mild asthmatic patients. *Lung.* 181*:*347-352.
12. Kaminsky, D. A., J. Mitchell, N. Carroll, A. James, R. Soultanakis, and Y. Janssen. 1999. Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J Allergy Clin Immunol.* 104*:*747-754.
13. Nadeem, A., S. K. Chhabra, A. Masood, and H. G. Raj. 2003. Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol.* 111*:*72-78.
14. Nadeem, A., H. G. Raj, and S. K. Chhabra. 2005. Increased oxidative stress in acute exacerbations of asthma. *J Asthma.* 42*:*45-50.
15. Loukides, S., D. Bouros, G. Papatheodorou, P. Panagou, and N. M. Siafakas. 2002. The relationships among hydrogen peroxide in expired breath condensate, airway inflammation, and asthma severity. *Chest.* 121*:*338-346.

16. Paredi, P., S. A. Kharitonov, and P. J. Barnes. 2002. Analysis of expired air for oxidation products. *Am J Respir Crit Care Med.* 166*:*S31-37.
17. Bucchieri, F., S. M. Puddicombe, J. L. Lordan, A. Richter, D. Buchanan, S. J. Wilson, J. Ward, G. Zummo, P. H. Howarth, R. Djukanovic, S. T. Holgate, and D. E. Davies. 2002. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol.* 27*:*179-185.
18. Hulsmann, A. R., H. R. Raatgeep, J. C. den Hollander, W. H. Bakker, P. R. Saxena, and J. C. de Jongste. 1996. Permeability of human isolated airways increases after hydrogen peroxide and poly-L-arginine. *Am J Respir Crit Care Med.* 153*:*841-846.
19. Hulsmann, A. R., H. R. Raatgeep, J. C. den Hollander, T. Stijnen, P. R. Saxena, K. F. Kerrebijn, and J. C. de Jongste. 1994. Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. *Am J Respir Crit Care Med.* 149*:*519-525.
20. Adler, K. B., W. J. Holden-Stauffer, and J. E. Repine. 1990. Oxygen metabolites stimulate release of high-molecular-weight glycoconjugates by cell and organ cultures of rodent respiratory epithelium via an arachidonic acid-dependent mechanism. *J Clin Invest.* 85*:*75-85.
21. Feldman, C., R. Anderson, K. Kanthakumar, A. Vargas, P. J. Cole, and R. Wilson. 1994. Oxidant-mediated ciliary dysfunction in human respiratory epithelium. *Free Radic Biol Med.* 17*:*1-10.
22. Shao, M. X., and J. A. Nadel. 2005. Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci U S A.* 102*:*767-772.
23. De Raeve, H. R., F. B. Thunnissen, F. T. Kaneko, F. H. Guo, M. Lewis, M. S. Kavuru, M. Secic, M. J. Thomassen, and S. C. Erzurum. 1997. Decreased Cu,Zn-SOD activity in asthmatic airway epithelium: correction by inhaled corticosteroid in vivo. *Am J Physiol.* 272*:*L148-154.
24. Comhair, S. A., W. Xu, S. Ghosh, F. B. Thunnissen, A. Almasan, W. J. Calhoun, A. J. Janocha, L. Zheng, S. L. Hazen, and S. C. Erzurum. 2005. Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. *Am J Pathol.* 166*:*663-674.
25. Ram, F. S., B. H. Rowe, and B. Kaur. 2004. Vitamin C supplementation for asthma. *Cochrane Database Syst Rev.*CD000993.
26. Pearson, P. J., S. A. Lewis, J. Britton, and A. Fogarty. 2004. Vitamin E supplements in asthma: a parallel group randomised placebo controlled trial. *Thorax.* 59*:*652-656.
27. Kilic, F. S., and K. Erol. 2003. The effects of vitamin E in ovalbumin-sensitized guinea pigs. *Methods Find Exp Clin Pharmacol.* 25*:*27-31.
28. Kloek, J., I. Van Ark, F. De Clerck, N. Bloksma, F. P. Nijkamp, and G. Folkerts. 2003. Modulation of airway hyperresponsiveness by thiols in a murine in vivo model of allergic asthma. *Inflamm Res.* 52*:*126-131.
29. Lee, Y. C., K. S. Lee, S. J. Park, H. S. Park, J. S. Lim, K. H. Park, M. J. Im, I. W. Choi, H. K. Lee, and U. H. Kim. 2004. Blockade of airway hyperresponsiveness and inflammation in a murine model of asthma by a prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid. *Faseb J.*
30. Chang, L. Y., and J. D. Crapo. 2003. Inhibition of airway inflammation and hyperreactivity by a catalytic antioxidant. *Chest.* 123*:*446S.
31. Masini, E., D. Bani, A. Vannacci, S. Pierpaoli, P. F. Mannaioni, S. A. Comhair, W. Xu, C. Muscoli, S. C. Erzurum, and D. Salvemini. 2005. Reduction of antigen-

induced respiratory abnormalities and airway inflammation in sensitized guinea pigs by a superoxide dismutase mimetic. *Free Radic Biol Med.* 39*:*520-531.
32. Li, G., Y. Chen, J. T. Saari, and Y. J. Kang. 1997. Catalase-overexpressing transgenic mouse heart is resistant to ischemia-reperfusion injury. *Am J Physiol.* 273*:*H1090-1095.
33. Kang, Y. J., Y. Chen, and P. N. Epstein. 1996. Suppression of doxorubicin cardiotoxicity by overexpression of catalase in the heart of transgenic mice. *J Biol Chem.* 271*:*12610-12616.
34. Xu, B., J. T. Moritz, and P. N. Epstein. 1999. Overexpression of catalase provides partial protection to transgenic mouse beta cells. *Free Radic Biol Med.* 27*:*830-837.
35. Schriner, S. E., N. J. Linford, G. M. Martin, P. Treuting, C. E. Ogburn, M. Emond, P. E. Coskun, W. Ladiges, N. Wolf, H. Van Remmen, D. C. Wallace, and P. S. Rabinovitch. 2005. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science.* 308*:*1909-1911.
36. Chen, X., H. Liang, H. Van Remmen, J. Vijg, and A. Richardson. 2004. Catalase transgenic mice: characterization and sensitivity to oxidative stress. *Arch Biochem Biophys.* 422*:*197-210.
37. Chen, X., J. Mele, H. Giese, H. Van Remmen, M. E. Dolle, M. Steinhelper, A. Richardson, and J. Vijg. 2003. A strategy for the ubiquitous overexpression of human catalase and CuZn superoxide dismutase genes in transgenic mice. *Mech Ageing Dev.* 124*:*219-227.
38. Poynter, M. E., C. G. Irvin, and Y. M. Janssen-Heininger. 2002. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol.* 160*:*1325-1334.
39. Tomioka, S., J. H. Bates, and C. G. Irvin. 2002. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol.* 93*:*263-270.
40. Hantos, Z., A. Adamicza, E. Govaerts, and B. Daroczy. 1992. Mechanical impedances of lungs and chest wall in the cat. *J Appl Physiol.* 73*:*427-433.
41. Lutchen, K. R., J. L. Greenstein, and B. Suki. 1996. How inhomogeneities and airway walls affect frequency dependence and separation of airway and tissue properties. *J Appl Physiol.* 80*:*1696-1707.
42. Junqueira, L. C., G. Bignolas, and R. R. Brentani. 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J.* 11*:*447-455.
43. Aebi, H. 1984. Catalase in vitro. *Methods Enzymol.* 105*:*121-126.
44. Koya, T., T. Kodama, K. Takeda, N. Miyahara, E. S. Yang, C. Taube, A. Joetham, J. W. Park, A. Dakhama, and E. W. Gelfand. 2005. Importance of Myeloid Dendritic Cells in Persistent Airway Disease After Repeated Allergen Exposure. *Am J Respir Crit Care Med.*
45. Tsuchiya, T., Y. Nishimura, T. Nishiuma, Y. Kotani, Y. Funada, S. Yoshimura, and M. Yokoyama. 2003. Airway remodeling of murine chronic antigen exposure model. *J Asthma.* 40*:*935-944.
46. Gao, Y., and P. M. Vanhoutte. 1993. Products of cyclooxygenase mediate the responses of the guinea pig trachea to hydrogen peroxide. *J Appl Physiol.* 74*:*2105-2111.
47. Gao, Y., and P. M. Vanhoutte. 1992. Effects of hydrogen peroxide on the responsiveness of isolated canine bronchi: role of prostaglandin E2 and I2. *Am J Physiol.* 263*:*L402-408.

