The anti-inflammatory potential of Adenosine - an experimental study with emphasis on ischemia-reperfusion injury

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The Anti-Inflammatory Potential of Adenosine

An experimental study with emphasis on ischemia-reperfusion injury

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. M.J. Cohen,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
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LIST OF ABBREVIATIONS

ADA	=	adenosine deaminase
ADP	=	adenosine diphosphate
AK	=	adenosine kinase
AMP	=	adenosine monophosphate
ATP	=	adenosine triphosphate
BCS	=	bovine calf serum
BSA	=	bovine serum albumin
2-CAdo	=	2-chloroadenosine
cAMP	=	cyclic AMP
CPA	=	N ⁶ -cyclopentyladenosine
dCF	=	2'deoxycoformycin
DMPX	=	3,7-dimethyl-1-propargylxanthine
DPCPX	=	1,3-dipropyl-8-cyclopentylxanthine
EHNA	=	erythro-9-(2-hydroxy-3-nonyl)adenine
ELISA	=	enzyme-linked immunosorbent assay
FMLP	=	formyl-Met-Leu-Phe
GP515	=	4-amino-1-(5-amino-5-deoxy-1-b-D-ribofuranosyl)-3-bromo-pyrazolo[3,4-d]pyrimidine
HUVEC	=	human umbilical vein endothelial cells
IB-MECA	=	N ⁶ -(3-iodobenzyl)-adenosine-5'-N-methyluronamide
IC ₅₀	=	50% inhibitory concentration
ICAM-1	=	intercellular adhesion molecule-1
IL-1	=	interleukin-1
IL-6	=	interleukin-6
IL-8	=	interleukin-8
LPS	=	lipopolysaccharide
LTB ₄	=	leukotriene B ₄
mAb	=	monoclonal antibody
NECA	=	5'-N-ethylcarboxamidoadenosine
OFR	=	oxygen free radical
PAF	=	platelet-activating factor
PBS	=	phosphate-buffered saline
PECAM-1	=	platelet endothelial cell adhesion molecule-1
PMN	=	polymorphonuclear leukocyte (neutrophil)
PST	=	8(<i>p</i> -sulphophenyl)theophylline
TNF α	=	tumor necrosis factor- α
TxA ₂	=	thromboxane A ₂
VCAM-1	=	vascular cell adhesion molecule-1
XAC	=	xanthine amine congener

CHAPTER 1

Introduction and Outline of the Thesis

Part of this chapter has been published as:

Bouma MG, Van den Wildenberg FAJM, Buurman WA

The anti-inflammatory potential of adenosine in ischemia-reperfusion injury:
established and putative beneficial actions of a retaliatory metabolite

Shock 8(5): 313-320, 1997

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dCF	=	2'deoxycoformycin
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1.1 PATHOPHYSIOLOGY OF ISCHEMIA-REPERFUSION INJURY

1.1.1 DEFINITION AND CLINICAL RELEVANCE

Ischemia-reperfusion injury is defined as the tissue injury that occurs after restoration of temporarily interrupted blood supply to an organ or tissue. While ischemia or hypoperfusion *per se* can induce necrotic cell death and tissue damage, the extent being dependent on both the duration and the severity of ischemia, reperfusion causes a paradoxical aggravation of ischemic injury. Among the first to describe this paradox were Bulkley and Hutchins (1) in 1977, who reported on myocardial necrosis in areas of successful revascularization by coronary artery bypass graft surgery. In essence, the sequence of ischemia and subsequent reperfusion elicits a non-specific inflammatory process, that results in tissue damage considerably in excess of that induced by ischemia alone. The cellular, biochemical, and microcirculatory characteristics of this inflammatory response will be outlined below. Clinically, ischemia-reperfusion injury is a frequently encountered event, resulting in impaired organ function. Presumably the most often occurring clinical manifestations of reperfusion injury result from myocardial and cerebral infarction, and consequently much of the research work on this phenomenon has been performed in ischemia-reperfusion models of the heart and the brain. In surgical practice, ischemia-reperfusion may occur accidentally after trauma and acute arterial obstruction of the extremities or visceral organs. Similarly, the application and subsequent release of vascular cross clamps during major vascular or cardiopulmonary bypass surgery, or of tourniquets during limb surgery, will result in an ischemia-reperfusion event. In transplantation surgery, ischemia-reperfusion will occur by definition, and may contribute to primary graft dysfunction or even nonfunction. Moreover, the inflammatory reaction induced by ischemia and reperfusion of the graft may form the onset of rejection, and as such be detrimental to the host.

While ischemia-reperfusion of different single organs or tissues results in local injury, it may also lead to injury of remote organs with intact perfusion. Particularly, when the mass of ischemic tissue is large, such as the intestine or the lower extremities, non-cardiogenic pulmonary edema may occur. Already in 1969, Stallone *et al.* (2) reported the development of pathological alterations in the lung following ischemia-reperfusion of the lower extremities. Nowadays, these pulmonary changes are considered to be features of the adult respiratory distress syndrome (ARDS), and to be a part of multiple organ failure (MOF). Moreover, global ischemia resulting from hypovolemic or septic shock, can

lead to a systemic inflammatory response syndrome (SIRS) culminating in multiple end-organ dysfunction with high mortality (3). While an initial massive ischemic insult may precipitate severe SIRS evolving directly into MOF, clinically the sequence of systemic priming of the inflammatory system, followed by a secondary activating event, seems to be more relevant (3, 4). In this so-called two-hit model, ischemia-reperfusion of the gut with subsequent bacterial translocation and release of neutrophil-activating substances, is thought to play a particularly important role (5, 6). Similarly, ischemia-reperfusion of the lower limbs has been shown to increase gut permeability with subsequent systemic endotoxemia (7). Thus, ischemia-reperfusion may cause local organ injury as well as, depending on its extent, a systemic inflammatory reaction with remote organ injury. The pathophysiological characteristics of either local or distant injury are, however, remarkably singular, and will be discussed below.

1.1.2 CENTRAL ROLE OF THE NEUTROPHIL

There is general consensus about the involvement of neutrophils as the primary effector cells in ischemia-reperfusion-induced injury (8, 9). Early and convincing evidence for such a central role of neutrophils originated from various animal studies using different models of ischemia-reperfusion, where neutrophil depletion largely prevented reperfusion injury (10-18). Similarly, prevention of neutrophil adhesion to the vascular endothelium has been shown to markedly reduce reperfusion injury in many experimental models (10, 19-28). The observation that neutrophil depletion and prevention of neutrophil adherence are equally effective in attenuating reperfusion injury, suggests that neutrophil adherence is the rate-limiting step in neutrophil-mediated microvascular injury (10, 29). The enhanced adhesive interactions between neutrophils and endothelial cells and the resulting increased microvascular permeability are largely confined to the postcapillary venules of the microcirculation (8, 30, 31).

There are several mechanisms by which neutrophils can induce postischemic microvascular damage. Neutrophils are part of the innate immune system and act as the first line of cellular defense against invading micro-organisms and other inflammatory stimuli under physiological conditions. Under pathological circumstances however, indiscriminate triggering of neutrophils may occur, resulting in a deleterious, self-destructive inflammatory process (32-34). In this respect, the accumulation of activated neutrophils in postischemic tissue hallmarks the inflammatory response to ischemia and reperfusion. The

neutrophil's armamentarium to mediate acute inflammatory damage is generally divided into two systems, according to their localization within the cell. First, the plasma membrane-associated NADPH oxidase system generates the superoxide radical ($O_2^{\cdot-}$), from which the secondary toxic oxygen metabolites hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are formed. Second, the cytosolic system of intracellular granules contains bactericidal proteins and proteolytic enzymes, such as elastase, gelatinase and collagenase, which are released by degranulation. Both systems act in a cooperative and concerted manner, thereby enhancing the destructive potential of the neutrophil (33, 34). As a result, essential components of the extracellular matrix are degraded leading to increased microvascular permeability, the key histopathological feature of reperfusion injury. However, as stated above, a prerequisite for neutrophil-mediated microvascular and parenchymal reperfusion injury is the sequence of neutrophil chemotaxis and activation, adhesion to the postcapillary vascular endothelium, and subsequent transendothelial migration into postischemic tissue.

Another neutrophil-mediated mechanism of reperfusion injury is the obstruction of capillaries by activated neutrophils adhering to each other, a process termed aggregation. *In vivo* neutrophil aggregation may lead to the formation of occlusive plugs within the microcirculation (35). This plugging of capillaries contributes to the "no-reflow" phenomenon, that is a progressive cessation of blood flow which can be observed within minutes after reperfusion of ischemic tissue, and which can obviously exacerbate the ischemic injury (16, 36, 37). Besides homotypic neutrophil aggregation, adherence of neutrophils to the postcapillary venular endothelium is a major factor contributing to postischemic capillary no-reflow (38, 39). Moreover, the ensuing vascular damage with endothelial cell edema and impaired ability to produce endothelial-derived vasodilating substances, such as nitric oxide (NO) and prostacyclin, plays an important role in the development of the no-reflow phenomenon (40-42).

In summary, neutrophils play a pivotal role in the pathogenesis of reperfusion injury, by contributing to the no-reflow phenomenon, and by adhesive interactions with endothelium of the postcapillary venules resulting in microvascular damage due to degranulation of hydrolytic enzymes and the production of toxic oxidants. The mechanisms involved in neutrophil recruitment into reperfused tissue are, however, very diverse and certainly not mutually exclusive. Nevertheless, a central pathogenetic mechanism, involving the generation of oxygen-derived free radicals (OFR), is generally considered to

play a crucial role in the initiation of the various inflammatory cascades that eventually lead to neutrophil-mediated reperfusion injury.

1.1.3 THE CONCEPT OF OXYGEN RADICAL-MEDIATED REPERFUSION INJURY

The concept that OFR form a link between reperfusion injury and inflammation was introduced by Granger and McCord (43-45). Central to their theory of OFR-mediated reperfusion injury is the conversion of the enzyme xanthine dehydrogenase (XD) to xanthine oxidase (XO) during ischemia (Figure 1.1), probably due to limited proteolysis as a result of activation of the cytosolic protease calpain (45, 46). Also during ischemia, massive catabolism of cellular ATP occurs, resulting in accumulation of its end-metabolite hypoxanthine.

Figure 1.1 Schematic diagram of neutrophil chemotaxis and activation, and subsequent endothelial adherence and transendothelial emigration in ischemia-reperfusion.

EC = endothelial cell; MΦ = macrophage/monocyte; SLe^x = sialyl-Lewis X tetrasaccharide; XD = xanthine dehydrogenase; XO = xanthine oxidase. For other abbreviations, see text and List of Abbreviations.

Upon reperfusion, when oxygen is re-introduced, XO converts hypoxanthine to xanthine with concomitant formation of large quantities of the superoxide anion ($O_2^{\cdot-}$) via univalent reduction of molecular oxygen. Additional toxic oxygen species, such as hydrogen peroxide (H_2O_2) and the aggressive hydroxyl radical ($HO^{\cdot}$), may subsequently be derived from superoxide by enzymatic and transition metal catalysis, respectively. Since the predominant localization of XO is within capillary endothelial cells (47), the vascular endothelium has been proposed as a central source of OFR generation during reperfusion (48, 49). This oxidative burst subsequently initiates a complex series of biochemical and microcirculatory events, that recruit neutrophils into reperfused tissue. In the course of these events, neutrophils become activated and generate OFR via the neutrophilic NADPH oxidase system, thus constituting a second important source of OFR production during reperfusion (10).

No-reflow phenomenon

The involvement of OFR in the initiation of the no-reflow phenomenon was demonstrated by Punch *et al.* (50) and by Suematsu and coworkers (51), who found that inhibition of XO activity by allopurinol or the competitive inhibitor BOF 4272 prevented no-reflow in skeletal muscle after 6 hours ischemia in the rat hind limb, and after 1 hour of hemorrhagic hypotension, respectively. In these studies endothelial damage occurred before leukocyte accumulation, suggesting the involvement of an endothelium-dependent XO-mediated mechanism in early no-reflow, rather than a neutrophil-dependent phenomenon. Nevertheless, after a latent period, neutrophils, activated either directly by OFR or by inflammatory mediators that may be released via OFR-mediated mechanisms, adhere to each other and to the vascular endothelium, thus contributing considerably to impairment of reflow (35). Therefore, impairment of postischemic endothelium-mediated vasodilation may be both leukocyte-dependent as well as leukocyte-independent (52).

Another factor contributing to the no-reflow phenomenon may be the formation of microvascular thrombi as a result of platelet aggregation. Similarly to neutrophils, platelets are potential targets for the potent inflammatory mediators platelet-activating factor (PAF) and thromboxane A_2 (TxA_2), which are known to be synthesized during reperfusion via an OFR-mediated mechanism (see below). Indeed, PAF-activated platelets display hyperaggregability and negatively affect coronary reflow during reperfusion (53). Furthermore, there is experimental evidence that *in vitro* hypoxia - presumably by an oxidant-mediated mechanism (54, 55) - induces expression of tissue factor (TF), now accepted as the major initiator of coagulation *in vivo*

(56, 57), which could contribute to the formation of occlusive platelet plugs, resulting in impairment of postischemic flow.

Microvascular spasm induced by vasoconstrictive mediators that are released in the setting of ischemia and reperfusion could also play a role in reduction of postischemic blood flow. For example, hypoxia can directly induce production and release of the vasoconstrictive autacoid endothelin-1 by endothelial cells (58), whereas products of OFR-mediated lipid peroxidation, such as leukotrienes, TxA_2 and PAF (see below), are also known to exert vasoconstrictive activity, particularly in the intestine and the lungs (59-61).

Neutrophil chemotaxis and activation

A relation between the production of OFR and neutrophil chemotaxis was elegantly demonstrated by Petrone *et al.* (62), who reported that superoxide activates a neutrophil chemotactic factor in human plasma, that was lipid-derived and bound to serum albumin. They suggested that this factor was analogous or even identical to arachidonic acid oxidation products. Indeed, breakdown products of arachidonic acid exert potent neutrophil chemotactic activity and are produced in enhanced concentrations upon reperfusion (61, 63-70). OFR induce peroxidation of membrane lipids, resulting in disturbance of intracellular calcium homeostasis and activation of phospholipase A_2 (71, 72), a ubiquitous cellular membrane enzyme which catalyzes the formation of arachidonic acid and lysophospholipids from membrane phospholipids. Arachidonic acid may subsequently be metabolized through either the lipoxygenase or the cyclo-oxygenase pathway, yielding the eicosanoids leukotriene B_4 (LTB_4) and TxA_2 , respectively. Alternatively, lysophospholipids may be remodeled into precursors of PAF. Both LTB_4 and TxA_2 , as well as PAF are well-recognized chemoattractants, and their role in neutrophil recruitment in reperfused tissues has been firmly established (13, 15, 61, 64-66, 73-76). Moreover, these substances are potent neutrophil agonists, triggering neutrophil toxicity by inducing release of OFR and toxic proteases. Furthermore, neutrophil activation during chemotaxis by these agonists results in upregulation of β_2 -integrin adhesion molecules, which are essential for subsequent neutrophil attachment to the endothelium (see below).

There is evidence to show that ischemia-reperfusion activates the alternative pathway of the complement cascade of plasma proteins (77-80), generating the chemotactic peptides C3a and C5a. Moreover, the classical complement cascade may also be activated during ischemia-reperfusion injury (81, 82). Although the link between OFR production and activation of the complement system during ischemia-reperfusion is poorly characterized, it is conceivable that OFR-

mediated loss of cell membrane integrity results in expression of neopeptides, that are not recognized as *self* by the immune system, thus inducing complement activation (84). Similar to the lipid-derived mediators described above, C5a is also capable of potentially triggering neutrophil respiratory burst activity and degranulation, as well as increasing neutrophil adhesiveness by upregulating the CD11b/CD18 integrin (78, 85, 86).

Several cytokines are known to be involved in neutrophil chemotaxis in inflammatory responses. Most notably, interleukin-8 (IL-8) has been identified as a very specific and potent neutrophil chemoattractant (87, 88). Furthermore, the macrophage-derived inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor- α (TNF α) may have a direct chemotactic potency (89, 90), but mainly promote chemotactic migration of neutrophils by inducing release of the chemokine IL-8 by endothelial cells (87) and fibroblasts (91). TNF α potentially activates oxidant release by neutrophils adherent to endothelium, through a CD11b/CD18-mediated signal (92), as well as via an endothelial cell-associated PAF-mediated mechanism (93). There is ample experimental and clinical evidence to support a role for these and other cytokines in the setting of ischemia-reperfusion, implicating the generation of OFR in cytokine production by mononuclear as well as endothelial cells (94, 95, 98, 99).