48. Rabe, K. F., G. Dent, and H. Magnussen. 1995. Hydrogen peroxide contracts human airways in vitro: role of epithelium. *Am J Physiol.* 269*:*L332-338.
49. Lauer, N., T. Suvorava, U. Ruther, R. Jacob, W. Meyer, D. G. Harrison, and G. Kojda. 2005. Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase. *Cardiovasc Res.* 65*:*254-262.
50. Polytarchou, C., and E. Papadimitriou. 2005. Antioxidants inhibit human endothelial cell functions through down-regulation of endothelial nitric oxide synthase activity. *Eur J Pharmacol.* 510*:*31-38.
51. Fischer, B. M., and J. A. Voynow. 2002. Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. *Am J Respir Cell Mol Biol.* 26*:*447-452.
52. Hashimoto, K., J. E. Durbin, W. Zhou, R. D. Collins, S. B. Ho, J. K. Kolls, P. J. Dubin, J. R. Sheller, K. Goleniewska, J. F. O'Neal, S. J. Olson, D. Mitchell, B. S. Graham, and R. S. Peebles, Jr. 2005. Respiratory syncytial virus infection in the absence of STAT 1 results in airway dysfunction, airway mucus, and augmented IL-17 levels. *J Allergy Clin Immunol.* 116*:*550-557.
53. Chen, Y., P. Thai, Y. H. Zhao, Y. S. Ho, M. M. DeSouza, and R. Wu. 2003. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem.* 278*:*17036-17043.
54. Williams, M. S., and J. Kwon. 2004. T cell receptor stimulation, reactive oxygen species, and cell signaling. *Free Radic Biol Med.* 37*:*1144-1151.
55. Matsue, H., D. Edelbaum, D. Shalhevet, N. Mizumoto, C. Yang, M. E. Mummert, J. Oeda, H. Masayasu, and A. Takashima. 2003. Generation and function of reactive oxygen species in dendritic cells during antigen presentation. *J Immunol.* 171*:*3010-3018.
56. Wu, Z., I. A. MacPhee, and D. B. Oliveira. 2004. Reactive oxygen species in the initiation of IL-4 driven autoimmunity as a potential therapeutic target. *Curr Pharm Des.* 10*:*899-913.
57. Rhee, S. G., K. S. Yang, S. W. Kang, H. A. Woo, and T. S. Chang. 2005. Controlled elimination of intracellular H(2)O(2): regulation of peroxiredoxin, catalase, and glutathione peroxidase via post-translational modification. *Antioxid Redox Signal.* 7*:*619-626.
58. Tolbert, N. E., and E. Essner. 1981. Microbodies: peroxisomes and glyoxysomes. *J Cell Biol.* 91*:*271s-283s.
59. Comhair, S. A., P. R. Bhathena, C. Farver, F. B. Thunnissen, and S. C. Erzurum. 2001. Extracellular glutathione peroxidase induction in asthmatic lungs: evidence for redox regulation of expression in human airway epithelial cells. *Faseb J.* 15*:*70-78.
60. Kelner, M. J., and R. Bagnell. 1990. Alteration of endogenous glutathione peroxidase, manganese superoxide dismutase, and glutathione transferase activity in cells transfected with a copper-zinc superoxide dismutase expression vector. Explanation for variations in paraquat resistance. *J Biol Chem.* 265*:*10872-10875.
61. Ceballos, I., A. Nicole, P. Briand, G. Grimber, A. Delacourte, S. Flament, J. L. Blouin, M. Thevenin, P. Kamoun, and P. M. Sinet. 1991. Expression of human Cu-Zn superoxide dismutase gene in transgenic mice: model for gene dosage effect in Down syndrome. *Free Radic Res Commun.* 12-13 Pt 2*:*581-589.
62. Przedborski, S., V. Jackson-Lewis, V. Kostic, E. Carlson, C. J. Epstein, and J. L. Cadet. 1992. Superoxide dismutase, catalase, and glutathione peroxidase activities in copper/zinc-superoxide dismutase transgenic mice. *J Neurochem.* 58*:*1760-1767.

CHAPTER 9

Discussion and future directions

Repression of IKK-NF-κB activation by oxidants

Because the DNA binding of NF-κB is critically dependent on its redox status, NF-κB has often been used as a cardinal example of a redox-sensitive transcription factor. Both S-nitrosylation and S-glutathionylation of cysteine 62 have been demonstrated to limit that DNA binding. In more recent studies however, oxidants have also been shown to limit the activation of the IKK complex, the prerequisite enzyme that controls the activation of NF-κB. A number of oxidants have been demonstrated to mediate the oxidation of IKKβ at cysteine 179, thereby preventing activation of the kinase activity (1, 2). Results in Chapters 3 and 5 expand the notion that oxidants can modulate IKK and NF-κB activity. Specifically, exposure of cells to either NO-donors or H_2O_2, led to a decreased ability of TNFα to activate IKK and NF-κB. In Chapter 3, S-nitrosylation of IKKβ was indicated to be responsible for the repression of IKK activity by NO-donors. More importantly, inhibition of NOS was demonstrated to cause denitrosylation of IKKβ and this was sufficient to induce kinase activity. This demonstrated that endogenously produced NO is an important suppressor of the basal activity of IKK. In Chapter 5, S-glutathionylation of cysteine 179 was found to occur after H_2O_2 treatment. Moreover, GRX1 was revealed to play a major role in controlling the extent of S-glutathionylation of IKKβ and the consequent repression of kinase activity as well as NF-κB transactivation after H_2O_2 treatment.
This work demonstrates for the first time that IKK and NF-κB can not only be affected by exposure to exogenous oxidants, but that there is in fact a role for endogenously produced oxidants in the regulation of the NF-κB signaling cascade. Furthermore, this work introduces a potential role of antioxidant systems like GRX in controlling the activity and activation profiles of NF-κB in addition to modulating the effects of oxidants.