Neutrophil adhesion and emigration

Once neutrophils have been directed to the reperfused tissue along chemotactical concentration gradients, they are sequestered to the site of inflammation as a result of adherence to the vascular endothelial lining. This adhesive process is generally considered to occur in distinct sequential stages. Initially, neutrophils "roll" along the vessel wall due to transient and reversible intermittent adhesive interactions with the activated endothelial cell surface, and subsequently progress to a stationary state of "sticking" as a result of firm, continuous adhesive interactions with the endothelium.

Leukocyte rolling is due to low-affinity adhesive interactions with the endothelium, mediated by glycoproteins that belong to the selectin family of cell adhesion receptors. P-selectin and E-selectin on endothelial cells, and L-selectin on leukocytes, interact with ligands containing the tetrasaccharide sialyl-Lewis X (SLe^x) or other sialylated, fucosylated structures, to mediate rolling. In particular, a role for endothelial P-selectin in ischemia-reperfusion-induced neutrophil adherence and subsequent reperfusion injury has been clearly demonstrated (19, 100-103). *De novo* expression of P-selectin by endothelial cells in this setting may be induced directly by OFR (104, 105), as well as by substances that are released during reperfusion, such as C5a (106),

TNF α (107), and IL-1 (108). It has been suggested that tethering of neutrophils by P-selectin facilitates their activation by endothelial-associated PAF, which may be co-expressed with P-selectin in a juxtacrine manner (109). The involvement of oxidants in PAF-mediated endothelial cell-dependent neutrophil adhesion (110, 111) supports the relevance of regulated coexpression of P-selectin and PAF to postischemic conditions. In several *in vitro* studies it has been hypothesized that OFR induce expression of endothelial E-selectin after anoxia-reoxygenation (104, 112), although others (113, 114) were unable to confirm this. As suggested by Seekamp *et al.* (115), participation of E-selectin in *in vivo* postischemic neutrophil-mediated tissue injury may be organ-specific and dependent on the production and release of cytokines, such as TNF α and IL-1, and may be confined to the lungs (115) and the heart (116). L-selectin, constitutively expressed on leukocytes, also seems to be required in the development of reperfusion injury (115, 117). The observation that treatment with soluble SLe^x, which binds to all three selectins, effectively reduces both local and remote reperfusion injury in different experimental models, may underscore the similar, and possibly redundant, role of E-, P-, and L-selectin in postischemic neutrophil adherence (19, 115, 118).

Leukocyte rolling evolves to a stationary state in which high-affinity adhesive interactions keep the leukocytes firmly attached to the endothelial surface. This stage is referred to as leukocyte sticking, and is mediated by leukocyte β_2 -integrins, particularly Mac-1 (CD11b/CD18), binding to the endothelial ligand intercellular adhesion molecule-1 (ICAM-1). The importance of integrin-mediated adhesion in the development of the inflammatory response to ischemia-reperfusion has been firmly established (10, 20-24, 110, 119, 121-125). Expression of the CD11/CD18 integrins on neutrophils can be induced directly by oxidants (126), but also indirectly by other inflammatory mediators that are produced during reperfusion, such as C5a, LTB₄, and PAF (127, 128). Upregulation of endothelial ICAM-1 expression is dependent on the action of cytokines released during reperfusion, and may also be induced by oxidants (129, 130). As a result of firm adherence, a microenvironment at the neutrophil-endothelial cell-cell interface is created, into which the activated neutrophils secrete their cytotoxic products, such as OFR and proteases, and cause endothelial injury, uninhibited by circulating anti-oxidants or anti-proteases (8). Eventually, neutrophils emigrate from the vasculature into the postischemic tissue by diapedesis between endothelial cells. A role for β_2 -integrins, endothelial cell-associated PAF and the platelet endothelial cell adhesion molecule-1 (PECAM-1) in neutrophil transendothelial migration has been described (73, 131-133). Several of the chemoattractants that are released

during reperfusion, such as PAF, LTB₄ and IL-8, potentially elicit leukocyte emigration (132, 134-136).

1.2 THERAPEUTIC APPROACHES TO ISCHEMIA-REPERFUSION INJURY

In view of the described complex pathogenetic mechanisms involved in local and systemic postischemic injury, theoretically there are multiple potential sites at which can be intervened to modulate the inflammatory response to ischemia and reperfusion. Indeed, a variety of approaches to the treatment of reperfusion injury exists, some of which have been implemented successfully in the experimental or clinical setting. In general, these therapeutic strategies are aimed at interfering at either the effector, the mediator or the tissue level of the inflammatory cascades elicited by ischemia and reperfusion (137). Although some of these strategies may have overlapping effects at more than one level, for reasons of clarity, they are discussed here separately.

1.2.1 THERAPEUTIC STRATEGIES AT THE EFFECTOR LEVEL

Inhibitors of OFR production, free radical scavengers and antioxidants

Given the central role of XO-generated OFR and neutrophil-derived oxidants as effectors of peroxidative endothelial injury, agents that interfere with either the production or the harmful actions of OFR, have become important therapeutic tools to protect from reperfusion injury. Clearly, based on the significance of OFR in initiating the release of various inflammatory mediators and the expression of endothelial adhesion molecules during reperfusion, as described earlier, such agents may also be effective at the mediator and tissue level of intervention.

Allopurinol, a structural analogue of hypoxanthine, competitively inhibits the enzyme xanthine-oxidase, and thus reduces the production of the superoxide anion during ischemia-reperfusion, as was demonstrated experimentally (138, 139) and clinically (140). Similarly, iron-chelating agents, such as deferoxamine and ceruloplasmin, interfere with iron-mediated catalysis of the production of the hydroxyl radical (Haber-Weiss reaction), and have demonstrated beneficial effects in ischemia-reperfusion (139, 141-144).

Free radical scavengers are agents that interact with reactive oxygen species and render them harmless. Superoxide dismutase and catalase are endogenous enzymes that act in combination to scavenge superoxide and hydrogen peroxide, thus protecting against oxidant-induced tissue injury *in vivo* in

ischemia-reperfusion syndromes as well as in other disease processes (43, 145, 146). Changes in serum levels of these antioxidant enzymes have even been demonstrated to effectively predict the development of ARDS in septic patients (147). Other, non-enzymatic free radical scavengers include dimethylsulphoxide (DMSO), dimethylthiourea (DMTU), and mannitol, which neutralize the hydroxyl radical and have been used clinically for many years to prevent oxidant-mediated injury (148-152). Lazaroids (21-aminosteroids and 2-methylaminochromans) are a relatively new class of compounds, designed and demonstrated to protect against tissue damage after trauma and ischemia by their ability to scavenge OFR and to inhibit superoxide-generating NADPH oxidase by neutrophils (153-155).

Antioxidants are agents that interrupt peroxidation, preventing the production of peroxides and terminating the chain reaction by which further free radicals are formed. Vitamin E (α -tocopherol) is an endogenous antioxidant present within cell membranes and plasma lipoproteins, that cooperates with vitamin C (ascorbic acid) to minimize the consequences of lipid peroxidation (156, 157), and exogenous administration of vitamin E has been shown to reduce reperfusion injury (158, 159). Similarly, treatment with coenzyme Q10 (ubiquinol-10), another antioxidant present in biological membranes (160), attenuates OFR-mediated reperfusion injury (161). Glutathione (GSH) peroxidase is an endogenous antioxidant enzyme, located in the cytosolic and mitochondrial cellular compartments, that decomposes hydrogen peroxide to H_2O at the expense of reducing equivalents from GSH (162). The antioxidant effect of the glutathione-glutathione peroxidase pathway can be effectively enhanced by replenishing intracellular GSH stores with the mucolytic agent *N*-acetylcysteine (Fluimucil®, Mucomyst®), a precursor of GSH, which has been used successfully in several models of OFR-mediated disease processes, including ischemia-reperfusion (163-166).

Specific inhibitors of neutrophil function

As stated earlier, neutrophils are commonly regarded as the main effector cells of reperfusion injury, and therefore several therapeutic modalities have been designed that specifically prevent or inhibit neutrophil OFR production and proteolytic enzyme activity, or interfere with neutrophil adherence to the endothelium.

While the free radical scavengers and antioxidants mentioned above will non-specifically attenuate peroxidative injury caused by neutrophil-derived OFR, adenosine has been recognized as a specific physiological modulator of superoxide release by neutrophils (167). This neutrophil-inhibitory feature of

adenosine will be discussed in more detail further on. Also, various calcium channel antagonists, including verapamil, can inhibit *in vitro* production of superoxide by stimulated neutrophils (168).

Several antiproteases inhibit neutrophil proteolytic enzyme activity, and thereby attenuate ischemia-reperfusion-induced neutrophil infiltration and increased microvascular permeability (169). Similarly, perfluorochemicals have been reported to suppress lysozyme degranulation by activated neutrophils during myocardial reperfusion (170), and to prevent neutrophil-mediated injury of endothelial cells after anoxia (171).

A very effective means of specifically inhibiting neutrophil function in the setting of ischemia-reperfusion, is the use of monoclonal antibodies (mAbs) directed against the neutrophil CD11b/CD18 (Mac-1) complex. Since the CD11b/CD18 integrin is involved in neutrophil activation as well as adherence, administration of mAbs against either the CD11b subunit or the CD18 subunit, has been shown to exert significant protective effects in many different *in vivo* models of reperfusion injury (10, 20-24, 110, 119-121).

1.2.2 THERAPEUTIC STRATEGIES AT THE MEDIATOR LEVEL

Inhibitors of generation of arachidonic acid metabolites

Activation of phospholipase A₂ is a proximal step in the generation of proinflammatory lipid mediators, and hence inhibition of this enzyme may prevent reperfusion damage (71, 72, 172), provided that the duration of the ischemic interval is limited, as suggested by Boros *et al.* (173). Inhibitors of the lipoxygenase and the cyclo-oxygenase pathway will reduce the generation of the chemoattractants LTB₄ and TxA₂, respectively, and thereby prevent neutrophil activation and migration into reperfused tissue, as has been demonstrated in various experimental models (13, 63, 66, 68-70, 136). However, as with phospholipase A₂ inhibitors, their efficacy may also depend on the length of the ischemic period (67). The generation of biologically active leukotrienes from arachidonic acid can be suppressed by the incorporation of *n*-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid contained in fish oil. A high dietary intake of fish oil has been demonstrated experimentally to protect from reperfusion injury (174), and has been shown to correlate with a low mortality from myocardial infarction in large epidemiological studies (175, 176).

PAF receptor antagonists

The significance of PAF in mediating neutrophil activation and adhesive interactions with the endothelium during reperfusion is underscored by the clearly demonstrated beneficial effects of PAF receptor antagonists in animal models of myocardial (53, 74, 75), hepatic (76), renal (177), and intestinal (73) ischemia-reperfusion, as well as in kidney (178), liver (179), and heart-lung (180) transplantation models.

Cytokine inhibitors

During recent years, advances in immunological research have led to the development of mAbs against a wide variety of cytokines, which have been used to dissect the role of these mediators in the host inflammatory response. Because $\text{TNF}\alpha$ and IL-1 have been identified as proximal mediators of the inflammatory response to ischemia-reperfusion, the use of specific inhibitors that block or neutralize their biological effects may be an important site of therapeutic intervention.

Neutralization of circulating $\text{TNF}\alpha$, using anti- $\text{TNF}\alpha$ polyclonal antisera or mAbs, has been shown to effectively attenuate both local and distant organ injury in different animal models of ischemia-reperfusion (181-184). While, clinically, the potential usefulness of mAbs to $\text{TNF}\alpha$ has been evaluated in the treatment of sepsis, with often disappointing results (185-187), their efficacy in human conditions of ischemia-reperfusion remains yet to be determined. As with $\text{TNF}\alpha$, the effects of circulating IL-1 in ischemia-reperfusion can also be neutralized with antibodies (188).

Alternatively, $\text{TNF}\alpha$ activity may be blocked by soluble TNF receptors that compete with cell surface receptors for TNF binding. Experimentally, administration of recombinant soluble TNF receptors has been an effective tool in preventing local and distant reperfusion injury (188, 189). Likewise, IL-1 receptor antagonists interfere with binding of IL-1 with its receptor, and have displayed protective effects in reperfusion injury of limbs (188) and brain (190), as well as in hemorrhagic shock and resuscitation (191).

While antagonism of the proximal inflammatory cytokines $\text{TNF}\alpha$ and IL-1 results in reduced leukocyte accumulation during reperfusion, neutrophil infiltration in postischemic tissue also can be prevented by administration of mAb or polyclonal antiserum against the chemotactic cytokine IL-8 (192, 193).

Transforming growth factor- β (TGF β) is an essentially ubiquitous cytokine found in many immunocompetent cell types, that has been recognized as an endogenous immunosuppressive agent (194). Among the immunomodulatory actions that have been invoked as potential protective mechanisms of

recombinant TGF β in reperfusion injury, are antagonism of TNF α effects and inhibition of TNF α release (195). In this context, it is also of interest that TGF β has been shown to induce release of the IL-1 receptor antagonist *in vitro* (196).

1.2.3 THERAPEUTIC STRATEGIES AT THE TISSUE LEVEL

MAbs against endothelial adhesion molecules

Where neutrophil accumulation in postischemic tissue is the hallmark of the inflammatory response to ischemia and reperfusion, and prevention of neutrophil-endothelial adhesion by blocking neutrophil adhesive receptors effectively blunts reperfusion injury (10, 20-28, 121), antagonism of the endothelial counterreceptors will obviously provide similar protective effects. As described previously, neutrophil-endothelial interactions consist of three steps of sequential events, *i.e.*, selectin-mediated rolling, tight adhesion mediated by neutrophil β_2 -integrins interacting with their endothelial ligand ICAM-1, and transendothelial migration. Consequently, selectin-blocking approaches, using either mAbs against endothelial P- and E-selectin (19, 100, 101, 103, 115, 197, 198), or SLe^x-containing oligosaccharides (19, 115, 118) have been successful in preventing from experimental reperfusion injury. Likewise, the use of mAbs against endothelial ICAM-1 has been shown to reduce neutrophil sequestration and concomitant injury in postischemic tissue (119, 120, 122-125). Recently, results from a study by Murohara *et al.* (199) have indicated that blockade of PECAM-1 with a polyclonal Ab significantly attenuates myocardial ischemia-reperfusion injury *in vivo*, presumably by inhibiting transendothelial migration of neutrophils.

1.3 PROTECTIVE ROLE OF ADENOSINE IN ISCHEMIA-REPERFUSION INJURY

1.3.1 ADENOSINE AND ADENOSINE RECEPTORS

During ischemia, cellular energy stores, particularly of ATP, are depleted, resulting in failure of cellular homeostasis characterized by a loss of transmembrane sodium, potassium and calcium ion balances, leading to cell necrosis (44). As a consequence of ATP breakdown, its metabolites adenosine, inosine and hypoxanthine accumulate in the ischemic tissue. The major pathway by which adenosine is formed under these conditions is by dephosphorylation of AMP catalyzed by 5'-nucleotidase (200). While normally produced during

physiological metabolic activity, adenosine can modulate a vast array of physiological processes in many mammalian systems. The numerous biological effects of adenosine on the central nervous, cardiovascular, respiratory, gastrointestinal, immune and metabolic system are mediated by activation of specific cell-surface adenosine receptors. Currently, four major subtypes of adenosine receptors have been classified on the basis of biochemical, functional and receptor-cloning studies (201). Activation of either A1, low-affinity A2a, high-affinity A2b, or A3 receptors, modulates the activity of different second messenger systems, coupled to the various receptors. However, an extensive discussion of these second messengers is beyond the scope of this thesis, and excellent comprehensive reviews on this subject are available (200-203). Since adenosine has a short half-life in the circulation, it is generally assumed that it modulates cellular function by interacting with its receptors in an autocrine or paracrine manner (202). While adenosine functions as an important homeostatic mediator in the physiology of many organ systems, its role in pathophysiological conditions, particularly in ischemia-reperfusion where adenosine formation is directly linked to ATP catabolism, has received growing appreciation. Moreover, increased understanding of the beneficial actions of adenosine on metabolic, cardiovascular and inflammatory aspects of reperfusion injury, has led to the development of pharmacological agents that interfere with adenosine metabolism under these circumstances, as a potential therapeutic strategy to prevent or attenuate the deleterious consequences of ischemia and reperfusion.

1.3.2 ADENOSINE AS A "RETALIATORY METABOLITE" IN ISCHEMIA-REPERFUSION

Under conditions of cellular metabolic stress, when the cellular energy state is depleted, intracellular adenosine levels acutely increase due to breakdown of ATP, and adenosine is released from the cell. Particularly during ischemia, the level of extracellular adenosine available for activating adenosine receptors increases dramatically, as adenosine formation is directly linked to the massive ATP catabolism. Because the net effect of adenosine released under these circumstances is to reduce metabolic demand and to adjust the cellular energy supply, thus retaliating against the external stimulus, *i.e.* ischemia, that causes excessive ATP breakdown, Newby proposed the name "retaliatory metabolite" to describe adenosine (204). While in his original proposal of adenosine as a retaliatory metabolite attention was focused mainly on its beneficial metabolic and cardiovascular actions, the rapidly expanding knowledge of its very diverse

anti-inflammatory effects, which may also counteract the deleterious consequences of ischemia-reperfusion, has added a new dimension to this concept. The beneficial "retaliatory" effects of adenosine in ischemia-reperfusion, as summarized in Table 1.1, will be discussed below with special emphasis on its anti-inflammatory actions.

Table 1.1 Established beneficial effects of adenosine in ischemia-reperfusion

	References
<i>Metabolic effects</i>	
purine salvaging	206-209
inhibition of lipolysis	200, 214
enhancement of glucose transport	200, 215
stimulation of gluconeogenesis	200
<i>Cardiovascular effects</i>	
vasodilation	200, 213, 240, 241
inhibition of platelet aggregation	203, 242
inhibition of vasoconstrictive mediators	63, 217
negative inotropy and dromotropy	203
preconditioning	219-221
<i>Anti-inflammatory effects</i>	
inhibition of neutrophil superoxide production	167, 217, 230
inhibition of neutrophil adhesion	134, 228, 232-237, 239

Metabolic actions

Adenosine, as substrate for the regeneration of high-energy phosphates, may play a role in preservation of the nucleotide pool, which is depleted during ischemia. Indeed, purine salvaging with enhanced recovery of ATP has been invoked to explain the beneficial actions of exogenous adenosine in ischemia-reperfusion (205-209). However, the importance of repletion of ATP in adenosine-mediated protection from reperfusion injury has been questioned (210-212). Consistent with the retaliatory metabolite concept, adenosine acting via A1 receptors inhibits catabolic hormone action, by antagonizing the effects of catecholamines in the heart and adipose tissue (200, 213). Furthermore, it plays a role in glucose metabolism by promoting the actions of the anabolic hormone insulin, and displaying insulin-like activity, such as inhibition of

lipolysis, stimulation of gluconeogenesis, and facilitating glucose transport (200, 214-216), thus increasing energy supply.

Cardiovascular actions

The most prominent cardiovascular action of adenosine is its vasodilatory effect mediated by A2a, A2b, and A3 receptors (201). By dilating pre-capillary sphincters and arteriolar resistance vessels, adenosine participates in the autoregulation of blood flow of organs, such as the heart, brain, gut, and skeletal muscle (203). As a result of vasodilation, the availability of substrate and oxygen during reperfusion is increased, and the no-reflow phenomenon is counteracted. Furthermore, via A2a receptor activation, adenosine inhibits platelet aggregation and adherence to the endothelium, thus preventing microvascular obstruction and no-reflow. Also, it may prevent microvascular spasm by reversing the effects of vasoconstrictive mediators, such as endothelin, leukotrienes, and PAF, that are released during reperfusion (63, 217). In the heart, adenosine exerts antiadrenergic, negative inotropic and dromotropic effects, and inhibits noradrenaline release from sympathetic nerve endings through activation of cardiac A1 receptors, thereby reducing myocardial oxygen demand (201, 203, 213, 218).

A special form of adenosine-mediated protection against ischemic injury, is "preconditioning". Particularly in the myocardium, brief, intermittent episodes of ischemia, render cardiac tissue less sensitive to a subsequent sustained ischemic insult. This effect is thought to be mediated primarily by adenosine (219), acting via A1 receptors (220) as well as A3 receptors (221), and may occur through inhibition of adrenergic stimulation and of noradrenaline release, and activation of ATP-sensitive potassium channels with reduction of calcium overload (220, 222). However, the precise mechanisms involved in cardioprotective preconditioning and the relative importance of adenosine in this phenomenon have not been fully elucidated, and may vary between species (223). While the predictability of ischemic events during cardiopulmonary bypass surgery and transplantation makes the preconditioning phenomenon clinically relevant to protect the ischemic-reperfused myocardium, recently adenosine-mediated preconditioning has also been shown to protect skeletal muscle from reperfusion injury (224), which could have therapeutic significance to other surgical fields, such as elective limb surgery.

Anti-inflammatory actions

One of the first clinical observations linking adenosine to the immune system was made in patients with a severe immunodeficiency disease, in whom an

autosomal inheritable deficiency of the enzyme adenosine deaminase was found (225). As a result of the inability to catabolize adenosine, these subjects suffered from a failure to thrive, diarrhea, malabsorption and recurrent infections. The highly elevated plasma levels of adenosine in these patients resulted in impaired cellular immunity, due to a cytotoxic effect on T-lymphocytes. More recently however, it has been demonstrated that adenosine is in fact a physiological endogenous modulator of inflammatory processes, exerting either pro-inflammatory or anti-inflammatory effects, depending on the specific adenosine receptors activated. In particular, the work of Cronstein (226-229), and subsequently that of others, has identified adenosine as an important modulator of various neutrophil functions. Given the central role of neutrophils in the pathogenesis of reperfusion injury, the anti-inflammatory effects of adenosine on activated neutrophils have been consistently implicated in its protective role in ischemia-reperfusion. First, adenosine acting via A2 receptors potently inhibits superoxide anion generation by activated neutrophils, thereby limiting OFR-mediated injury of endothelial cells (167, 226, 230, 231). Second, it can reduce neutrophil-mediated cellular injury by inhibiting neutrophil-endothelial adhesion, as a result of A2 receptor engagement on neutrophils (228). Several *in vivo* studies have demonstrated that adenosine inhibits ischemia-reperfusion induced adhesion (232-234), as well as adhesive interactions elicited by PAF (134, 235). It has been established that adenosine can interfere with both stages of the adhesion process, that is with selectin-mediated rolling (232, 236) as well as integrin-mediated sticking (232, 237-239) of neutrophils. Also, interference of adenosine with leukocyte emigration has been reported (134, 233, 235). Thus, the anti-inflammatory effects of adenosine on activated neutrophil function have been firmly established and confirmed in *in vivo* ischemia-reperfusion models.

1.4 OUTLINE OF THE THESIS

From the previous introduction it can be concluded that several distinct immunocompetent cell types play a prominent role in the pathophysiology of the inflammatory response to ischemia and reperfusion. First, neutrophils have been undisputably identified as the primary effector cells of reperfusion injury. Second, cells of mononuclear lineage, such as monocytes and macrophages, are believed to be responsible for the excessive production and release of pro-inflammatory cytokines, as observed during ischemia-reperfusion. Third, the vascular endothelium is the center-stage where the inflammatory events take

place, and is actively involved in the chemotaxis and activation of neutrophils and their guidance into target tissue.

As discussed previously, Cronstein was the first to recognize adenosine as an immunomodulatory agent, by describing its effects on superoxide generation by activated human neutrophils and demonstrating the involvement of specific adenosine receptors. Based on his observations, and given the ubiquitous presence of adenosine in the body, he proposed that adenosine functions as an important endogenous physiological anti-inflammatory molecule. Elaborating this concept further, we hypothesized that adenosine might modulate the inflammatory function of other immunocompetent cell types of human origin.

The main objective of this thesis, therefore, was to investigate whether adenosine affects relevant inflammatory features of the various different cell types involved in the host response to ischemia and reperfusion, and to determine the role of specific adenosine receptors in this context. Furthermore, the effects of agents that interfere with adenosine metabolism thereby enhancing endogenous adenosine release were evaluated *in vitro* as well as *in vivo*, in an attempt to assess the potential therapeutic relevance of such "adenosine-regulating" agents to inflammatory disorders, specifically ischemia-reperfusion.

In *Chapter 2* we study the effects of both exogenous and endogenous adenosine on the degranulation of activated human neutrophils, as determined by the release of neutrophil elastase, bactericidal/permeability-increasing protein and defensins, in an *ex vivo* whole blood model, and investigate the involvement of specific adenosine receptors.

In *Chapter 3* the actions of adenosine on release of the pro-inflammatory cytokines $\text{TNF}\alpha$, IL-6 and IL-8 by activated human monocytes *in vitro* are determined and linked to specific adenosine receptors.

Similarly, in *Chapter 4* the effects of exogenous and endogenous adenosine and receptor-specific adenosine analogues on activation of cultured human endothelium, as determined by the expression of the adhesion molecules E-selectin, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), as well as the release of the cytokines IL-6 and IL-8, are evaluated.

Finally, the possible relevance of the adenosine-regulating agent GP515, an adenosine kinase inhibitor, to the treatment of ischemia-reperfusion-induced inflammation is assessed in an *in vitro* and an *in vivo* model of ischemia-reperfusion in *Chapter 5* and *Chapter 6*, respectively.

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CHAPTER 2

Adenosine Inhibits Neutrophil Degranulation in Activated Human Whole Blood

Involvement of adenosine A2 and A3 receptors

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ABSTRACT

Adenosine acting via A2 receptors is a potent inhibitor of neutrophil oxidative burst, but its effects and mechanisms of action on neutrophil degranulation have been less well-characterized. We therefore investigated the effects of adenosine and its receptor-specific analogues on neutrophil degranulation in stimulated human whole blood. Adenosine dose-dependently inhibited LPS- and TNF α -induced release of the azurophilic granule proteins bactericidal/permeability-increasing protein, elastase, and defensins to approximately the same extent, with a maximum inhibition of 70-80%, and IC₅₀ ranging from 14 to 24 μ M. The inhibitory effects of adenosine were partially blocked by the A2 receptor antagonist 3,7-dimethyl-1-propargylxanthine, the A1/A2 antagonist 8(*p*-sulphophenyl)theophylline (PST), and the A1/A3 antagonist xanthine amine congener (XAC), but not by the A1 antagonist 1,3-dipropyl-8-cyclopentylxanthine. The highly selective A3 agonist N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA) and the non-selective agonist 2-chloroadenosine (2-CAdo) reduced degranulation more potently than the A1 agonist N⁶-cyclopentyladenosine. The inhibitory effects of IB-MECA and 2-CAdo were strongly reversed by XAC, but were not affected by PST. In addition, the adenosine kinase inhibitor GP515 also attenuated degranulation via an adenosine-mediated mechanism. These data indicate that adenosine acts via A2 as well as A3 receptors to inhibit human neutrophil degranulation, and add to the anti-inflammatory potential of adenosine and adenosine-regulating agents towards neutrophil-mediated tissue injury.

2.1 INTRODUCTION

Activation of neutrophils (PMN) by appropriate stimuli with subsequent release of a wide range of more or less toxic secretory products is a crucial component of the physiological inflammatory response. These neutrophil products include preformed granule constituents, such as lytic enzymes, other proteins and glycosaminoglycans, as well as nonproteinaceous nongranule materials, such as phospholipids and reactive oxygen species (1). Whereas controlled degranulation and secretion by locally accumulated neutrophils contributes to a protective, limited tissue inflammatory response, exaggerated and uncontrolled systemic activation of neutrophils in various inflammatory conditions, such as sepsis (2) and ischemia/reperfusion (3), may cause a detrimental systemic inflammatory process, resulting in pathological tissue injury.

Adenosine has been recognized as an important modulator of neutrophil function, particularly with regard to neutrophil superoxide production and neutrophil adherence and cytotoxicity to the vascular endothelium (4-10), and may therefore be protective against neutrophil-mediated tissue injury. Specifically, adenosine is strongly protective against reperfusion damage (11). Also, adenosine-regulating agents, such as acadesine, AICA riboside (5-amino-1- β -D-ribofuranosylimidazole-4-carboxamide), adenosine deaminase inhibitors, and an adenosine kinase (AK) inhibitor, provide protection against reperfusion injury (12-15) and endotoxin challenge (16, 17) *in vivo*.

To date, four different receptors have been identified (18), that mediate the effects of adenosine on many cells (A1, A2a, A2b, and A3). Neutrophils have been shown to express adenosine receptors of both A1 and A2 types, which have opposing effects on various neutrophil functions. Ligation of neutrophil A1 receptors by adenosine at picomolar concentrations enhances neutrophil chemotaxis and adherence (10, 19), thereby exerting a pro-inflammatory effect. In contrast, adenosine in micromolar concentrations has been shown to inhibit production of superoxide anion, the presumed parent compound of various reactive oxygen species, via occupation of A2 receptors (4, 9). Also, adenosine inhibits neutrophil adherence to endothelium (6, 10) and cardiac myocytes (7), acting via A2 receptors on the neutrophil cell surface. Thus, ligation of A2 receptors by adenosine has anti-inflammatory consequences. Similarly, A3 receptors have been implicated in the anti-inflammatory actions of adenosine by inhibiting release of TNF α by human macrophages (20), but expression of A3 receptors on human neutrophils has thus far not been demonstrated.

While in previous studies by Cronstein and coworkers (21, 22) it was demonstrated that adenosine strongly reduced superoxide anion generation, at the same time it exerted little effect on the release of beta-glucuronidase and lysozyme by FMLP-stimulated human neutrophils *in vitro*. Similarly, others (23) have reported that secretion of granule proteins from neutrophils was only modestly affected by adenosine. Thus, while the inhibitory actions of adenosine on neutrophil respiratory burst are well-documented, its effects and mechanism of action on degranulation are less clear.

Therefore, this study was undertaken to evaluate whether adenosine affects release of the granule proteins bactericidal/permeability-increasing protein (BPI), human neutrophil elastase (HNE), and defensins (HNP) by neutrophils in an *ex vivo* human whole blood model, and to assess the role of specific adenosine receptors. In addition, the effects of the adenosine-regulating agent GP515, a novel AK inhibitor, on neutrophil degranulation in this model were determined.

2.2 MATERIALS AND METHODS

2.2.1 REAGENTS

Adenosine, 2-chloroadenosine (2-CAdo), N⁶-cyclopentyladenosine (CPA), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), FMLP and LPS (from *Escherichia coli*, serotype 055:B5, catalog no. L2880) were all obtained from Sigma Chemical (St. Louis, MO). 3,7-dimethyl-1-propargylxanthine (DMPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), 8(*p*-sulfophenyl)theophylline (PST), and xanthine amine congener (XAC) were purchased from Research Biochemicals Incorporated (Natick, MA). The novel AK inhibitor 4-amino-1-(5-amino-5'-deoxy-1-β-D-ribofuranosyl)-3-bromo-pyrazolo[3,4-d]pyrimidine (GP515) was kindly provided by Dr. K.M. Mullane (Gensia, Inc., San Diego, CA). Recombinant human TNFα was kindly provided by BASF/Knoll (Ludwigshafen, Germany).

2.2.2 BLOOD COLLECTION AND WHOLE BLOOD STIMULATION

Blood, obtained from healthy volunteers after informed consent for research was given, was anticoagulated in evacuated blood collection tubes (Sherwood Medical, St. Louis, MO), containing 0.1 ml 500 IU/ml Leo Heparin (Leo Pharmaceutical Products B.V., Weesp, The Netherlands). Whole blood was incubated in polypropylene tubes (Costar, Cambridge, MA) at 37°C with reagents as indicated. In experiments where the effects of adenosine or GP515 were tested, 100 μM EHNA was added to the cultures to prevent breakdown of adenosine by endogenous adenosine deaminase. After 2 h incubation, plasma was separated from blood cells by centrifugation for 5 min at 1500 x *G*, aliquotted, and stored at -20°C until analysis by ELISA.