In this thesis, S-nitrosylation and S-glutathionylation of cysteine 179 of IKKβ are described as mechanisms for oxidant repression of IKK activity. The key question that remains to be addressed is how these oxidative modifications inhibit IKK enzymatic activity. In Chapter 3 it was demonstrated that despite enhanced S-nitrosylation and inhibition of the kinase activity after treatment with L-CSNO, IKK remains phosphorylated. Therefore, at least S-nitrosylation and phosphorylation do not appear to be mutually exclusive events. However, it remains to be elucidated whether the phosphorylation status of IKKβ is impacted by S-glutathionylation. On the other hand, the possibility exists that oxidation of cysteine 179 would interfere with the recruitment and binding of other members of the kinase complex and/or substrates. Unpublished observations as well as studies performed by other investigators do not indicate altered recruitment of at least the IκB substrates. Since there are numerous proteins that form the IKK complex, some even still unknown, more thorough investigations are warranted.

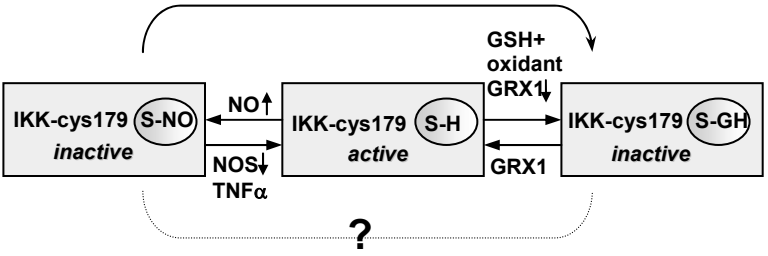


Figure 1 S-nitrosylation and S-glutathionylation are described as mechanisms of oxidative repression of IKK activity. S-nitrosylation could represent an intermediate oxidation step to the formation of the mixed disulfide with glutathione (solid arrow). It remains to be determined to what extent these two post-translational modifications are linked and whether an interplay exists (dotted line).

Two oxidant dependent posttranslational modifications have been described separately in IKKβ after exposure to two different oxidants, with essentially the same outcome (Figure 1). Cells in intact organs will however be exposed to multiple oxidants at the same time and these oxidants could furthermore interact. It is therefore not easy to predict which modification would be more common under physiological conditions. S-nitrosylation is a posttranslational modification that requires less energy to be established then S-glutathionylation (3). Additionally, published reports suggest that S-nitrosylation is an intermediate step to the

formation of mixed disulfides with GSH (3, 4), analogous to the sulfenic acid intermediate that was described in Chapter 5. S-glutathionylation furthermore could be an intermediate to oxidations that require even more energy for their formation, like sulfonic acids. Again, the basal oxidation state of IKKβ and the relative importance of S-nitrosylation versus S-glutathionylation likely differs between cell types depending on the expression levels of oxidant producing enzymes, antioxidant enzymes and neighboring cells. To address these questions and to map the relative importance of these oxidations, additional studies need to be undertaken. For instance, the dissociation constants (K_M) could be calculated for different oxidation products of IKKβ. Additionally, the sensitivity of IKK kinase activity to different oxidants could be examined.

In our studies, we never observed any effect of oxidant treatment per se on the basal level of IKK or NF-κB activity. Other studies have however demonstrated that oxidants themselves are capable of activating the NF-κB signaling cascade. For instance, H_2O_2 was shown to lead to the phosphorylation and activation of both IKKα and IKKβ and additionally prolonged IKK activity after TNFα stimulation (5). Furthermore, oxidative stress has been reported to lead to the activation of the tyrosine kinase, casein kinase (CK)2 (6), which is associated with the phosphorylation of IκBα at tyrosine 42. In contrast to serine phosphorylation of IκBα, this will target the inhibitory molecules for degradation by calpain proteases, resulting in NF-κB activation. In addition, H_2O_2 has been shown to cause Syk-dependent tyrosine phoshorylation of IκBα (7). This was demonstrated to cause nuclear translocation of p65 without degradation of the inhibitory molecules.

Studies on the effects of oxidants on the NF-κB pathway are clearly conflicting. However, the inhibitory effects of oxidants can mostly be attributed to the oxidation of IKKβ (cysteine 179), as well as p50 (cysteine 62). Activation on the other hand is mainly described to occur through the activation of upstream kinases via largely undefined oxidative mechanisms. It is also important to keep in mind that multiple oxidations could be occurring at the same time, and that the net-outcome of oxidative stress is likely to be determined by the co-stimulus, the concentration, time frame and type of oxidant, the redox status of the cell and the cell type under investigation. Collectively, these factors could help to explain the conflicting results of current publications.

New methods to detect S-nitrosylation and S-glutathionylation in situ

Because of the lack of reliable methods to demonstrate the occurrence of S-nitrosylation and S-glutathionylation *in vivo*, the physiological relevance of these redox-dependent posttranslational modifications has often been questioned. Antibodies to these oxidative modifications have been generated and have been used to localize general S-nitrosylation and S-glutathionylation patterns. However, because of the small size of the epitopes and the relative unstability of these modifications their use is considered problematic. To circumvent these problems, an existing protocol that relies on chemical derivatization and labeling was adapted to visualize S-nitrosylation in Chapter 4 and S-glutathionylation in Chapter 6. Labeling of S-nitrosylated proteins is dependent on the reduction of SNO groups by ascorbate, whereas the catalytic activity of GRX1 is utilized to label S-glutathionylated proteins. These methods have been demonstrated to be relatively specific and will allow for the detection of patterns of these oxidative modifications in healthy tissues as well as the study of alterations during disease processes. These could have very important applications, since S-nitrosylation and S-glutathionylation are known to influence the function of a growing number of proteins and alterations in both posttranslational modifications are known to occur in multiple diseases. For instance altered S-nitrosylation was found in lung diseases like asthma (8, 9), cystic fibrosis (10), pneumonia (11) and pulmonary hypertension (12). Changes in S-glutathionylation have so far not been demonstrated in pulmonary diseases, but are known to occur in a variety of other pathological conditions like diabetes mellitus (13, 14) and HIV (15).

GRX in allergic airway disease

A number of antioxidant enzymes have been demonstrated to be altered in patients with asthma. Some of these include enzymes that are involved in glutathione homeostasis like GPx, γGCS and GSTs. GRX is a relatively newly discovered enzyme with the unique capacity to regulate proteins S-glutathionylation. In Chapter 7, GRX1 expression was found to be increased in the lungs of mice with allergic airway disease. Concomitantly, GRX activity was found to be elevated. Enhanced GRX activity could result in enhanced deglutathionylation activity and would liberate more GSH from proteins in order to restore lung GSH levels. This could overall be viewed as a means to combat the oxidative stress that is

associated with the inflammatory response. In addition, alterations in the S-glutathionylation status of proteins could play an important role in the disease process by influencing signaling cascades, like for instance NF-κB. As was demonstrated in Chapter 5, increased GRX1 expression was able to prevent inhibition of IKK activity by H_2O_2 and even resulted in enhanced NF-κB transcriptional activity in response to TNFα. It remains to be determined whether an etiological link exists between GRX expression and NF-κB activity in allergic airway disease. In order to start addressing this question, it would first need to be established whether alterations in S-glutathionylation indeed occur in asthma. In a second phase, protein targets for altered S-glutathionylation will need to be identified. Studies aimed at addressing the functional significance of the regulation of NF-κB by GRX1 will likely involve the use of GRX1 knock-out mice and the generation of GRX1 transgenic mice.