For determination of purine levels by HPLC, supernatants were deproteinized with 0.4 M perchloric acid on ice, centrifuged at 1200 rpm for 10 min at 4°C, and neutralized with 0.4 M K₂HPO₄. Samples were kept at -70 °C until determination of purine levels.

2.2.3 PMN ISOLATION AND STIMULATION

PMN were isolated from buffy coats kindly provided by the Red Cross Blood Bank Zuid Limburg (Maastricht, The Netherlands). Blood was donated by healthy volunteers after informed consent for research was obtained. PMN were

isolated using a standard procedure. In short, blood cells were separated by density gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway). The interphase containing the mononuclear leukocytes was removed, and the remaining mixture of PMN and red blood cells was treated for 30 min with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.4) to lyse the erythrocytes. The remaining PMN were washed twice. The PMN had a purity of more than 98%, and consisted mainly of neutrophils (>95%). Isolated PMN ($5 \times 10^6/\text{ml}$) were resuspended in RPMI-1640, preincubated with 5 $\mu\text{g}/\text{ml}$ cytochalasin-B, and subsequently incubated in polypropylene tubes at 37°C with reagents as indicated. In experiments where the effects of adenosine or GP515 were tested, 100 μM EHNA was added to the cultures to prevent breakdown of adenosine by endogenous adenosine deaminase. After 2 h incubation, the supernatant was separated from PMN by centrifugation for 5 min at 1500 x *G*, and stored at -20°C until analysis by ELISA. Cell viability during the experiment was tested by lactate dehydrogenase release, which was always below 2% (data not shown).

2.2.4 REVERSE TRANSCRIPTION PCR OF ADENOSINE A3 RECEPTOR MRNA

Thirty ml blood was obtained from healthy donors with heparinized vacutainers. Fresh whole blood was mixed with Hespan (DuPont Pharmaceuticals, Boston, MA) in 50 ml conical centrifuge tubes and the erythrocytes were allowed to settle for 30 min. Following sedimentation, the supernatant was layered on a 75% Percoll gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 2000 rpm for 50 min at 4°C. The neutrophil pellet was resuspended in Heinz-HEPES buffer pH 7.4. The cells were washed briefly in Heinz-HEPES buffer, and lysed with RNASAT-60 (Tel Test, Friendswood, TX). Total RNA was isolated as per the manufacturers instructions and dissolved in DEPC-treated water. First strand cDNA was synthesized using the RNA PCR Core Kit (Perkin Elmer, Foster City, CA) according to the manufacturers instructions. The amplification primers were as follows: 5'-AACGTGCTGGTCATCTGCGTGGTC-3', and 5'-GTAGTCCATTCTCATGACGGAAAC-3'. Primers were synthesized and HPLC-purified by Operon Technologies (Alameda, CA). PCR was performed for 33 cycles and consisted of denaturing at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 45 sec. PCR products from the A3 receptor reaction were reamplified using nested amplification primers, *i.e.* the 5' primer described above, and the additional 3' A3 primer 5'-CTGCAGACCACCACCTTCTATTTC-3'. PCR conditions were not changed

for the reamplification. Reaction products were resolved on a 1% agarose gel, and visualized with ethidium bromide under UV light illumination.

2.2.5 BPI ELISA

BPI concentrations were determined using a sandwich-ELISA as described previously (24). In short, 96-well plates (Immuno-Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with human BPI-specific mAb 4E3, and free sites were blocked with PBS-1% BSA. Wash and dilution buffer consisted of 80mM MgCl₂, 50mM TrisHCl pH7.4, 150mM NaCl, 0.1% BSA, and 0.05% Tween 20. The plates were washed 5 times after each incubation step. Human rBPI (a kind gift from Dr. M. Marra, Incyte, Palo Alto, CA) was used for standard titration curves. Test samples, diluted in assay buffer, were added to the plates and incubated for 2 h at room temperature. Next, 5 µg/ml biotinylated polyclonal rabbit anti-human BPI IgG was added as detection antibody and incubated for 1 h at room temperature, followed by a 1-h incubation period with peroxidase-conjugated streptavidin. Finally, the plates were washed with distilled water containing 0.1% Tween 20, and 3,3',5,5'-tetramethylbenzidine (TMB) was added as substrate for peroxidase. Photospectrometry at 450 nm was performed using a micro-ELISA autoreader. The detection limit of the assay was 200 pg/ml.

2.2.6 ELASTASE ELISA

Human neutrophil elastase (HNE) was detected with a sandwich-ELISA generously provided by Dr. P.S. Hiemstra, Department of Pulmonology, University Hospital Leiden, Leiden, The Netherlands. Briefly, microwells (Immulon 4; Dynatech Laboratories, Chantilly, VA) were coated overnight at room temperature with the IgG fraction of polyclonal rabbit anti-human elastase. HNE standard, purified from purulent sputum (25), as well as test samples were diluted in PBS containing 0.05% (v/v) Tween 20 and 1% (v/v) heat-inactivated newborn calf serum, and were incubated for 1 h at 37°C. Bound elastase was detected using biotinylated rabbit anti-human elastase IgG during a 1 h incubation period at 37°C. Next, peroxidase-conjugated streptavidin was incubated for 1 h at 37°C, followed by TMB. The lower detection limit of the assay was less than 0.4 ng/ml. Crossreactivity in this ELISA with highly purified preparations of the neutrophil proteinases cathepsin G and proteinase 3 was less than 0.3% and 0.01%, respectively (26).

2.2.7 DEFENSIN ELISA

Human defensins HNP 1-3 were determined by ELISA, as originally described by Panyutich *et al.* (27), with minor modifications. Microtiter plates (Nunc, Denmark) were coated overnight at 4°C with 4 µg/ml anti-HNP-1 mAb D1-1 in 0.1 M Na₂CO₃, pH 9.6. After washing the plates with 20 mM Tris-HCl, HNP standard and experimental samples were diluted in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4, containing 0.01% hexadecyltrimethylammonium bromide (TBS-CETAB), and incubated for 50 min at room temperature. Standard titration curves were made in plasma from 10 healthy donors, diluted to the same concentration as the experimental samples. Next, biotin-labeled anti-HNP mAb D1-11, diluted in TBS-CETAB, was added to the wells, and incubated for 40 min at room temperature, followed by a 40-min incubation period with streptavidin-peroxidase in TBS-CETAB. Finally, the plates were developed with TMB, and photospectrometry was performed at 450 nm.

2.2.8 STATISTICAL ANALYSIS

All data are presented as mean ± SEM. Statistical analysis was performed as indicated by Student *t*-test or by one-way analysis of variance, followed by Bonferroni's *t*-test when overall variation was significant. Differences were considered statistically significant at $p < 0.05$.

2.3 RESULTS

2.3.1 EFFECTS OF ADENOSINE ON NEUTROPHIL DEGRANULATION IN STIMULATED WHOLE BLOOD AND BY ISOLATED PMN

Control levels of BPI, HNE, and HNP in unstimulated whole blood incubated for 2 h, were 8.4 ± 0.6 ng/ml, 150 ± 20 ng/ml, and 150 ± 15 ng/ml, respectively (Figure 2.1). Incubation of whole blood with 10 ng/ml LPS for 2 h resulted in a 5-fold increase of BPI, a 6.5-fold increase of HNE, and a 9-fold increase of HNP compared to control levels. Similarly, stimulation of whole blood with 10 ng/ml TNFα potently induced release of BPI (55.7 ± 3.6 ng/ml), HNE ($2,040 \pm 360$ ng/ml), and HNP ($1,735 \pm 115$ ng/ml). LPS and TNFα were used as

activating agents since they represent physiological stimuli, and have been shown to be very potent and sensitive inducers of BPI and elastase¹.

Figure 2.1 Control levels of BPI, HNE, and HNP in unstimulated and stimulated whole blood

Whole blood was incubated with either 10 ng/ml LPS or 10 ng/ml TNFα, or without stimuli for 2 h at 37°C. At the end of the incubation period, supernatants were collected as described, and BPI (*open bars*), HNE (*hatched bars*), and HNP (*cross-hatched bars*) content were determined by specific ELISA. Results are shown as mean + SEM of four separate experiments.

When LPS- and TNFα-stimulated whole blood cultures were coincubated with a concentration range of adenosine, a dose-dependent inhibition of the release of BPI as well as HNE and HNP was observed (Figure 2.2). For both LPS- and TNFα-induced BPI, maximum inhibition was approximately 60-70%, whereas maximum inhibition of HNE was about 80%. Release of HNP induced by LPS or TNFα was also attenuated by adenosine in an identical fashion as described for BPI and HNE. In addition, adenosine dose-dependently inhibited release of BPI, HNE, and HNP by whole blood stimulated with other microbial agents, such as FMLP, and serum-treated zymosan (STZ), to a similar extent (Table 2.1).

¹Dentener MA, Francot GJM, Hiemstra PS, Tool ATJ, Verhoeven AJ, Vandenabeele P, Buurman WA. Bactericidal/permeability-increasing protein release in whole blood ex vivo: strong induction by lipopolysaccharide and tumor necrosis factor-α. *J Infect Dis* 175: 108-117, 1997.

Figure 2.2. Adenosine inhibits the release of BPI (○), HNE (●), and HNP (▲) in stimulated whole blood

Whole blood was incubated for 2 h at 37°C with either 10 ng/ml LPS (A), or 10 ng/ml TNFα (B) and a concentration range of 0-100 μM adenosine (Ado) in the presence of 100 μM EHNA. At the end of the incubation period, supernatants were collected as described, and BPI, HNE, and HNP were determined by specific ELISA. Results are shown as mean ± SEM of four separate experiments. Error bars are omitted if they fall within the symbol. All data presented differed significantly from control cultures incubated without adenosine ($p < 0.05$ by Student *t*-test).

Table 2.1 Inhibitory effects of adenosine on release of BPI and HNE in stimulated whole blood^a

Treatment	BPI		HNE	
	Maximum Inhibition (%) ^b	IC ₅₀ (μM) ^c	Maximum Inhibition (%)	IC ₅₀ (μM)
LPS	72.0 ± 6.6	20.2 ± 4.7	75.0 ± 10.3	14.2 ± 5.8
TNFα	71.5 ± 3.5	23.6 ± 5.1	71.0 ± 7.5	21.2 ± 2.9
FMLP	80.5 ± 3.3	26.9 ± 7.5	82.8 ± 0.7	26.5 ± 1.8
STZ	59.1 ± 5.6	24.5 ± 10.6	57.6 ± 19.3	17.6 ± 7.5

^a Whole blood was incubated for 2 h at 37°C with either 10 ng/ml LPS, 10 ng/ml TNFα, 10⁻⁷ M FMLP, or 100 μg/ml STZ, with or without a concentration range of 1-100 μM adenosine in the presence of 100 μM EHNA. Results are given as mean ± SEM of four separate experiments. All data presented differed significantly from stimulated control cultures incubated without adenosine ($p < 0.05$ by Student *t*-test). ^b Percentage inhibition was calculated as 100% minus experimental value as % of control. ^c IC₅₀, i.e. the concentration of adenosine producing 50% of maximal inhibition, was determined by extrapolation from dose-response curves of four separate experiments.

In comparison, the effects of adenosine on LPS-induced release of BPI and HNE by isolated PMN were determined (Table 2.2, *upper panel*). At a concentration of 10 μM adenosine, release of HNE by isolated PMN was reduced more potently than in whole blood (38.6 ± 2.2% of control vs. 77.4 ± 4.6% of control, respectively), whereas inhibition of BPI in whole blood and isolated PMN was not significantly different. At a concentration of 100 μM of adenosine however, reduction of BPI as well as HNE was identical in whole blood compared to isolated PMN. Release of BPI and HNE by isolated PMN stimulated with either FMLP, STZ, and PMA was attenuated in a similar fashion as described above for whole blood (data not shown).

2.3.2 EFFECTS OF ADENOSINE RECEPTOR-SPECIFIC AGONISTS ON NEUTROPHIL DEGRANULATION IN WHOLE BLOOD AND BY ISOLATED PMN

To determine the role of specific adenosine receptors in the observed inhibition of neutrophil degranulation, the effects of the non-metabolizable adenosine analogues CPA, 2-CAdo, and IB-MECA on stimulated release of BPI and HNE in whole blood were compared. CPA is a highly selective A1 receptor agonist (18, 28), whereas 2-CAdo binds to A1 and A3 receptors (18, 20), and at higher

micromolar concentrations also exerts A₂-mediated effects (6). IB-MECA has recently been described as a highly potent and selective A₃ receptor agonist *in vitro* and *in vivo* (29, 30).

Figure 2.3 Effects of adenosine receptor-specific agonists on release of BPI and HNE in stimulated whole blood

Whole blood was incubated for 2 h with either 10 ng/ml LPS (*A and B*) or 10 ng/ml TNFα (*C and D*), and a concentration range of either 1 -100 μM 2-CA do (○), CPA (●), or IB-MECA (▲). Results are shown as mean ± SEM of at least four different experiments. Error bars are omitted if they fall within the symbol. * indicates a statistically significant difference versus CPA, # indicates a statistically significant difference versus 2-CA do ($p < 0.05$ by one-way ANOVA). All data presented differed significantly from control cultures incubated without adenosine agonists.

As illustrated in Figures 2.3A and 2.3B, 2-CAdo attenuated LPS-induced BPI in whole blood somewhat more potently than CPA, although statistical significance was not reached. Except at a 10 μ M concentration, where 2-CAdo had a significantly stronger inhibitory effect, CPA was also approximately equipotent to 2-CAdo in reducing release of BPI induced by TNF α (Figures 2.3C and 2.3D). However, at concentrations of 10 μ M and higher, IB-MECA reduced LPS- as well as TNF α -induced BPI significantly stronger than CPA. At these concentrations, IB-MECA also reduced BPI more than 2-CAdo, but significance was not consistently reached. In contrast to the results obtained with stimulated release of BPI, 2-CAdo at concentrations of 10 μ M and higher was significantly more effective than CPA in inhibiting release of HNE after stimulation with either LPS or TNF α (Figure 2.3).

Table 2.2 Inhibitory effects of adenosine and adenosine receptor-specific agonists on release of BPI and HNE by LPS-stimulated PMN in whole blood and isolated PMN^a

	whole blood		isolated PMN	
	BPI	HNE	BPI	HNE
<i>Adenosine</i>				
10 μ M	86.3 $\pm$ 4.9	77.4 $\pm$ 4.6 [#]	61.0 $\pm$ 5.2	38.6 $\pm$ 2.2
100 μ M	40.7 $\pm$ 7.8	20.1 $\pm$ 1.5	33.6 $\pm$ 5.4	28.8 $\pm$ 4.7
<i>2-CAdo</i>				
10 μ M	42.0 $\pm$ 5.2	29.1 $\pm$ 4.6 [*]	60.1 $\pm$ 6.1 [*]	45.8 $\pm$ 6.9 [*]
100 μ M	34.0 $\pm$ 12.0	13.5 $\pm$ 1.9 [#]	45.8 $\pm$ 4.3	46.4 $\pm$ 2.5
<i>CPA</i>				
10 μ M	58.2 $\pm$ 5.1 [#]	64.7 $\pm$ 13.4	80.7 $\pm$ 5.9	70.8 $\pm$ 3.6
100 μ M	52.0 $\pm$ 17.0	24.1 $\pm$ 2.7 [#]	48.8 $\pm$ 10.9	60.4 $\pm$ 5.7

^aWhole blood or cytochalasin-B-pretreated isolated PMN in suspension were incubated for 2 h at 37°C with 10 ng/ml LPS with either adenosine, 2-CAdo or CPA at the indicated concentrations. Data are presented as % of release by control cultures stimulated with 10 ng/ml LPS without adenosine or receptor-specific agonists. All data presented differed significantly from stimulated control cultures incubated without adenosine or receptor-specific agonists ($p < 0.05$ by Student *t*-test). Results are given as mean $\pm$ SEM of six separate experiments. * indicates a statistically significant difference compared to effect of an equimolar concentration of CPA in the same group ($p < 0.05$ by oneway-ANOVA, followed by Bonferroni's *t*-test), # indicates a statistically significant difference compared with release by isolated PMN ($p < 0.05$ by Student *t*-test).