Role of endogenous H_2O_2 in allergic airway disease

Elevated amounts of H_2O_2 have been found in exhaled breath condensates of patients with asthma, and this is associated with the degree of pulmonary inflammation (16). H_2O_2 is not only considered a by-product of the inflammatory process, but is furthermore thought to play an etiological role in the disease process through its damaging properties. Through the use of catalase transgenic mice, which have an enhanced capacity to detoxify H_2O_2, we investigated the role of H_2O_2 in allergic airway disease in a more direct manner. In Chapter 8 we described that overexpression of catalase surprisingly does not protect against the development of allergic airway inflammation. Catalase transgenic and C57BL/6 control mice displayed comparable airway and tissue inflammation, and identical increases in pro-collagen 1 mRNA expression, following sensitization and challenge with OVA. Furthermore, catalase transgenic mice demonstrated enhanced mucus metaplasia compared to strain controls. In addition, intrinsic as well as allergen-induced hyperresponsiveness to methacholine were observed in catalase transgenic mice. This study indicates that endogenously produced H_2O_2 is a repressor of mucus production and plays a role in the maintenance of normal lung function. The absence of any deleterious role and the unexpected protective involvement of H_2O_2 in allergic airway disease stand in stark contrast to the positive correlations that have been made between oxidants and disease parameters. However, this study might highlight the fine balance that exists between oxidants

and antioxidants. Since oxidants have been demonstrated to act as regulators of important physiological processes like cell growth and wound repair, any interference with the basal level of oxidants could potentially have adverse effects. It remains puzzling however, that no protection towards the development of cellular inflammation was provided by the overexpression of catalase. It is important to keep in mind that other enzymes exist that can detoxify H_2O_2, like GPx and peroxiredoxins. Their likely importance in the degradation of H_2O_2 would need to be addressed in future studies.

Future directions

In recent years, the field of redox-regulated signal transduction has made the leap from studying oxidations of isolated proteins and functional alterations to identifying targets for oxidations and modes of oxidation in intact cells or tissues. However, to date only few studies have been undertaken that unequivocally determined the functional significance of specific protein oxidations in *in vivo* models of diseases. Thus, the challenge that lies ahead in this field is to establish an etiological link between specific protein oxidations and disease pathology. In order to advance the field new tools will need to be generated to reliably and sensitively identify protein oxidations *in vivo*. The most current proteomic screens of protein oxidations often identify the same highly abundant proteins, like actin (17, 18). More sensitive approaches will hopefully make it possible to identify less abundant, but equally important targets.

One such promising new approach encompasses the generation of antibodies against a stable oxidation of a specific protein, analogous to phospho-specific proteins. Antibodies generated based on this principle so far, were raised against the sulfonic acid forms of peroxiredoxins and PTP1B (19, 20). This approach however will likely only be applicable towards stable forms of oxidations. For unstable epitopes like SNO moieties, antibodies might fail to be specific. Unfortunately, the modifications that appear to be the most relevant to signal transduction are coincidentally the most unstable and most difficult to detect.
On the other hand, transgenic mice could be generated that lack the amino acid residues that are targeted by oxidations. Knock-in approaches in which the oxidation prone cysteine is replaced by a non-oxidizable amino acid like alanine could provide valuable insights into the importance of these cysteine oxidations. This approach has so far only been carried out

for the protein Ref-1, which facilitates the DNA binding of both AP-1 and NF-κB (21, 22). Specifically, cysteine 64 of Ref-1 has been demonstrated to be critical for the reduction of oxidized cysteines in these transcription factors. Experiments using a Ref-1 mouse where cysteine 64 was replaced with alanine however did not confirm the importance of cysteine 64 in the DNA binding of AP-1 (23).

Once protein modules have been established that regulate the redox-dependent posttranslational modifications, like GRX, they can be manipulated *in vivo* in order to investigate the relevance of the modification. These antioxidant proteins could additionally be explored as possible therapeutic targets. When embarking on these approaches, it is important to consider that most antioxidant enzyme systems are interconnected, and that some degree of compensation is often revealed when knock-out approaches are utilized. This concept of redundancy also holds true for NOS enzymes, based upon reports demonstrating that compared to controls the total amount of NO produced was not necessarily different in the various isoform-knock-outs. Therefore, the use of inducible transgenic or knock-out approaches would be more informative and would overcome this potential pitfall. If a certain organ or cell type is of particular interest, the use of tissue or cell specific promoters to drive the transgene or loxP-flanked allele furthermore will allow for very precise information on the relevance of redox signaling within that compartment.

Concluding remarks

Most research so far has focused on investigating correlations between oxidants and inflammation in asthmatic patients. A positive relationship has indeed been established between these two parameters and is now being explored as a means to monitor the disease process as well as therapeutic effects. Although oxidants are not believed to be merely markers of inflammation, and are thought to play an etiological role in the disease process, few studies have unraveled mechanisms whereby oxidants could potentially contribute to asthma pathology. The work presented here demonstrates that mild oxidants can play an anti-inflammatory role by suppressing the activity of IKK and NF-κB through inflicting posttranslational modifications of cysteine 179 of IKKβ. The oxidative stress that hallmarks asthma will cause irreversible damaging oxidations, and will thereby likely attenuate the beneficial actions of

oxidants on this inflammatory signaling cascade. Taken together with the *in vivo* studies, the work presented here illustrates the importance of understanding the regulatory functions of oxidants in cell and organ function in order to start to elucidate their contributions in disease pathologies.

REFERENCES

1. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M. & Santoro, M. G. (2000) *Nature* **403,** 103-8.
2. Kapahi, P., Takahashi, T., Natoli, G., Adams, S. R., Chen, Y., Tsien, R. Y. & Karin, M. (2000) *J Biol Chem* **275,** 36062-6.
3. Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E. & Stamler, J. S. (2005) *Nat Rev Mol Cell Biol* **6,** 150-66.
4. Adachi, T., Weisbrod, R. M., Pimentel, D. R., Ying, J., Sharov, V. S., Schoneich, C. & Cohen, R. A. (2004) *Nat Med* **10,** 1200-7.
5. Kamata, H., Manabe, T., Oka, S., Kamata, K. & Hirata, H. (2002) *FEBS Lett* **519,** 231-7.
6. Schoonbroodt, S., Ferreira, V., Best-Belpomme, M., Boelaert, J. R., Legrand-Poels, S., Korner, M. & Piette, J. (2000) *J Immunol* **164,** 4292-300.
7. Takada, Y., Mukhopadhyay, A., Kundu, G. C., Mahabeleshwar, G. H., Singh, S. & Aggarwal, B. B. (2003) *J Biol Chem* **278,** 24233-41.
8. Gaston, B., Sears, S., Woods, J., Hunt, J., Ponaman, M., McMahon, T. & Stamler, J. S. (1998) *Lancet* **351,** 1317-9.
9. Dweik, R. A., Comhair, S. A., Gaston, B., Thunnissen, F. B., Farver, C., Thomassen, M. J., Kavuru, M., Hammel, J., Abu-Soud, H. M. & Erzurum, S. C. (2001) *Proc Natl Acad Sci U S A* **98,** 2622-7.
10. Grasemann, H., Gaston, B., Fang, K., Paul, K. & Ratjen, F. (1999) *J Pediatr* **135,** 770-2.
11. Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J. & et al. (1993) *Proc Natl Acad Sci U S A* **90,** 10957-61.
12. McMahon, T. J., Ahearn, G. S., Moya, M. P., Gow, A. J., Huang, Y. C., Luchsinger, B. P., Nudelman, R., Yan, Y., Krichman, A. D., Bashore, T. M., Califf, R. M., Singel, D. J., Piantadosi, C. A., Tapson, V. F. & Stamler, J. S. (2005) *Proc Natl Acad Sci U S A* **102,** 14801-6.
13. Niwa, T., Naito, C., Mawjood, A. H. & Imai, K. (2000) *Clin Chem* **46,** 82-8.
14. Al-Abed, Y., VanPatten, S., Li, H., Lawson, J. A., FitzGerald, G. A., Manogue, K. R. & Bucala, R. (2001) *Mol Med* **7,** 619-23.
15. Ghezzi, P., Romines, B., Fratelli, M., Eberini, I., Gianazza, E., Casagrande, S., Laragione, T., Mengozzi, M. & Herzenberg, L. A. (2002) *Mol Immunol* **38,** 773-80.
16. Emelyanov, A., Fedoseev, G., Abulimity, A., Rudinski, K., Fedoulov, A., Karabanov, A. & Barnes, P. J. (2001) *Chest* **120,** 1136-9.
17. Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E. & Ghezzi, P. (2002) *Proc Natl Acad Sci U S A* **99,** 3505-10.
18. Martinez-Ruiz, A. & Lamas, S. (2004) *Arch Biochem Biophys* **423,** 192-9.
19. Woo, H. A., Kang, S. W., Kim, H. K., Yang, K. S., Chae, H. Z. & Rhee, S. G. (2003) *J Biol Chem* **278,** 47361-4.
20. Persson, C., Sjoblom, T., Groen, A., Kappert, K., Engstrom, U., Hellman, U., Heldin, C. H., den Hertog, J. & Ostman, A. (2004) *Proc Natl Acad Sci U S A* **101,** 1886-91.
21. Nishi, T., Shimizu, N., Hiramoto, M., Sato, I., Yamaguchi, Y., Hasegawa, M., Aizawa, S., Tanaka, H., Kataoka, K., Watanabe, H. & Handa, H. (2002) *J Biol Chem* **277,** 44548-56.
22. Xanthoudakis, S. & Curran, T. (1992) *Embo J* **11,** 653-65.

23. Ordway, J. M., Eberhart, D. & Curran, T. (2003) *Mol Cell Biol* **23,** 4257-66.

CHAPTER 10

Summary

The aim of the first part of this thesis was to investigate if and how the oxidants nitric oxide ($NO^{\cdot}$) and hydrogen peroxide (H_2O_2) modulate the activation of Inhibitory kappa B kinase (IKK) and the transcription factor NF-κB.

In the past it has been shown that $NO^{\cdot}$ possesses anti-inflammatory properties. For instance, studies have demonstrated that $NO^{\cdot}$ can bind to a specific cysteine amino acid in the p50 subunit of NF-κB. This binding is termed S-nitrosylation and as a result, NF-κB displayed reduced DNA binding activity. Consequently, transcription of inflammatory genes from DNA to mRNA and proteins was attenuated. We demonstrated in Chapter 3 that $NO^{\cdot}$ can also bind to cysteine 179 of the β subunit of the IKK complex, the prerequisite enzyme that controls the activation of NF-κB. S-nitrosylation of IKKβ resulted in repressed IKK activity, and consequently to attenuated NF-κB transactivation. Furthermore, by inhibiting $NO^{\cdot}$ producing enzymes, we demonstrated that endogenously produced $NO^{\cdot}$ is a repressor of basal IKK activity. This study reveals IKKβ as an important target for redox regulation of NF-κB by endogenous as well as exogenous $NO^{\cdot}$, providing an additional mechanism for its anti-inflammatory properties (Figure 1).

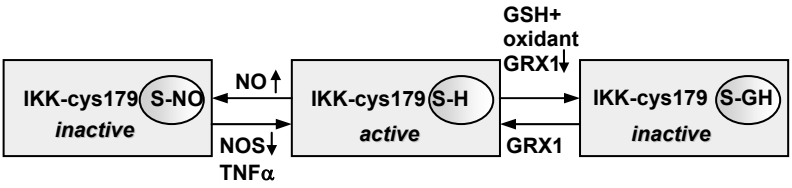


Figure 1 S-nitrosylation and S-glutathionylation are described as mechanisms whereby oxidants can influence the activity of the IKK complex.

S-nitrosylation is a very unstable posttranslational modification. Demonstrating S-nitrosylation of proteins is therefore not easily accomplished. For instance, reliable antibodies are not available. For those reasons, some methods to detect S-nitrosylation rely on derivatization

techniques. In Chapter 4 we adapted such a published derivatization approach to visualize S-nitrosylation in intact cells and tissues. The multi-step procedure is represented schematically in the bottom part of Figure 2. This method relies on the blocking of free sulfhydryl groups (SH) in proteins and the subsequent reduction of S-nitrosylated sulfhydryl groups to free sulfhydryl groups by vitamin C. De newly formed free sulfhydryl groups can then be labeled with biotin and visualized using fluorescent streptavidin. This new method will allow for S-nitrosylated proteins to be localized in healthy tissues as well as permit the study of alterations in protein S-nitrosylation in different pathologies.

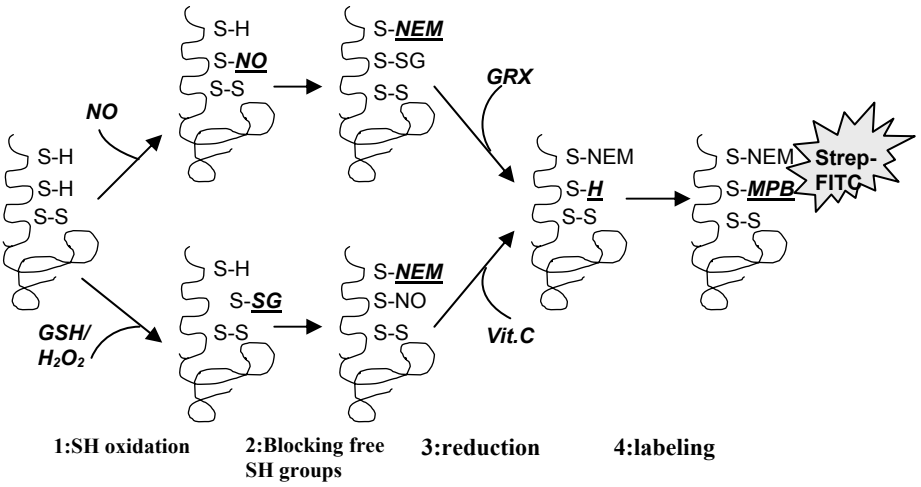


Figure 2 In Chapters 4 and 6, two new methods are described that allow visualization in intact cells of S-nitrosylation and S-glutathionylation respectively.