Similarly, IB-MECA attenuated stimulated HNE release much more potently than CPA. Compared with 2-CAdo, IB-MECA exerted a somewhat more potent inhibitory effect on HNE, but significance was not always reached. However, at concentrations of 1 and 10 μ M, a difference between IB-MECA and 2-CAdo with regard to inhibition of TNF α -induced HNE was indeed observed. Release of HNE was maximally reduced to approximately 10 to 15% of control by 2-CAdo, whereas release of BPI into the same supernatants was reduced maximally to approximately 35% of control. Maximum inhibition of either BPI or HNE by IB-MECA ranged from 2.5 to 7% of control. When the effects of CPA and 2-CAdo on LPS-induced BPI and HNE by isolated PMN were compared, a significant difference between the two agonists was observed (Table 2.2). At 10 μ M, 2-CAdo inhibited both BPI and HNE more potently than CPA.

2.3.3 EFFECTS OF ADENOSINE RECEPTOR-SPECIFIC ANTAGONISTS ON NEUTROPHIL DEGRANULATION IN STIMULATED WHOLE BLOOD

To further examine the role of specific adenosine receptors, the effects of the A1-selective antagonist DPCPX, the A2-selective antagonist DMPX, the non-selective A1/A2 receptor antagonist PST, and the A1/A3 receptor antagonist XAC on the observed inhibition of neutrophil degranulation by adenosine were determined (Figure 2.4). The inhibitory effects of adenosine on LPS- and TNF α -induced release of BPI were partially blocked by the A1/A3 antagonist XAC. Similarly, the A1/A2 antagonist PST partially reversed adenosine-mediated inhibition of BPI in whole blood stimulated with LPS (adenosine + PST: $52.5 \pm 3.5\%$ of control *vs.* adenosine alone: $31.2 \pm 4.4\%$ of control), and TNF α (adenosine + PST: $42.6 \pm 5.5\%$ of control *vs.* adenosine alone: $30.4 \pm 1.7\%$ of control). Moreover, while the A2-selective antagonist DMPX was equally effective to PST in blocking the effect of adenosine, the A1-selective antagonist DPCPX was completely without effect on adenosine-mediated inhibition of degranulation. Furthermore, the combination of XAC and PST did have an additive blocking effect on adenosine-mediated inhibition of TNF α -stimulated release of BPI (XAC + PST: $67.1 \pm 3.6\%$ of control *vs.* XAC alone: $53.3 \pm 4.8\%$ of control), whereas for LPS-induced BPI no additive effect of the combination of both antagonists compared with either antagonist alone was observed.

Figure 2.4 Effects of adenosine receptor-specific antagonists on inhibition of BPI release by adenosine in stimulated whole blood

Whole blood was preincubated for 15 min with 50 μ M of either DPCPX, DMPX, PST, XAC, or a combination of PST and XAC, and subsequently coincubated with 50 μ M adenosine (Ado) and either 10 ng/ml LPS (A), or 10 ng/ml TNF α (B) for 2 h at 37 $^{\circ}$ C. Control cultures were incubated with either LPS or TNF α alone. Results are shown as mean + SEM of three separate experiments with duplicate determinations. * indicates a statistically significant difference ($p < 0.05$ by Student *t*-test) compared with stimulated cultures incubated with adenosine without antagonists.

Figure 2.5 Effects of adenosine receptor-specific antagonists on inhibition of BPI release by 2-CAdo and IB-MECA in stimulated whole blood

Whole blood was preincubated for 15 min with 50 μ M of either PST, XAC, or a combination of both antagonists, and subsequently coincubated with either 50 μ M 2-CAdo or IB-MECA and either 10 ng/ml LPS (*A and B*), or 10 ng/ml TNF α (*C and D*) for 2 h at 37 $^{\circ}$ C. Control cultures were incubated with either LPS or TNF α alone. Results are shown as mean + SEM of three separate experiments with duplicate determinations. * indicates a statistically significant difference ($p < 0.05$ by Student *t*-test) compared with stimulated cultures incubated with 2-CAdo or IB-MECA without antagonists.

Next, the effects of PST and XAC on 2-CAdo- and IB-MECA-mediated inhibition of degranulation were assessed (Figure 2.5). The inhibitory effect of 2-CAdo on release of BPI in LPS-activated whole blood was largely abrogated by XAC (2-CAdo + XAC: $65.9 \pm 5.3\%$ of control vs. 2-CAdo alone: $37.3 \pm 3.3\%$ of control), and 2-CAdo-mediated inhibition of $\text{TNF}\alpha$ -induced BPI was almost completely reversed by XAC (2-CAdo + XAC: $85.6 \pm 6.5\%$ of control vs. 2-CAdo alone: $35.7 \pm 3.2\%$ of control). In contrast, PST did not affect the suppression of either LPS- or $\text{TNF}\alpha$ -induced release of BPI by 2-CAdo. In line with this, the combination of XAC and PST reversed the effects of 2-CAdo to a similar extent as XAC alone. Similar results were obtained with IB-MECA-mediated inhibition of stimulated BPI release, where XAC strongly antagonized its effect, whereas PST, either alone or combined with XAC, was completely without effect. The actions of PST and XAC on inhibition of HNE release by 2-CAdo and IB-MECA, were identical to those described here for release of BPI (data not shown).

Figure 2.6 Presence of A3 receptor message in human neutrophils

Neutrophils from two different donors were isolated, and PCR analysis was performed as described in *Materials and Methods*. Some neutrophil suspensions were treated with 10^{-7} M FMLP for 10 min prior to PCR analysis. *Lane 1*: A3 cDNA control plasmid. *Lane 2*: negative control (PCR without template). *Lane 3*: 4×10^6 resting PMN. *Lane 4*: 4×10^6 FMLP-stimulated PMN. *Lane 5*: 4×10^6 FMLP-stimulated PMN. *Lane 6*: 8×10^6 FMLP-stimulated PMN. *Lane 7*: 8×10^6 resting PMN. *Lane 8*: Size marker.

The finding that 2-CAdo and IB-MECA exert potent inhibitory effects on neutrophil degranulation, combined with the reversal of these effects by the A1/A3 antagonist XAC, strongly suggested the involvement of an A3 receptor on neutrophils in these observations. However, the presence of A3 receptors on human neutrophils had thus far not been demonstrated. Therefore, non-quantitative RT-PCR of A3 receptor mRNA was performed in human neutrophils, and confirmed that A3 receptor message was indeed present in these cells (Figure 2.6).

2.3.4 EFFECTS OF THE AK-INHIBITOR GP515

To evaluate the relevance of the results described above to the potential therapeutic benefits of adenosine-regulating agents, the effect of GP515 on neutrophil degranulation in the *ex vivo* whole blood model was tested. GP515 is a novel AK inhibitor, that has been proven effective in animal models of endotoxic (17) and hemorrhagic shock². Coincubation of LPS- or TNF α -stimulated whole blood with a concentration range of 1 to 100 μ M GP515 dose-dependently reduced release of BPI (Figure 2.7) and HNE (not shown), maximally to approximately 50% of control. To confirm that GP515 increased endogenous adenosine levels, adenosine concentrations in the culture supernatants were determined by HPLC. While in LPS-stimulated whole blood incubated without GP515 2.5 ± 0.4 μ M endogenous adenosine was present, coincubation of LPS-stimulated whole blood with 100 μ M GP515 enhanced endogenous adenosine levels significantly to 12.5 ± 0.7 μ M (Figure 2.8). Similarly, GP515 elevated adenosine concentrations in unstimulated whole blood to a comparable level of 13.6 ± 0.5 μ M (data not shown). To confirm that inhibition of degranulation by GP515 was indeed mediated by endogenous adenosine, the effects of coincubation with the adenosine receptor antagonists DMPX and XAC were determined. As depicted in Figure 2.9, the inhibitory actions of GP515 on LPS-induced BPI and HNE were significantly reduced by XAC, whereas DMPX had a mild, but significant, antagonizing effect on GP515-mediated inhibition of BPI, but not of HNE.

²Bauer C, Bouma MG, Herrmann I, Van den Wildenberg FAJM, Firestein GS, Marzi I, Buurman WA. The adenosine kinase inhibitor GP515 attenuates leukocyte-endothelial interactions in the liver after hemorrhagic shock and resuscitation in the rat. *Am J Physiol (Gastrointest Liver Physiol)*: In press.

Figure 2.7 Effect of the AK inhibitor GP515 on release of BPI in stimulated whole blood

Whole blood was incubated with either 10 ng/ml LPS (○) or 10 ng/ml TNF- α (●), and a concentration range of 1-100 μ M GP515 in the presence of 100 μ M EHNA for 2 h at 37°C. At the end of the incubation period, supernatants were collected, and BPI concentrations were determined by specific ELISA. Results are shown as mean $\pm$ SEM of three experiments with duplicate determinations. Error bars are omitted if they fall within the symbol. All data presented differed significantly from stimulated control cultures incubated without GP515 ($p < 0.05$ by Student t -test).

Figure 2.8 Effect of the AK inhibitor GP515 on purine levels in LPS-stimulated whole blood

Whole blood was stimulated with 10 ng/ml LPS either without (*open bars*) or with 100 μ M GP515 (*hatched bars*) in the presence of 100 μ M EHNA for 2 h at 37 °C. At the end of the incubation period, plasma samples were processed as described in *Materials and Methods*, and purine levels were determined by HPLC. Results are shown as mean + SEM of four different donors. * indicates a statistically significant difference compared with stimulated cultures incubated without GP515 ($p < 0.05$ by Student t -test).

Figure 2.9 Effects of adenosine receptor-specific antagonists on inhibition of release of BPI (A) and HNE (B) by GP515 in LPS-stimulated whole blood

Whole blood was preincubated for 15 min with 50 μ M of either DMPX or XAC, and subsequently coincubated with 100 μ M GP515 in the presence of 100 μ M EHNA, and 10 ng/ml LPS for 2 h at 37 °C. Control cultures were incubated with LPS alone. Results are shown as mean + SEM of three separate experiments with duplicate determinations. * indicates a statistically significant difference ($p < 0.05$ by Student *t*-test) compared with stimulated cultures incubated with GP515 without antagonists.

2.4 DISCUSSION

While there is a large body of evidence to show that adenosine, acting via A2 receptors, is a potent inhibitor of the neutrophil respiratory burst (4, 9, 21, 22), its reported effects on degranulation by isolated neutrophils, either adherent or in suspension, have been contradictory. Therefore, the present study was undertaken to investigate the effects of adenosine and its receptor-specific analogues on the release of neutrophil granule constituents in an *ex vivo* human whole blood system. As parameters of neutrophil degranulation, the release of the azurophilic granule constituents BPI, HNE, and HNP in response to LPS and TNF α was determined.

Here, we demonstrate that adenosine dose-dependently inhibited neutrophil degranulation in human whole blood activated with either LPS or TNF α , and that it attenuated the release of each of the three azurophilic granule proteins determined to approximately the same extent. Furthermore, the inhibitory effect of adenosine was of a comparable magnitude for two other inflammatory agents tested, *i.e.* FMLP and STZ, with IC₅₀ ranging from approximately 15 to 25 μ M. Our observation that degranulation, induced by agents that activate different signal transduction pathways, was inhibited by adenosine to a similar extent, suggests a final common inhibitory pathway. The reported stimulus-specificity for modulation of neutrophil oxygen free radical generation (23) may therefore not apply to neutrophil degranulation. In relation to this it must be noted that, in the present study and in that of McGarrity *et al.* (23), the concentrations of adenosine required to inhibit degranulation, are higher than those that effectively attenuate respiratory burst, which is consistent with reported differences in threshold effects of cAMP involved in regulation of neutrophil enzyme release and superoxide production (31).

The reported lack of effect of adenosine on degranulation by isolated PMN in previous *in vitro* studies (21-23) has been attributed to the use of cytochalasin-B to enhance secretion from neutrophils in suspension (32). On the other hand, it has been suggested that adherence of neutrophils to biological surfaces might be a prerequisite for the inhibitory actions of adenosine on both respiratory burst (33) as well as degranulation (32). We therefore selected LPS and TNF α as activating agents in our whole blood experiments, since they are very potent and selective inducers of azurophilic granule release in whole blood, without the requirement for cytochalasin-B or adherence as additional triggering signals, as was recently demonstrated by our group^(see footnote 1). However, in the present study, comparison of the inhibitory effects of adenosine on degranulation in whole blood and isolated, cytochalasin-B-pretreated PMN

in suspension, revealed no major differences. Likewise, the efficacy of 2-CAdo to reduce degranulation was largely identical in whole blood and isolated PMN. Thus, our data do not support the hypothesis that adherence or the use of cytochalasin-B is of critical importance when assessing the effects of adenosine or its analogues on neutrophil degranulation. In line with our observations, others have also demonstrated inhibitory effects of adenosine (34) and 2-CAdo (35) on exocytotic release of granule constituents in the presence of cytochalasin-B. The reason for these conflicting results with cytochalasin-B treated PMN is not clear, but may be related to other factors, such as PMN isolation procedure, agonist concentration, length of incubation, or intrinsic differences in PMN responsiveness.

The results obtained with the adenosine receptor-specific agonists and antagonists suggest that inhibition of degranulation is partly dependent on A₂-receptor activation as well as on A₃ receptor activation. The latter conclusion is based on the observation that IB-MECA, a highly selective A₃ agonist, as well as 2-CAdo, an agonist that binds to all of the adenosine receptor subtypes and has the greatest affinity for A₁ and A₃ receptors (18, 20), reduced degranulation, particularly of HNE, significantly stronger than CPA, a highly selective A₁-receptor agonist. Moreover, the mixed A₁/A₃ antagonist XAC strongly reduced IB-MECA- as well as 2-CAdo-mediated inhibition of LPS- and TNF α -induced degranulation, whereas the non-selective A₁/A₂ antagonist PST did not affect the inhibitory actions of both agonists on degranulation. Together, these observations, combined with the demonstrated presence of A₃ receptor message in human PMN, strongly suggest the involvement of A₃ receptor activation in suppression of neutrophil degranulation. However, A₂ receptor ligation also seems to be involved in this process, because the inhibitory effects of adenosine were partially reversed by the highly A₂ receptor-specific antagonist DMPX, and the A₁/A₂ receptor antagonist PST. Moreover, the highly selective A₁ antagonist DPCPX was completely without effect on adenosine-mediated inhibition of degranulation. In line with the suggested role for A₃ receptors, XAC also partially blocked the actions of adenosine.

The observed involvement of A₂ receptors in the inhibition of degranulation is consistent with the known A₂ receptor-mediated actions of adenosine on neutrophil superoxide generation and adhesion (4, 6-10). A₂ receptors are thought to couple unifocally to adenylate cyclase, with activation resulting in enhanced generation of intracellular cAMP (36). Enhancement of cAMP is therefore regarded as a major effector system of the anti-inflammatory actions of adenosine, although it is unlikely to be the sole mechanism by which its

inhibitory effects on neutrophil function are exerted (22, 37, 38). The reported inhibitory effects of cAMP-raising agents on neutrophil degranulation *in vitro* (32), and *in vivo* (39) support our observation that A2 receptors are, at least in part, involved in attenuation of degranulation.

The involvement of A3 receptor ligation in inhibition of human neutrophil degranulation, as observed in the present study, is in sharp contrast with reports that A3 receptor activation facilitates degranulation of mast cells in the rat (40, 41). Although the cell types are different, we have currently no explanation for these opposing effects of A3 receptor activation on degranulation between both species. In this context however, it is of importance to note that the A3 receptor exhibits unusually large differences in structure, tissue distribution and pharmacological properties between species (41), which severely hampers the extrapolation of the properties of this receptor between species. In line with this, Sajjadi *et al.* (42), describing an alternatively spliced rat A3 receptor variant, for which no human homologue could be identified, stated that the relevance of non-human models to human A3 function should be viewed with caution. The suggested intracellular mechanisms involved in A3 receptor-mediated responses in the rat include activation of phospholipase C with elevation of calcium and inositol 1,4,5-triphosphate levels (43), and generation of nitric oxide with subsequent elevation of cGMP (44). In contrast, in human macrophages A3-mediated inhibition of TNF α expression has recently been shown to be independent of phospholipase C activation (20), indeed illustrating the functional diversity of A3 receptors between species. Therefore, the underlying mechanisms involved in A3 receptor-mediated inhibition of human neutrophil degranulation have yet to be defined.