H_2O_2 is an oxidant that is constantly being produced at low levels, similar to $NO^{\cdot}$. Previous studies have demonstrated that H_2O_2 can prevent the activation of IKK by inflammatory cytokines. In Chapter 5, we expanded on these studies and determined that H_2O_2 can induce the binding of glutathione to cysteine 179 in IKKβ. This protein modification by glutathione is termed S-glutathionylation and was shown to be responsible for the inhibition of IKK activity by H_2O_2. Additionally, this study demonstrated that S-glutathionylation of IKKβ is regulated by the antioxidant enzyme glutaredoxin 1 (GRX1). Overexpression of GRX1 protected IKK against inhibition by H_2O_2 and even enhanced NF-κB transactivation. Knock-down of GRX1 on the other hand greatly sensitized IKK and NF-κB to inhibition by H_2O_2 and attenuated basal activity levels of both IKK and NF-κB. This study demonstrates that the activities of

IKK and NF-κB are controlled by the antioxidant enzyme GRX1 (Figure 1).

S-glutathionylation is a more stable protein modification, but similar to S-nitrosylation, few methods are available that allow its visualization. Analogous to the method that was developed in Chapter 4, Chapter 5 introduced a derivatization approach to visualize S-glutathionylation. This method is represented in the upper part of Figure 2 and again relies on the blockage of free sulfhydryl groups in proteins. It next utilizes the catalytic deglutathionylating activity of GRX1 to specifically revert S-glutathionylated moieties to free sulfydryl groups. Identical to Chapter 4, these free sulfhydryl groups can then be labeled with biotin and visualized using fluorescent streptavidin. Given the growing importance of S-glutathionylation this new method will be useful for the study and localization of this protein modification in healthy tissues as well as its relation to disease pathologies.

In the second part of this thesis the aims were to demonstrate the role of endogenously produced H_2O_2 in a mouse model of asthma as well as to investigate the expression of the antioxidant enzyme glutaredoxin in this animal model.

The previous studies demonstrated the potential importance of GRX1-regulated S-glutathionylation as a redox-dependent posttranslational modification that can influence inflammation through IKK and NF-κB. Therefore, the expression of GRX enzymes was investigated in a mouse model of asthma in Chapter 7. In this study the expression of GRX1, but not GRX2 was found to be elevated in the lungs during allergic airway inflammation. This was associated with elevated levels of GRX activity in the lungs. The cytokine IFNγ furthermore increased the expression of GRX1 and GRX activity in primary cultures of tracheal epithelial cells, while TGFβ decreased the expression of GRX1 and GRX activity. This study reveals GRX1 as part of the antioxidant response of the lung during allergic airway inflammation. Consequent alterations in protein S-glutathionylation could in addition play an important role in the disease process.

Studies that tested antioxidant supplementation in mice have indicated that H_2O_2 could play an etiological role in the pathogenesis of allergic airway disease. To more directly address this possibility, we tested whether mice

that overexpress the H_2O_2 detoxifying enzyme catalase would be protected against the development of allergic airway disease in Chapter 8. These mice were found to mount a normal immune response against the ovalbumin antigen. Furthermore, catalase transgenic mice did not display any protection against the development of pulmonary inflammation in response to ovalbumin. In contrast, these mice demonstrated signs of enhanced mucus production as well as enhanced airway hyperresponsiveness to methacholine. Moreover, increased airway hyperresponsiveness was observed in both naïve catalase transgenic mice and catalase transgenic mice with allergic airway disease. This study demonstrates that the overexpression of catalase does not protect against the development of allergic airway inflammation, but in contrast enhances mucus production and airway hyperresponsiveness.

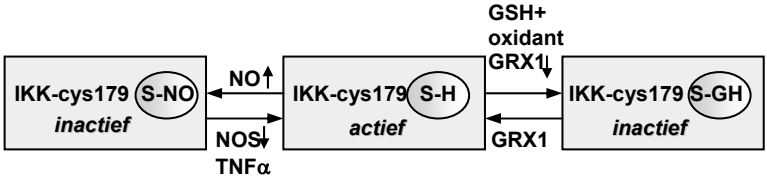
In conclusion, this thesis has demonstrated that both $NO^{\cdot}$ and H_2O_2 exert an important control over the activation of IKK and NF-κB. These oxidants can therefore play an important role in the modulation of inflammatory processes.

HOOFDSTUK 11

Samenvatting

De doelstelling in het eerste deel van dit proefschrift was om de modulatie van de activering van inhibitory kappa B kinase (IKK) en de transcriptie factor NF-κB door stikstofoxide en waterstofperoxide te onderzoeken.

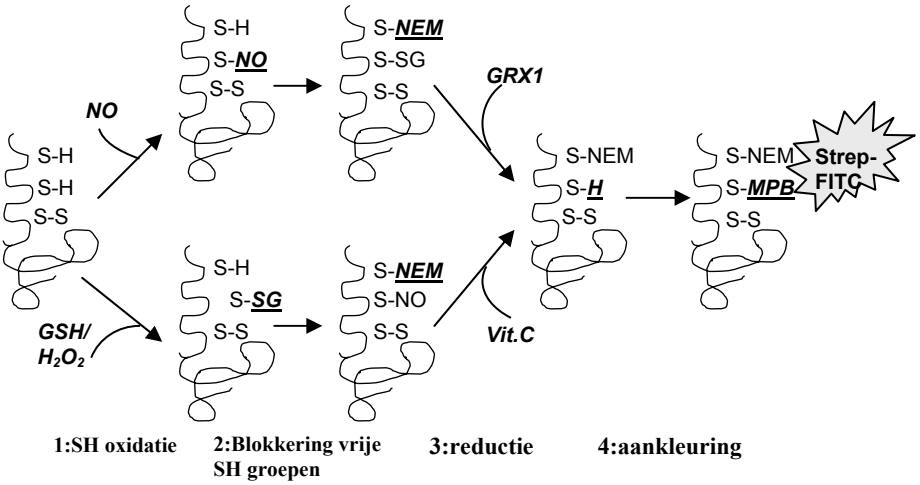
In het verleden werd al aangetoond dat stikstofoxide ontstekingsremmende eigenschappen vertoont. Ondermeer werd in studies aangetoond dat stikstofoxide zich kan binden aan een bepaald cysteine aminozuur in het p50 eiwit dat deel uitmaakt van NF-κB. Deze binding wordt ook S-nitrosylatie genoemd. Het heeft als resultaat dat NF-κB minder aan DNA kan binden en er bijgevolg minder ontstekingsgenen van DNA naar mRNA en eiwitten worden omgezet. In hoofdstuk 3 van dit proefschrift wordt de binding van stikstofoxide ook aangetoond in cysteïne 179 van IKK, het eiwit dat de activering van NF-κB controleert. Dit heeft tot gevolg dat de activiteit van IKK wordt geremd en daardoor ook verminderde activering van NF-κB plaatsvindt. Voorts werd er door stikstofoxide producerende enzymen te blokkeren, aangetoond dat endogeen geproduceerd stikstofoxide de activiteit van IKK remt. Deze studie toont aan dat stikstofoxide een belangrijke rol speelt in de controle van IKK activatie (Figuur 1).



Figuur 1 In dit proefscrhift worden S-nitrosylatie en S-glutathionylatie beschreven als mechanismen waardoor oxidanten de activiteit van IKK kunnen remmen.

Omdat S-nitrosylatie een zeer onstabiele modificatie van eiwitten is, is het aantonen van S-nitrosylatie niet evident. Zo zijn er bijvoorbeeld geen goede antilichamen beschikbaar. De meest gebruikte methoden berusten daardoor op derivatisatie - technieken. In hoofdstuk 4 werd zo een derivatisatie- techniek aangepast om S-nitrosylatie van eiwitten visueel te

kunnen aantonen. Deze techniek wordt in het onderste deel van Figuur 2 schematisch weergegeven en berust op het blokkeren van vrije zwavel (S-) groepen in eiwitten en vervolgens de selectieve omzetting van S-nitrosylaties tot vrije S-groepen met behulp van vitamine C. De nieuw gevormde vrije S-groepen kunnen dan gemarkeerd worden met biotine en gevisualiseerd met fluorescent streptavidine. Deze nieuwe methode zal het mogelijk maken om S-nitrosylatie van eiwitten te lokaliseren in gezonde weefsels alsook om veranderingen te bestuderen in verscheidene ziekteprocessen.



Figuur 2 In hoofdstukken 4 en 6 worden twee nieuwe methodes beschreven om respectievelijk S-nitrosylatie en S-glutathionylatie aan te kleuren in intacte cellen.

Waterstofperoxide is een oxidant dat net als stikstofoxide in beperkte mate voortdurend wordt aangemaakt door residentiële cellen. Onderzoek heeft aangetoond dat waterstofperoxide de activering van IKK door inflammatoire eiwitten kan beperken. In hoofdstuk 5 werd in dit proefschrift aangetoond dat waterstofperoxide de binding van glutathione aan cysteïne 179 in IKK induceert. Deze modificatie van eiwitten door glutathione wordt S-glutathionylatie genoemd en is verantwoordelijk voor de inhibitie van IKK door waterstofperoxide. Voorts werd aangetoond dat dit proces wordt gereguleerd door het antioxidant enzyme glutaredoxine 1. Overexpressie van glutaredoxine 1 beschermt IKK tegen inactivering door waterstofperoxide en verhoogt zelfs NF-κB activiteit. Aan de andere kant leidt verlaagde expressie van glutaredoxine 1 tot een verlaagde basale activiteit van IKK en NF-κB. Tevens zijn de activiteiten van zowel IKK

als NF-κB meer gevoelig voor inhibitie door waterstofperoxide als de expressie van glutaredoxine 1 is verlaagd. Deze studie toont aan dat de activiteit van IKK en NF-κB afhankelijk is van het antioxidant enzyme glutaredoxine 1 (Figuur 1).

S-glutathionylatie is een meer stabiele eiwit modificatie, maar toch zijn er net als voor S-nitrosylatie weinig methoden beschikbaar om dit te visualiseren. Naar analogie met de methode die werd ontwikkeld in hoofdstuk 4, wordt in het vijfde hoofdstuk een derivatisatie techniek geïntroduceerd om S-glutathionylatie aan te kleuren. Deze techniek wordt in het bovenste deel van Figuur 2 schematisch weergegeven en berust opnieuw op de blokkering van vrije S-groepen in eiwitten, maar maakt vervolgens gebruik van de catalytische deglutathionylatie activiteit van glutaredoxine om S-glutathionylatie specifiek om te zetten in vrije S-groepen. Net als in hoofdstuk 4 kunnen deze vrije S-groepen vervolgens worden gemarkeerd met biotine en gevisualiseerd met een fluorescent streptavidine. Aangezien het belang dat aan S-glutathionylatie wordt gehecht sterk is toegenomen in de afgelopen jaren, zal deze nieuwe methode zeer nuttig zijn voor het besturen en lokaliseren van deze eiwit modificatie in gezonde weefsels alsook in relatie tot verscheidene pathologiëen.

Het tweede deel van dit proefschrift had tot doelstelling een rol aan te tonen voor endogeen waterstofperoxide in een muizenmodel voor astma en ook om de expressie van het antioxidant eiwit glutaredoxine in dit muizenmodel te onderzoeken.

De voorgaande studies toonden het potentiële belang aan van glutaredoxine gereguleerde S-glutathionylatie als een redox afhankelijke posttranslationele modificatie die inflammatie kan beïnvloeden. Daarom werd de expressie van de glutaredoxine enzymen in hoofdstuk 7 onderzocht in het muizenmodel voor astma. In deze studie werd aangetoond dat enkel de expressie van glutaredoxine 1, niet GRX2, is verhoogd in de longen gedurende allergische luchtwegontsteking in de muis. Dit leidt tot een verhoogde activiteit van GRX in de longen. Het cytokine interferon γ verhoogde de expressie van GRX1 en de GRX activiteit in een primaire kweek van luchtwegepitheelcellen, terwijl behandeling met het cytokine TGFβ de expressie van GRX1 en de GRX activiteit verlaagde. Deze studie toont aan dat GRX1 deel uitmaakt van de antioxidant respons tijdens allergische luchtwegontsteking en dat

bijgevolg veranderde S-glutathionylatie een rol zou kunnen spelen in het ziekteproces.

Studies in muizen waarbij supplementatie met antioxidanten werd getest wijzen erop dat waterstofperoxide een etiologische rol zou kunnen spelen in het ontstaan van allergische luchtwegontsteking. Om dit verband direct aan te tonen werd in hoofdstuk 8 onderzocht of muizen die het waterstofperoxide detoxifiërend enzyme catalase tot overexpressie brengen (catalase transgene muizen), beschermd zijn tegen het ontwikkelen van allergische luchtwegontsteking. Ten eerste werd gevonden dat deze muizen in staat zijn om een normale immuunrespons tegen het ovalbumine allergen te genereren. Voorts bleek dat catalase transgene muizen niet beschermd zijn tegen inflammatie. Integendeel zelfs, deze muizen vertoonden tekenen van verhoogde slijm (mucus) produktie. Ook onverwachts vertoonden deze muizen een verhoogde reactiviteit van de luchtwegen na het toedienen van metacholine. Deze verhoogde reactiviteit werd waargenomen in controle muizen alsook in muizen met allergische luchtwegontsteking. Uit deze studie kan de conclusie getrokken worden dat overexpressie van catalase niet leidt tot bescherming tegen allergische luchtwegontsteking, maar integendeel verhoogde slijmproduktie en overgevoeligheid van de longen veroorzaakt.

In dit proefschrift wordt aangetoond dat zowel stikstof- als waterstofperoxide een belangrijke controle uitoefenen over de activering van IKK en NF-κB. Deze oxidanten kunnen bijgevolg een belangrijke rol vervullen in de modulatie van ontstekingsprocessen.

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LIST OF PUBLICATIONS

FULL PAPERS

Janssen-Heininger YM, Persinger RL, Korn SH, Pantano C, McElhinney B, Reynaert NL, Langen RC, Ckless K, Shrivastava P, Poynter ME. Reactive nitrogen species and cell signaling: implications for death or survival of lung epithelium. *Am J Respir Crit Care Med* 2002 Dec 15;166(12 Pt 2):S9-S16.

Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide represses inhibitory kappa B kinase through S-nitrosylation. *Proc Natl Acad Sci U S A* 2004 Jun 15;101(24):8945-8950.