The results obtained with the AK inhibitor GP515 point to a further therapeutical benefit of this agent, and possibly other adenosine-regulating agents, in inflammatory disorders. The strategy underlying the use of adenosine-regulating agents is to increase endogenous adenosine levels at sites of inflammation, thus enhancing the beneficial effects of adenosine. Indeed, GP515 significantly enhanced endogenous adenosine levels in unstimulated and stimulated whole blood in this study, while previous studies have indicated that GP515 augments release of adenosine from resting endothelial cells *in vitro* (6), and enhances adenosine concentrations in cell free exudates in a murine air pouch model, using carrageenan as pro-inflammatory stimulant (45). While GP515 did augment the concentration of endogenous adenosine, the corresponding level of inhibition of degranulation was higher than the degree of inhibition obtained with a similar concentration of exogenous adenosine. However, since it has been postulated that endogenous adenosine modulates

cellular function by interacting with its receptors in an autocrine or paracrine manner (36), it is conceivable that lower concentrations of endogenous adenosine, compared with the amount of exogenously added adenosine, are required to obtain a similar degree of inhibition. The observation that the inhibitory effects of GP515 were antagonized by adenosine receptor antagonists, further supports the contention that its effects are indeed mediated by endogenous adenosine.

In conclusion, we report here that adenosine at micromolar concentrations and the AK inhibitor GP515 inhibit *ex vivo* human neutrophil degranulation, partly via A2- and A3 receptor-mediated mechanisms. As mentioned earlier, release of preformed granule-stored proteins is an important feature of neutrophil activation, contributing to neutrophil-mediated tissue injury in a variety of infectious and inflammatory conditions. Next to the inhibitory effects of adenosine on cytokine release (16, 17, 46, 47), expression of endothelial adhesion molecules (47), neutrophil adhesion (6, 7, 10) and superoxide anion generation (9, 21), these findings may therefore represent an additional mechanism by which adenosine exerts its anti-inflammatory actions, and add to the therapeutic potential of adenosine-regulating agents towards neutrophil-mediated tissue injury in inflammatory conditions, such as sepsis and ischemia-reperfusion.

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CHAPTER 3

Differential Regulatory Effects of Adenosine on Cytokine Release by Activated Human Monocytes

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ABSTRACT

Adenosine is an endogenous nucleoside that can modulate the function of cells involved in the inflammatory response, such as polymorphonuclear granulocytes (PMN) and monocytes. Production and release of cytokines by activated mononuclear phagocytes is an important event in the pathogenesis of ischemia-reperfusion injury, a pathological phenomenon that is associated with excessive ATP-catabolism and subsequent local release of adenosine. The "retaliatory metabolite" adenosine has been shown to interfere with PMN function, thereby attenuating the deleterious consequences of ischemia and reperfusion. In this study we demonstrate that adenosine inhibits the production of $\text{TNF}\alpha$, IL-6 and IL-8 by LPS-activated human monocytes with a differential potency. The A2 receptor-specific adenosine analogues 2-chloroadenosine (2-CAdo) and 5'-N-ethylcarboxamidoadenosine (NECA) were most effective in attenuating LPS-induced cytokine production, while the A1-selective adenosine analogue N⁶-cyclopentyladenosine (CPA) was less effective, indicating that inhibition of cytokine production by adenosine is primarily an A2 receptor-mediated event. The observed inhibitory effects were not restricted to endotoxin-induced cytokine production, since adenosine also inhibited $\text{TNF}\alpha$ production by monocytes stimulated with the proinflammatory cytokine IL-1 β . Again, 2-CAdo and NECA reduced IL-1 β -induced $\text{TNF}\alpha$ production more potently than CPA. In contrast, adenosine enhanced production of IL-6 and IL-8 by monocytes stimulated with IL-1 β . Furthermore, only 2-CAdo, but not NECA, strongly inhibited cytokine-induced IL-6 and IL-8 production. These results suggest an additional A2 receptor-mediated mechanism of "retaliatory" action of adenosine under pathological conditions, where cytokine production by activated mononuclear phagocytes is involved, such as ischemia-reperfusion injury and septic shock.

3.1 INTRODUCTION

Adenosine is a purine nucleoside, that is released extracellularly under normal circumstances at submicromolar concentrations after intracellular degradation of ATP (1). However, under conditions of metabolic stress, such as ischemia, a rapid and massive depletion of intracellular ATP occurs, eventually leading to accumulation of AMP, which in turn is degraded to adenosine. During ischemia and subsequent reperfusion, adenosine and its physiologically inactive metabolites inosine and hypoxanthine, are released locally in the micromolar

concentration range (2-4). Through binding to specific receptors (5), adenosine exerts many modulating effects on various physiological systems, such as the cardiovascular system and the immune system (6, 7).

The most prominent cardiovascular action of adenosine is its vasodilating effect, presumably mediated by binding to A2 receptors on vascular smooth muscle, thereby increasing local blood flow in various tissues like heart, brain and skeletal muscle, during ischemia or enhanced physical activity (7, 8). Due to this beneficial effect of adenosine under metabolically unfavorable circumstances, which induce its formation by excessive ATP catabolism, it has been termed a "retaliatory metabolite" (9).

The known immunomodulatory actions of adenosine in inflammatory processes are to a large extent mediated through its effects on leukocyte function. Adenosine inhibits, through occupancy of A2 receptors, superoxide anion generation by activated PMN *in vitro* (10-12). Furthermore, adenosine and the adenosine analogue 3-deazaadenosine have been shown to interfere with adherence of PMN to the vascular endothelium (13-16), a crucial event in acute inflammatory processes. There is evidence that inhibition of leukocyte-endothelium interaction also occurs via A2 receptor activation (15). In contrast, occupancy of the adenosine A1 receptor has been shown to promote neutrophil chemotaxis (1, 17), and adherence to endothelium or gelatin-coated surfaces (18), thereby exerting a pro-inflammatory effect. Finally, it was reported recently that adenosine in the micromolar range acting via A2 receptors inhibits upregulation of CD11b/CD18 expression on FMLP-stimulated neutrophils (19). By virtue of its vasodilating action as well as its A2 receptor-mediated anti-inflammatory effects on PMN, the key mediators of tissue injury in acute inflammatory conditions, adenosine has been recognized as an important endogenous substance that protects against the deleterious consequences of ischemia-reperfusion (13, 20-23).

In analogy to the described actions of adenosine on PMN function, similar modulatory effects on the phagocytic function of mononuclear phagocytes have been described. Ligation of A1 receptors by picomolar concentrations of adenosine is pro-inflammatory as a result of enhanced Fc γ receptor-mediated phagocytosis, whereas A2 receptor ligation by adenosine at micromolar concentrations inhibits Fc γ receptor-mediated phagocytosis, providing an anti-inflammatory effect (24).

Secretion of inflammatory mediators, such as cytokines, is another important function of mononuclear phagocytes, that plays an essential regulatory role in inflammatory responses. Production and release of cytokines by endotoxin-activated mononuclear phagocytes is an important event in the development of

septic shock (25, 26). Furthermore, secretion of cytokines by activated mononuclear phagocytes has been implicated in the pathogenesis of ischemia-reperfusion injury, both in *in vitro* and *in vivo* experimental models (27-36). Given the concept of an adenosine-mediated general immunoregulatory feedback mechanism, as mentioned above, we hypothesized that adenosine might be an endogenous modulator of cytokine production and release by activated monocytes. Indeed, this hypothesis was supported by data recently reported by Parmely *et al.* (37), who found that adenosine selectively inhibits TNF α release by LPS-activated mouse peritoneal macrophages and mouse macrophage-like cell lines *in vitro*. The present study was undertaken to investigate the effects of adenosine on production and release of TNF α , IL-6 and IL-8 by activated human monocytes *in vitro*, and to assess the role of specific adenosine receptors in this context.

3.2 MATERIALS AND METHODS

3.2.1 REAGENTS

Adenosine, 2-chloroadenosine (2-CAdo), 2'-deoxyadenosine, CPA, NECA, and LPS (from *Escherichia Coli*, serotype 055:LB5, catalog nr. L-2880) were all obtained from Sigma Chemicals, Inc. (St. Louis, MO). Deoxycoryformycin (CN81,565, lot nr. R) was from Warner-Lambert Comp. (Ann Arbor, MI). R 88021 (Draflazine), the active L-enantiomer of the nucleoside transport inhibitor R 75231, was kindly provided by Janssen Pharmaceutica (Beerse, Belgium). Recombinant human TNF α was kindly provided by BASF/Knoll (Ludwigshafen, Germany). Recombinant human IL-1 β was a kind gift of Dr. S. Gillis (Immunex, Seattle, WA). RPMI 1640 was obtained from Gibco (Paisley, UK). BCS, purchased from Hyclone (Logan, UT), was heat-inactivated for 30 minutes at 56 °C and stored at 4 °C.

3.2.2 CELL ISOLATION AND STIMULATION

Buffy coats were kindly provided by the Red Cross Blood Bank Zuid Limburg, Maastricht, The Netherlands. Blood was donated by healthy volunteers, after informed consent for research was obtained. Human monocytes were isolated from buffy coats, essentially according to the method described by Graziano *et al.* (38). Briefly, mononuclear cell suspensions obtained after density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) were allowed to

clump by low speed centrifugation at 4 °C. Cell clumps, consisting for 80 - 95% of monocytes, were separated by sedimentation through ice-cold BCS resulting in cell suspensions consisting of more than 85% of monocytes. Monocytes were resuspended in RPMI 1640 supplemented with 10% BCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, plated on 24-well tissue culture plates (Costar, Cambridge, MA) at 1×10^6 cells per well to an end-volume of 1 ml and incubated at 37 °C. The cells were stimulated with either LPS (10 ng/ml), or rhIL-1 β (100 U/ml) for the indicated time periods, in the presence of adenosine or its analogues as indicated, after which the supernatants were harvested and stored at -20 °C until use in ELISA. In each experiment, the incubations with the various concentrations of adenosine or its analogues were performed at least in triplicate macrowells per concentration used. In experiments designed to investigate the effects of nucleoside transport inhibition, monocytes were preincubated with a concentration range of R 88021 for 5 minutes at 37 °C and subsequently coincubated with the reagents as described above. Viability of the cells, as measured by trypan blue exclusion, was not significantly affected by any of the reagents used (data not shown).

3.2.3 CYTOKINE-ASSAYS

Cytokine concentrations in culture supernatants were determined using previously described sandwich-ELISA's for TNF α (39), IL-6 (40), and a newly developed ELISA for IL-8. In short, 96-well Immuno Maxisorp plates (Nunc, Roskilde, Danmark) were coated overnight at 4 °C with cytokine-specific murine mAb 61E71 (anti-TNF α), 5E1 (anti-IL-6) and a newly developed IL-8-specific murine mAb, designated HM5 (IgG₁). Recombinant hTNF α , rhIL-6 and rhIL-8 were used for standard titration curves. Test samples were added and incubated for 2 to 3 h at room temperature. Each experimental sample was assayed at least in duplicate. Polyclonal rabbit anti-human TNF α , anti-human IL-6 and biotinylated polyclonal rabbit anti-human IL-8 antibodies were incubated at room temperature for 1 h, followed by a 1 h incubation with peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA) for TNF α and IL-6, and peroxidase-labelled streptavidin for IL-8. Finally, 3, 3',5, 5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added as a substrate. The reaction was stopped after 15 minutes with 1M H₂SO₄ and photospectrometry was performed at 450 nm. The lower detection limits of the ELISA's were 1 pg/ml for TNF α , 10 pg/ml for IL-6 and 5 pg/ml for IL-8.

To exclude the possibility that the presence of adenosine or its tested analogues interfered with detection of cytokines in the ELISA's, cytokine-containing control samples were diluted in PBS-0.1% BSA with a 100 μ M concentration of adenosine or analogues, and cytokine recovery as compared to control samples diluted in PBS-0.1% BSA was determined. No interference with cytokine detection occurred in any of the ELISA's used (data not shown).

3.2.4 STATISTICAL ANALYSIS

All data are presented as mean values $\pm$ SEM. Differences between the various treatments were tested by one-way analysis of variance, followed by Bonferroni's *t*-test when overall variation was significant. Differences were considered statistically significant at $p < 0.05$.

3.3 RESULTS

3.3.1 EFFECTS OF ADENOSINE ON LPS-INDUCED CYTOKINE RELEASE

Stimulation of monocytes with a concentration range of LPS for 6 h resulted in a concentration-dependent release of TNF α , IL-6 and IL-8. Coincubation with adenosine inhibited LPS-induced TNF α , IL-6 and IL-8 release in a dose-dependent manner (Figure 3.1 and Figure 3.2). During stimulation with 10 ng/ml LPS, the inhibitory activity of adenosine was most pronounced on TNF α -release, with maximum inhibition of 85 % (Table 3.1). Release of IL-6 and IL-8 was inhibited to a lesser extent with maximum inhibition of 67 % and 43 %, respectively. While IC₅₀, *i.e.* the concentration of adenosine producing 50 % of maximal inhibition, was 3 μ M for TNF α , IC₅₀ for IL-6 and IL-8 were 11 and 40 μ M, respectively, indicating a differential susceptibility to the inhibitory action of adenosine. The inhibitory effect was dependent on the LPS-concentration used, since even stronger inhibitory effects were seen on cytokine release by monocytes stimulated with 1 ng/ml LPS, with an almost complete abolishment of TNF α .

To determine whether the effect of adenosine was sustained over a longer period of time, monocytes were stimulated with LPS in the presence of adenosine during a 24 h incubation period. As shown in Figure 3.3, reduction of cytokine release was maintained throughout the 24 h incubation period, indicating that adenosine does not delay cytokine release, but actually attenuates the release of cytokines.

Figure 3.1 Adenosine inhibits $TNF\alpha$ (A), IL-6 (B), and IL-8 release (C) by LPS-stimulated human monocytes

Monocytes were stimulated with a concentration range of LPS in the absence (*open bars*) or presence of 1 (*hatched bars*), 10 (*double-hatched bars*) or 100 μM (*solid bars*) adenosine. After a 6 h incubation period, the supernatants were collected and cytokine release was determined by ELISA. The results of one experiment, representative of four experiments, are shown. Data are expressed as mean + SEM of four determinations. Error bars are omitted if they fall within the bars. * indicates a statistically significant difference compared to controls.

Figure 3.2 Inhibition of LPS-induced cytokine release by adenosine and receptor-specific adenosine analogues

Monocytes were incubated with LPS (10 ng/ml) and a concentration range of either adenosine (■), NECA (○), 2-CAdo (●), CPA (▽) or 2'-deoxyadenosine (□) for 6 h. Control cultures were incubated with LPS alone. The supernatants were collected and cytokine release was determined by ELISA. Control cytokine levels were 507 ± 46 pg/ml for TNF α , 139.3 ± 27.8 ng/ml for IL-6, and 181.4 ± 25.9 ng/ml for IL-8. Percentage inhibition was calculated as $100\% - \% \text{ of control}$. Results are shown as mean + SEM of four cultures. Error bars are omitted if they fall within the symbol. * indicates a statistically significant difference compared to adenosine.

Figure 3.3 Inhibition of LPS-induced TNF α (A), IL-6 (B) and IL-8 (C) by adenosine is maintained throughout a 24 h incubation period

Monocytes were incubated with LPS (10 ng/ml) in the absence ($\square$) or presence of 100 μ M adenosine ($\blacksquare$) for 24 h. At the indicated timepoints supernatants were collected and cytokine release was determined by specific ELISA. The results of one experiment, representative of three, are shown. Data are expressed as mean $\pm$ SEM of triplicate determinations. Error bars are omitted if they fall within the symbol.

Table 3.1 Inhibitory effects of adenosine on cytokine release by monocytes stimulated with LPS^a

cytokine	max.inhibition (%) ^b	IC ₅₀ (μM) ^c
TNFα	85.1 ± 2.8	3.3 ± 2.3
IL-6	67.2 ± 7.4	11.3 ± 9.6
IL-8	43.3 ± 6.1	40.0 ± 15.3

^a Monocytes were stimulated with LPS (10 ng/ml) in the absence (control) or presence of adenosine (0.1 - 200 μM). The supernatant was collected after an incubation period of 6 h and release of TNFα, IL-6 and IL-8 was determined by ELISA. Control cytokine levels were 480 ± 35 pg/ml for TNFα, 147.1 ± 29.3 ng/ml for IL-6, and 190.0 ± 40.6 ng/ml for IL-8.

^b Percentage inhibition was calculated as 100 % - (% of control). ^c IC₅₀ were determined by extrapolation from dose-response curves of four separate experiments. Data are expressed as mean ± SEM of four separate experiments.