Karina Ckless, Niki L Reynaert, Douglas J Taatjes, Karen M Lounsbury, Albert van der Vliet, Yvonne Janssen-Heininger. *In situ* detection and visualization of S-nitrosylated proteins following chemical derivatization: identification of Ran GTPase as a target for S-nitrosylation. *Nitric Oxide* 2004 Nov 11(3): 216-227.

Reynaert NL, Ckless K, Wouters EF, van der Vliet A, Janssen-Heinger YM. Nitric oxide and redox signaling in allergic airway inflammation. *Antioxid Redox Signal* 2005 Jan-Feb;7(1-2):129-43.

Janssen-Heininger Y, Ckless K, Reynaert N, van der Vliet A. SOD Inactivation in Asthma: Bad News or NO News? *Am J Pathol* 2005 Mar;166(3):649-52.

Juanita HJ Vernooy, Niki Reynaert, Tim GAM Wolfs, Roy HE Cloots, Astrid Haegens, Bart de Vries, Mieke A Dentener, Wim A Buurman, Emiel FM Wouters. Rapid pulmonary expression of acute-phase reactants after local lipopolysaccharide exposure in mice is followed by an interleukin-6 mediated systemic acute-phase response. *Exp Lung Res* 2005;31(9-10): 855-871.

Niki L. Reynaert, Karina Ckless, Amy S. Guala, Emiel F.M. Wouters, Albert van der Vliet and Yvonne M.W. Janssen-Heininger. *In situ* detection of S-glutathionylated proteins following glutaredoxin-1

catalyzed cysteine derivatization. *Biochim Biophys Acta* 2006;1760:380-387.

Niki L. Reynaert, Albert van der Vliet, Milena Hristova, Cristen Pantano, Nicholas H. Heintz, John Heim, Dwight E. Matthews, Emiel F.M. Wouters, and Yvonne M.W. Janssen-Heininger. Dynamic redox control of NF-κB through glutaredoxin-regulated S-nitrosylation of inhibitory kappa B kinase. *Proc Natl Acad Sci U S A*. In press.

Niki L. Reynaert, Toby McGovern, Amy Brown, Emiel F.M. Wouters, Charles G. Irvin, and Yvonne M.W. Janssen-Heininger. Catalase overexpression does not attenuate inflammation and increases airway hyperresponsiveness in a murine model of allergic airway disease. *J Immunol.* In revision.

Niki L Reynaert, Emiel FM Wouters and Yvonne Janssen-Heininger. Modulation of glutaredoxin expression in a mouse model of allergic airway disease. *Am J Respir Cell Mol Biol.* Submitted.

Cristen Pantano, Niki L. Reynaert, Albert van der Vliet and Yvonne M.W. Janssen-Heininger. Redox sensitive kinases of the nuclear factor kappa B signaling pathway. *Antioxid Redox Signal*. Submitted.

BOOK CONTRIBUTIONS

Involvement of oxidants in the aetiology of chronic airway diseases. Proteomic approaches to identify redox processes in epithelial cell signaling and inflammation. Albert van der Vliet, Niki L Reynaert, Peter Bove, Karina Ckless, Anne-Katrin Greul, Milena Hristova, Yvonne Janssen-Heininger. *Redox Proteomics: from Protein Modifications to Cellular Oxidative/Nitrosative Stress* (I. Dalle-Donne, A. Scaloni, and A. Butterfield, Eds.), John Wiley & Sons, Inc., July 2006.

SELECTED ABSTRACTS

Niki Reynaert, Karina Ckless, Albert van der Vliet, Solange H Korn, Nanda Vos, Emiel FM Wouters and Yvonne Janssen-Heininger. Nitric oxide inhibits inhibitory kappa B kinase through S-nitrosation and

prevents activation of nuclear factor kappa B (NF-κB). *FRBM* 2002, 33(sup2): S374.

NL Reynaert, K Ckless, A van der Vliet, S Korn, N Vos, EFM Wouters, Y Janssen-Heininger. Nitric oxide inhibits I kappa B kinase (IKK) through S-nitrosation and S-glutathiolation and prevents activation of Nuclear Factor Kappa B (NF-κB). Awarded best abstract presentation at the Taormina 2003 ERS Lung Science Conference.

Niki Reynaert, Karina Ckless, Albert van der Vliet, Emiel FM Wouters and Yvonne Janssen-Heininger. Inactivation and S-glutathiolation of IKKβ by H_2O_2. *FRBM* 2003, 35(sup1): S72.
NL Reynaert, K Ckless, A van der Vliet, S Korn, N. Vos, EFM Wouters, Y Janssen-Heininger.Inhibition of I Kappa B Kinase (IKK) by S-nitrosation and S-glutathiolation prevents activation of Nuclear Factor Kappa B. *Am J Respir Crit Care Med* 2003, 167(7): A796.

N.L. Reynaert, K. Ckless, A. van der Vliet, E.F.M. Wouters, Y. Janssen-Heininger. Inactivation and Glutathiolation of Inhibitory kappa B kinase by H_2O_2. *Am J Respir Crit Care Med* 2004, 169(7): A425.

Niki Reynaert, Karina Ckless, Cristen Pantano, Dwight Matthews, Albert van der Vliet, Emiel Wouters and Yvonne Janssen-Heininger. Inhibition of inhibitory kappa B kinase β by H_2O_2: A role for S-glutathionylation and irreversible oxidations. *FRBM*. 2004, 37(sup1): S70. Travel Award.

Niki Reynaert, A van der Vliet, EFM Wouters and Y Janssen-Heininger. Glutaredoxins in allergic airway disease and regulation by H_2O_2 *in vivo*. ERS 2005, Cell and Molecular Biology Young Scientist Travel Award.

PATENTS

Yvonne M. Janssen-Heininger, Albert van der Vliet, Karina Ckless, Niki Reynaert. Detection of nitrosylated proteins. US publication number 2005-023874. International publication number WO2005/101019.

Yvonne M. Janssen-Heininger, Albert van der Vliet, Karina Ckless, Niki Reynaert. Diagnostic detection of S-nitrosylated proteins. Provisional patent application serial number 11/104,387.

CURRICULUM VITAE

Niki Reynaert was born on April 4th 1979 in Sint-Truiden, Belgium. In 1997 she finished high school at the Royal Atheneum I in Hasselt and started the study of biomedical sciences at the Free University of Brussels. The last year of the study consisted of an internship that she performed at the Department of Respiratory Medicine at Maastricht University. The topic of the study was "The role of hyaluronan fragments in chronic inflammation and lung pathology: studies into the pathogenesis of COPD". After receiving her Master in Science degree Magna Cum Laude in 2001, she continued working at the Department of Respiratory Medicine Maastricht as a PhD. student. During four years the actual work was performed in the laboratory of Dr.Yvonne Janssen-Heininger at the Department of Pathology at the University of Vermont, U.S.A. In March 2003 she was awarded best abstract presentation and a travel award at the First European Respiratory Society Lung Science Conference. In 2004 she received a travel award from the Society for Free Radical Biology and Medicine. At the ERS Annual Congress in 2005 she was awarded the Cell and Molecular Biology Young Scientist Travel award. The title of her thesis is "Redox regulation of Inhibitory kappa B kinase. Oxidants in the pathogenesis of inflammatory lung disease". She returned to Maastricht in April of 2006 and will continue to investigate oxidant-mediated signal transduction in the context of COPD.