3.3.2 EFFECTS OF ADENOSINE ON IL-1β-INDUCED CYTOKINE RELEASE

Next, to investigate whether the observed inhibition by adenosine was specific for endotoxin-induced cytokine release, or that adenosine might also act as an inhibitor of cytokine release by cytokine-activated monocytes, the effects of adenosine on IL-1β-induced cytokine release were determined. Adenosine also attenuated release of TNFα by monocytes activated with 100 U/ml IL-1β, but to a lesser extent than LPS-induced TNFα release (Figure 3.4). However, adenosine up to a concentration of 200 μM was ineffective in reducing IL-6 and IL-8 production after stimulation with IL-1β for 6 h. On the contrary, adenosine in a concentration of 10 μM and higher, significantly stimulated release of IL-6 ($p < 0.05$ vs. control), while no significant effects on IL-8 release were apparent. However, at 24 h the presence of 100 μM adenosine markedly enhanced IL-1β-induced IL-6 as well as IL-8 release (Figure 3.5). Similar results were obtained with monocytes stimulated with TNFα, where adenosine also enhanced the release of IL-6 and IL-8 during a 24 h incubation period (data not shown).

Figure 3.4 Modulation of IL-1 β -induced cytokine release by adenosine and receptor-specific adenosine analogues

Monocytes were incubated with rhIL-1 β (100 U/ml) and a concentration range of either adenosine (■), NECA (○), 2-CAdo (●) or CPA (▽) for 6 h. Control cultures were incubated with rhIL-1 β alone. The supernatants were collected and cytokine release was determined by ELISA. Control cytokine levels were 78 ± 25 pg/ml for TNF α , 16.3 ± 2.8 ng/ml for IL-6, and 34.8 ± 10.8 ng/ml for IL-8. Percentage inhibition was calculated as $100\% - \% \text{ of control}$. Results are shown as mean $\pm$ SEM of four cultures. Error bars are omitted if they fall within the symbol. * indicates a statistically significant difference compared to adenosine.

Figure 3.5 Adenosine enhances release of IL-6 (A) and IL-8 (B) after stimulation with IL-1 β

Monocytes were incubated with rhIL-1 β (100 U/ml) in the absence (□) or presence of 100 μ M adenosine for 24 h. At the indicated timepoints supernatants were collected and cytokine release was determined by ELISA. The results of one experiment, representative of three, are shown. Data are expressed as mean $\pm$ SEM of six determinations. Error bars are omitted if they fall within the symbol.

3.3.3 EFFECT OF A1 AND A2 RECEPTOR-SPECIFIC ADENOSINE ANALOGUES ON CYTOKINE RELEASE

After having established that adenosine affected cytokine release by stimulated monocytes, we investigated whether these effects could be related to occupancy

of specific adenosine receptors. Since establishing the potency order of receptor-specific agonists has become the preferred method for classification of adenosine receptors (19), we compared the inhibitory capacity of adenosine with that of its A1 and A2 analogues. The A2 receptor-specific agonist 2-CAdo exerted a much stronger inhibitory effect than adenosine on LPS-induced cytokine release (Figure 3.2). NECA, a weak A2-selective adenosine agonist (41), was equally effective as 2-chloroadenosine in attenuating TNF α release, but was less potent in reducing IL-6 and IL-8 release in response to endotoxin. However, NECA in a concentration range of 0.1 to 10 μ M still attenuated IL-6 and IL-8 release more potently than equimolar concentrations of adenosine. In contrast, the A1-specific agonist CPA was less effective than the A2 receptor agonists in attenuating LPS-induced cytokine release, while the naturally occurring adenosine analog 2'-deoxyadenosine, which is modified in the ribose moiety, was ineffective. The observed order of potency of adenosine and its analogues to inhibit cytokine release was 2-CAdo $\geq$ NECA $\geq$ adenosine $\geq$ CPA $>$ 2'-deoxyadenosine, which indicates that the inhibition is primarily an A2 receptor-mediated event. An almost similar order of potency was observed with inhibition of IL-1 β -induced TNF α release (Figure 3.4), although CPA at a concentration of 10 μ M and higher inhibited TNF α release more potently than adenosine. Contrary to these observations, only the A2 receptor agonist 2-CAdo was effective in reducing cytokine-induced IL-6 and IL-8 release. Like adenosine, NECA as well as CPA at concentrations of 10 μ M and higher significantly enhanced release of IL-1 β -induced IL-6 release ($p < 0.05$ vs. control). NECA and CPA also tended to increase cytokine-induced IL-8 release, but no statistically significant difference compared to controls was reached at any of the concentrations used (Figure 3.4).

Adenosine as well as 2'-deoxyadenosine are substrates for degradation to inosine or 2'-deoxyinosine by the cytosolic enzyme adenosine deaminase, whereas the synthetic receptor-specific analogues are not. Since endogenous adenosine deaminase might be induced in activated monocytes, an event which could hamper the comparison of the activity of adenosine or 2'-deoxyadenosine with that of its synthetic analogues, the effects of the adenosine deaminase inhibitor deoxycoformycin, on the observed actions of adenosine and 2'-deoxyadenosine on cytokine release were determined. Addition of 10 - 100 μ M deoxycoformycin did not significantly alter adenosine-mediated inhibition of LPS-induced TNF α release (Figure 3.6) and IL-1 β -induced TNF α release (data not shown), indicating that the activity of exogenously added adenosine is not substantially affected by endogenous adenosine deaminase. In line with these observations, inhibition of LPS-induced IL-6 and IL-8 was slightly, but non-

significantly, enhanced in the presence of deoxycoformycin, while potentiation of IL-1 β -induced IL-6 and IL-8 by exogenous adenosine was not altered by adenosine deaminase inhibition (data not shown). In the absence of adenosine or 2'-deoxyadenosine, 10 μ M deoxycoformycin inhibited release of LPS-induced TNF α by $23.8 \pm 14.9\%$, although statistical significance was not reached (Figure 3.6). Nevertheless, this finding suggests that there is endogenous adenosine activity, which, in absence of endogenous adenosine deaminase activity, results in attenuation of TNF α release. Finally, during inhibition of endogenous adenosine deaminase by deoxycoformycin, cytokine levels in the presence of 2'-deoxyadenosine did not differ from controls (data not shown), which confirms that, despite inhibition of its breakdown, 2'-deoxyadenosine has no effects on cytokine release by activated monocytes.

Figure 3.6 *Effects of inhibition of endogenous adenosine deaminase on TNF α release*

Monocytes were incubated with LPS (10 ng/ml) and 0 to 100 μ M adenosine in the absence (■) or presence of 10 (○) or 100 μ M (▽) deoxycoformycin for 6 h. The supernatants were collected and cytokine release was determined by ELISA. Data are expressed as mean + SEM of three cultures. Error bars are omitted if they fall within the symbol.

3.3.4 EFFECT OF NUCLEOSIDE UPTAKE INHIBITION

To test whether cellular uptake of adenosine by monocytes was required for the inhibitory effects on cytokine release, the nucleoside transport inhibitor R 88021 (Draflazine) was coincubated with adenosine and LPS. To this end, cells were preincubated with R 88021 for 5 minutes, and next adenosine and LPS were added. R 88021 is the active L-enantiomer of R 75231, a racemic mixture with a very specific and effective capacity to inhibit adenosine transport across cellular membranes (42). As shown in Figure 3.7, R 88021 did not affect inhibition of LPS-induced $\text{TNF}\alpha$ release by adenosine. These findings indicate that uptake of exogenously added adenosine by monocytes is not a prerequisite for the inhibitory effects described above.

Figure 3.7 Nucleoside uptake blockade does not affect inhibition of $\text{TNF}\alpha$ release by adenosine

Monocytes were preincubated for 5 min with the nucleoside transport inhibitor R 88021 at the indicated concentrations and subsequently coincubated with LPS (10 ng/ml) in the absence (■) or presence of either 10 (○), 100 (△) or 200 μM (□) adenosine for 6 h. The supernatants were collected and cytokine release was determined by ELISA. The results of one experiment, representative of four, are shown. Data are expressed as mean $\pm$ SEM of triplicate determinations. Error bars are omitted if they fall within the symbol.

3.4 DISCUSSION

Adenosine is an endogenous nucleoside that exerts a wide variety of biological effects via distinct cell-surface receptors (5, 6). Since formation and release of adenosine is closely linked to ATP catabolism during increased metabolic demands, Newby introduced the term "retaliatory metabolite" to describe the actions of adenosine under these circumstances (9). Adenosine has been shown to attenuate reperfusion injury following ischemia of the myocardium (21), brain (23), and spinal cord (20), while Marts *et al.* recently reported a protective effect of the adenosine analogue 2-CAdo on reperfusion injury of the lung (22). The protective actions of adenosine on the deleterious consequences of ischemia and reperfusion injury are considered to be mediated by several distinct mechanisms. Adenosine restores the ischemia-induced disturbance of energy metabolism (9). Furthermore, adenosine participates in the autoregulation of blood flow in various organs and tissues (7). It induces vasodilatation in heart, brain and skeletal muscle, thereby increasing oxygen supply during ischemia and counteracting the "no-reflow" phenomenon. Moreover, the anti-inflammatory role of adenosine during ischemia and reperfusion is of specific importance. Formation of oxygen free radicals by activated neutrophils and enhanced adhesion of neutrophils to endothelial cells are crucial events in the pathogenesis of reperfusion injury. Adenosine inhibits superoxide anion generation by activated neutrophils (10-12), and reduces neutrophil adherence to endothelial cells (13, 15), thereby providing protective anti-inflammatory effects on reperfusion injury.

In this study, we have shown that adenosine and A2 receptor-specific adenosine analogues inhibit the release of $\text{TNF}\alpha$, IL-6 and IL-8 by activated monocytes. The conclusion that adenosine, and not its metabolites, is responsible for these effects, is based on two observations. First, when deamination of exogenous adenosine to inosine by endogenous adenosine deaminase was prevented using the adenosine deaminase inhibitor deoxycoformycin, the effects on cytokine release were not altered. This strongly suggests that indeed adenosine and not its metabolite inosine is responsible for these actions. Second, to further confirm this, the effects of exogenous inosine on cytokine release were tested. Inosine, used in a concentration range of 1 to 200 μM , had no effects on either LPS- or IL-1 β -induced cytokine release (data not shown).

In addition to the already described actions on leukocyte function, the inhibitory effects of adenosine on cytokine release by activated monocytes may constitute another important mechanism of its anti-inflammatory action. Besides

neutrophils, mononuclear phagocytes also play an important role in acute inflammatory responses, mainly by producing and secreting inflammatory cytokines upon activation with specific stimuli. Excessive cytokine production by mononuclear phagocytes is associated with severe clinical conditions, such as septic shock and ischemia-reperfusion injury. Immuno-neutralization of TNF α has been shown to have protective effects in animal models of hepatic (31, 43), hind limb (27, 28), intestinal (32), pulmonary (29) and myocardial (30) ischemia-reperfusion injury. Likewise, a role for IL-6 has been suggested in hepatic (43), hindlimb (27) and myocardial (44) ischemia and reperfusion. Also, there is evidence that functional inhibition of IL-8 by specific antibodies prevents lung reperfusion injury (33, 34). Although in these *in vivo* studies the exact cellular sources of cytokine production under such conditions have not always been established with certainty, mononuclear phagocytes are very likely candidates. Indeed, several *in vitro* studies have indicated that anoxia or hypoxia followed by reoxygenation can directly stimulate monocytes and macrophages to release cytokines (45-48). Reported beneficial effects of experimental interventions with A2-specific adenosine analogues (22) or compounds that increase or prolong the biological availability of endogenously released adenosine at local sites of injury, such as acadesine (8, 49), nucleoside transport inhibitors (42) or adenosine deaminase inhibitors (50), might therefore be attributed, at least in part, to the inhibition of cytokine production and release. Whether, as a result of intervention with these substances, cytokine levels during ischemia and reperfusion are actually diminished by the described inhibitory actions of adenosine on mononuclear phagocytes, remains to be investigated.

We have demonstrated in this study that adenosine in the micromolar concentration range attenuates TNF α release and, to a lesser extent, also IL-6 and IL-8 release by endotoxin-stimulated human peripheral blood monocytes. This observation suggests that, under these conditions, adenosine acts via a common inhibitory pathway. Thus far, the inhibitory pathway of adenosine in PMN and mononuclear phagocytes has not been completely revealed. It has been established that adenosine, via A2 receptors, increases intracellular cAMP through stimulation of adenylate cyclase. Since LPS-induced expression of genes encoding for TNF α , as well as IL-6 and IL-8 is commonly controlled by the inducible transcription factor NF- κ B (51), it could be hypothesized that interference of adenosine with activation or mobilization of NF- κ B is responsible for the common inhibitory effects on LPS-induced cytokine-production, as observed in this study. Interestingly, Strieter *et al.* (52) reported that an increase in cAMP interferes with endotoxin-induced transcription of the

TNF α gene. However, cAMP-raising agents such as pentoxifylline and theophylline, selectively inhibit LPS-induced synthesis of TNF α , but do not affect the production of IL-6 *in vitro* (53). These observations make the existence of a cAMP-mediated common inhibitory pathway for TNF α and IL-6 doubtful. Therefore, the exact molecular mechanisms of action of adenosine involved in the observed specificity of cytokine-inhibition (TNF α > IL-6 > IL-8) remain to be elucidated.

Our finding that adenosine attenuates TNF α release by endotoxin-stimulated monocytes is supported by data recently reported by Parmeley *et al.* (37), who found selective inhibition of TNF α production by LPS-activated mouse peritoneal macrophages and mouse macrophage-like cell lines *in vitro*. In their experiments a 100 μ M concentration of adenosine inhibited TNF α production by more than 80% with IC₅₀ ranging from 1 to 10 μ M, findings which correspond well with our observations. These authors suggested that inhibition of TNF α production may be independent of specific adenosine receptor ligation, since the synthetic adenosine analogue MDL201112, which binds with only low affinity to classical A1 and A2 receptors, potently reduced LPS-induced TNF α -levels both *in vitro* and *in vivo*. These data however, do not preclude the possibility of inhibition of cytokine release by specific adenosine-receptors. In our hands, the A2 receptor-specific adenosine analogues 2-CAdo and NECA exerted stronger inhibitory effects on endotoxin-induced cytokine release than adenosine or the A1 receptor-specific agonist CPA, which suggests that this inhibition is primarily an A2 receptor-mediated event. The finding that the A1-selective agonist CPA inhibited TNF α release to the same extent as adenosine is compatible with non-specific A2 receptor binding of CPA at higher concentrations (41), and further substantiates that endotoxin-induced TNF α release is highly sensitive to A2 receptor-mediated inhibition.

In contrast with the broad inhibitory effects of adenosine on LPS-induced cytokine release, adenosine up to 200 μ M was ineffective in reducing IL-6 and IL-8 release by monocytes stimulated with IL-1 β . Moreover, we found that adenosine significantly increased IL-6 levels in the culture supernatants after 6 h of stimulation with IL-1 β . This potentiating effect was evident for IL-6 as well as IL-8 during a 24 h incubation period. Enhancement of IL-6 and IL-8 release was also found during stimulation of monocytes with TNF α (data not shown). These findings show that adenosine has opposing effects on the release of IL-6 and IL-8 by activated monocytes, depending on the specific stimulus used. In the same supernatants however, IL-1 β -induced TNF α release was inhibited by adenosine in a similar manner as TNF α release in response to endotoxin, albeit that maximal inhibition was only 60%. This discrepancy between the inhibitory

effects of adenosine on TNF α release on the one hand, and the potentiating effects on IL-6 and IL-8 release on the other hand, further argues against a common inhibitory mechanism.

The potency order of receptor-specific agonists with regard to inhibition of TNF α during stimulation with IL-1 β was almost identical to that observed with LPS-induced TNF α , although CPA at concentrations of 10 μ M and higher inhibited TNF α release even stronger than adenosine, which is consistent with the aforementioned non-specific A2 receptor ligation of CPA at higher concentrations. The A2-specific agonist 2-CAdo strongly inhibited IL-1 β -induced IL-6 and IL-8, whereas the weaker A2-specific agonist NECA as well as the A1-selective agonist CPA potentiated the release of these cytokines. Since equivalent concentrations of CPA had similar effects as NECA, being approximately equipotent at A1 and A2 receptors, the potentiating effects of NECA can not be ascribed to A2 receptor-mediated processes (41). It is tempting to speculate that enhancement of IL-1 β -induced cytokine release may be a result of A1 receptor binding, but further study is warranted to substantiate this.

Finally, our observation that a synthetic A2-specific adenosine analogue, such as 2-CAdo, inhibited endotoxin-induced cytokine production more potently than the A1-specific agonist CPA, coupled to the fact that adenosine uptake blockade did not affect this inhibition, suggests that freshly isolated monocytes express functional A2 receptors on their surface membrane. The expression of both adenosine A1 and A2 receptors has been reported however, to be a characteristic of macrophages, derived from *in vitro* culture of human peripheral blood monocytes (24, 54). These studies focused on the inhibitory effects of adenosine on phagocytic function of mononuclear phagocytes. Contrary to these observations however, but in agreement with the data we present here, Baker *et al.* (55) demonstrated that human peripheral blood monocytes were sensitive to the inhibitory effects of adenosine and NECA on zymosan-induced release of thromboxane and lysozymal enzymes, suggesting the presence of functional A2 receptors on freshly isolated human monocytes.

In conclusion, we have shown that adenosine in micromolar concentrations strongly inhibits TNF α release by activated monocytes. Release of IL-6 and IL-8 can be either inhibited or potentiated by adenosine, depending on the stimulus. Furthermore, our data strongly indicate that specific A2 receptor ligation by 2-CAdo potently reduces the release of TNF α , IL-6 and IL-8 by monocytes, stimulated with either LPS or the inflammatory cytokines IL-1 β and TNF α .

This modulating effect on cytokine release is consistent with the already established role of adenosine as a physiological endogenous regulator of leukocyte function. In this study a consistent, albeit relatively small, effect of endogenous adenosine on cytokine release by activated monocytes was observed, when its breakdown by adenosine deaminase was inhibited with deoxycytosine. The modulatory effects of endogenous adenosine were comparable to the effects of approximately 1 μ M of exogenous adenosine, a concentration that resembles levels found *in vivo* (2-4).

Based on the observations described above, we hypothesize that, specifically during ischemia-reperfusion, adenosine may act to attenuate injury by inhibiting cytokine release. In this context, adenosine might be a component of the normal host defense in mild ischemic foci. In more severe ischemic events, when local release of endogenous adenosine might be insufficient to prevent tissue damage, the use of A2-selective adenosine agonists or substances that retain endogenously released adenosine at local sites of injury could potentiate the inhibitory effects of adenosine on cytokine release.

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CHAPTER 4

Adenosine Inhibits Cytokine Release and Expression of Adhesion Molecules by Activated Human Endothelial Cells

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ABSTRACT

Ischemia induces excessive ATP catabolism with subsequent local release of its metabolite adenosine, an autacoid with anti-inflammatory properties. Since activation of the vascular endothelium is critical to the inflammatory host response during ischemia and reperfusion, the effects of adenosine on two major determinants of endothelial cell activation, *i.e.* the release of pro-inflammatory cytokines and the expression of adhesion molecules, were studied. Adenosine dose-dependently inhibited the release of IL-6 and IL-8 by stimulated human umbilical vein endothelial cells (HUVEC). Expression of E-selectin and VCAM-1, but not ICAM-1, by activated HUVEC was also reduced by adenosine. Inhibition of endogenous adenosine deaminase activity by EHNA or 2'-deoxycoformycin strongly enhanced the inhibitory effects of exogenous adenosine on cytokine release and expression of E-selectin and VCAM-1. However, a clear role for specific adenosine-receptors in the described inhibitory events could not be established. Taken together, these data imply that the vascular endothelium constitutes an important target for the anti-inflammatory actions of adenosine.

4.1 INTRODUCTION

During recent years it has been recognized that the physiological functions of the vascular endothelium extend well beyond its passive primary barrier function between the bloodstream and tissue. It is now known that the endothelium is an active "organ", that plays important regulatory roles in a variety of physiological homeostatic processes, such as inflammation, coagulation and regulation of vascular tone (1).

In the early stages of inflammation, pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF α), as well as bacterial endotoxin can activate endothelial cells to produce a wide array of vasoactive, thrombogenic and inflammatory mediators (for review see Ref. 2). Amongst the inflammatory mediators elaborated by endothelial cells upon stimulation are the pleiotropic cytokines IL-1 and IL-6 as well as the chemokine IL-8 (3). Moreover, stimulation of endothelial cells by cytokines or endotoxin results in *de novo* expression or upregulation of different classes of adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on their cell surface (4). Endothelial cell expression of adhesion molecules interacting with counterstructures on

leukocytes is essential in the development of an inflammatory response. Thus, as a result of endothelial cell activation, leukocytes are attracted to local sites of inflammation, become activated, adhere to the endothelium and finally transmigrate into the target tissue. Since chemotaxis and subsequent adherence of circulating leukocytes to the vascular endothelium are crucial events in the development of inflammatory responses, they constitute important possible targets for therapeutical intervention in a variety of inflammatory disorders.

Adenosine is an endogenous intermediate of purine nucleotide metabolism with anti-inflammatory properties. Particularly during ischemia, local tissue levels of adenosine rise, as a consequence of ATP catabolism. Several *in vivo* studies have indicated that adenosine has protective effects on tissue injury following reperfusion after a preceding period of ischemia. In this context, Newby has introduced the term "retaliatory metabolite" to describe the actions of adenosine during ischemia and reperfusion (5).

The anti-inflammatory actions of adenosine have been ascribed mainly to its modulatory effects on neutrophil function. In various *in vitro* experimental models, adenosine has been shown to affect neutrophil superoxide generation (6). Furthermore, adenosine interferes with neutrophil-endothelial adhesion (7-10), but it is still controversial whether the anti-adhesive activity of adenosine is exerted through its actions on polymorphonuclear leukocytes (PMN) alone, or that it may also result from its effects on endothelial cells. Finally, adenosine interferes with the phagocytic function and with the release of inflammatory mediators by mononuclear phagocytes (11-13). Recently, we and others reported that adenosine affects cytokine release by activated mononuclear phagocytes (14, 15).

In view of the important role of the vascular endothelium in the inflammatory host response, particularly in ischemia-reperfusion injury, we investigated the effects of adenosine on *in vitro* endothelial cell activation, as determined by the release of cytokines and the expression of adhesion molecules.

4.2 MATERIALS AND METHODS

4.2.1 REAGENTS

Adenosine, 2-chloroadenosine (2-CAdo), *N*⁶-cyclopentyladenosine (CPA), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), inosine, hypoxanthine and LPS (from *Escherichia coli*, serotype 055:LB5, catalog no. L-2880) were all

obtained from Sigma Chemical Co. (St. Louis, MO). 2'Deoxycoformycin (dCF; CN81,565, lot no. R) was obtained from Warner-Lambert Co. (Ann Arbor, MI). Recombinant human TNF α was kindly provided by BASF/Knoll (Ludwigshafen, Germany). Recombinant human IL-1 β was a kind gift of Dr. S. Gillis (Immunex, Seattle, WA). RPMI 1640 was obtained from Life Technologies, Inc. (Paisley, UK). Bovine calf serum (BCS), purchased from HyClone Laboratories (Logan, UT), was heat-inactivated for 30 min at 56°C and stored at 4°C. Human serum (HS), obtained from healthy blood donors at the Red Cross Blood Bank Zuid Limburg (Maastricht, The Netherlands), after informed consent for research was given, was pooled, heat-inactivated, and stored at 4°C.

4.2.2 ENDOTHELIAL CELL CULTURE

HUVEC were isolated from fresh human umbilical cords by collagenase treatment and were seeded into fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue-culture flasks (Costar Corp., Cambridge, MA) in complete medium consisting of RPMI 1640 supplemented with 50 μ g/ml heparine (Sigma), 2 mM L-glutamine, 100 IU penicillin and 100 μ g/ml streptomycin, 30 μ g/ml Endothelial Cell Growth Supplement (Collaborative Research Inc., Bedford, MA), 10% BCS and 10% HS. Endothelial cells were characterized by their pavement-like monolayer morphology, by positive staining with mAb hec7 (generously provided by Dr. W.A. Muller, Rockefeller University, NY) directed against PECAM-1 (16), and by positive staining with anti-E-selectin mAb ENA-1 (17) after 4 hours of incubation with TNF α . Cultures never contained more than 1% non-endothelial cells.

For experiments, HUVEC passage 2-3 were plated onto 24-well or 96-well tissue culture plates (Costar Corp., Cambridge, MA) in complete media. When monolayers were confluent, the cells were rinsed once with RPMI-3% BCS and incubated with the various reagents as indicated in RPMI-10% BCS supplemented with glutamine and antibiotics for the indicated time periods. In all experiments, adenosine and its analogues were added simultaneously with either IL-1 β , TNF α or LPS. In experiments designed to test the effects of adenosine deaminase inhibition, cells were preincubated with either EHNA or dCF for 15 min and subsequently coincubated with the reagents as indicated. At the end of the indicated incubation periods, supernatants were collected and stored at -20 °C until determination of cytokine levels by ELISA. For determination of purine levels by High Performance Liquid Chromatography,

supernatants were deproteinized with 0.4 M perchloric acid on ice, centrifuged at 1200 rpm for 10 min at 4°C, and neutralized with 0.4 M K₂HPO₄. Samples were kept at -70 °C until determination of purine levels.

Viability of the cells was determined by cellular morphology on microscopic examination as well as by trypan blue exclusion. Cytotoxicity of the various treatments with adenosine or its analogues was assessed by methylthiazol-tetrazolium (MTT) test (18).

4.2.3 CYTOKINE ASSAYS

Cytokine concentrations in culture supernatants were determined using previously described sandwich-ELISA's for IL-6 (19) and IL-8 (14). In short, 96-well Immuno Maxisorp plates (Nunc, Inc., Roskilde, Denmark) were coated overnight at 4 °C with cytokine-specific murine mAbs 5E1 (anti-IL-6) and HM5 (anti-IL-8). Recombinant human IL-6 and rhIL-8 were used for standard titration curves. Test samples were added and incubated for 2 to 3 h at room temperature. Each experimental sample was assayed at least in duplicate. Polyclonal rabbit anti-human IL-6 and biotinylated polyclonal rabbit anti-human IL-8 Abs were incubated at room temperature for 1 h, followed by a 1-h incubation with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for IL-6, and peroxidase-labelled streptavidin for IL-8. Finally, 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, MD) was added as substrate. The reaction was stopped after 15 min with 1M H₂SO₄ and photospectrometry was performed at 450 nm. The lower detection limits of the ELISA's were 10 pg/ml for IL-6 and 5 pg/ml for IL-8. To exclude the possibility that the presence of adenosine or its tested analogues interfered with detection of cytokines in the ELISA's, cytokine-containing control samples were diluted in PBS-0.1% BSA with a 100 µM concentration of adenosine or analogues, and cytokine recovery as compared to control samples diluted in PBS-0.1% BSA was determined. No interference with cytokine detection occurred in any of the ELISA's used (data not shown).

4.2.4 EXPRESSION OF ADHESION MOLECULES

HUVEC passage 2-3 were plated onto fibronectin-coated 96-well flat-bottom cell culture plates (Costar Corp., Cambridge, MA) in complete medium two days prior to experiments. Confluent monolayers were incubated for the indicated time periods with the various reagents as described above in RPMI-10% BCS supplemented with glutamine and antibiotics. The cells were rinsed

three times with PBS supplemented with calcium and magnesium, and fixed with 0.025% glutaraldehyde for 10 min at room temperature. Finally, the fixed monolayers were rinsed five times with PBS, the wells were incubated with 1% bovine serum albumin in PBS, and kept at 4°C until use in ELISA. Cell-surface expression of E-selectin, ICAM-1 and VCAM-1 was detected by incubation for 2 h at room temperature with mAbs ENA-1 (anti-E-selectin) (20), RR-1 (anti-ICAM-1, a kind gift of Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT) and 1G11 (anti-VCAM-1, a kind gift of Dr. D.O. Haskard, Hammersmith Hospital, Department of Rheumatology, London, U.K.) (20) respectively, followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h. TMB was added as substrate, and the reaction was stopped after 15 min with 1M H₂SO₄. Photospectrometry was performed at 450 nm.

4.2.5 STATISTICAL ANALYSIS

All data are presented as mean $\pm$ SEM. Differences between the various treatments were tested as indicated by Wilcoxon signed rank test, Student *t*-test or by one-way analysis of variance, followed by Bonferroni's *t*-test when overall variation was significant. Differences were considered to be statistically significant at $p < 0.05$.

4.3 RESULTS

4.3.1 EFFECTS OF ADENOSINE ON CYTOKINE RELEASE

Stimulation of HUVEC with concentration ranges of either IL-1 β (1-100 U/ml), TNF α (0.1-100 ng/ml) or LPS (1-100 ng/ml) induced a dose-dependent release of IL-6 and IL-8 (data not shown). Coincubation of these cultures with a 100 μ M concentration of adenosine resulted in inhibition of the release of both cytokines, the rate of which was dependent on the concentration of the specific stimulus used (Figure 4.1). When HUVEC were activated with a low dose of either IL-1 β (1 U/ml), TNF α (0.1 ng/ml) or LPS (1 ng/ml), 100 μ M adenosine did not significantly alter release of IL-6 as compared to control cultures stimulated in the absence of adenosine. Similarly, no significant effect of adenosine on release of IL-8 was observed during activation with low doses of the pro-inflammatory stimuli.

Figure 4.1 Inhibitory effects of adenosine on cytokine release are dependent on the concentration of the stimulus

HUVEC were activated with concentration ranges of rhIL-1 β (A), rhTNF α (B), or LPS (C) in the presence of 100 μ M adenosine. Control cultures were incubated with rhIL-1 β , rhTNF α or LPS alone. After a 5-h incubation period, the supernatants were collected and release of IL-6 (open bars) and IL-8 (hatched bars) was determined by ELISA. Percentage inhibition was calculated as 100% - (experimental value as % of control). Results are shown as mean + SEM of at least four separate experiments. * indicates a statistically significant difference compared to activation with the lowest concentration of the specific stimulus used ($p < 0.05$ by Student *t*-test).

However, when HUVEC were stimulated with higher concentrations of IL-1 β (10 U/ml), TNF α (1 and 10 ng/ml), and LPS (10 ng/ml), a significant inhibition of IL-6 by approximately 20% was observed in the presence of 100 μ M adenosine, whereas release of IL-8 was inhibited by about 10 to 20% under these conditions, depending on the stimulus used. Finally, inhibition by 100 μ M adenosine of cytokine release by maximally activated HUVEC, ranged from $29.3 \pm 5.3\%$ to $42.2 \pm 4.7\%$ for IL-6, and from $17.4 \pm 2.4\%$ to $27.2 \pm 6.3\%$ for IL-8.

After having established that the effects of adenosine on cytokine release were most pronounced when HUVEC were maximally activated, we determined the effects of a 10 to 250- μ M concentration range of adenosine under such conditions (Figures 4.2A and 4.2B, *solid circles*). During coincubation with 10 μ M adenosine, no significant effects on the release of IL-6 and IL-8 by IL-1 β -activated HUVEC occurred as compared to control cultures without adenosine. While 10 μ M adenosine was also without effect on LPS-induced IL-8 release, it significantly increased IL-6 release by $15.1 \pm 5.3\%$ during stimulation with endotoxin ($p < 0.05$ vs. LPS-treated controls without adenosine by Wilcoxon signed rank test). However, at higher concentrations adenosine dose-dependently reduced release of both cytokines. At the highest concentration of adenosine used, the release of IL-6 was inhibited by approximately 50%, whereas release of IL-8 by IL-1 β - and LPS-activated HUVEC was diminished by $29.9 \pm 4.3\%$ and $16.0 \pm 5.7\%$, respectively. Pretreatment with 100 μ M of adenosine did not enhance the inhibitory effects on cytokine release by activated HUVEC in comparison with the effects observed when cells were cotreated with adenosine during activation by inflammatory stimuli (data not shown). To rule out the possibility that the observed inhibitory effects were due to cellular toxicity of adenosine, particularly at higher concentrations, viability was tested in several ways. Coincubation with adenosine up to 250 μ M did not alter the normal cobblestone appearance of the activated endothelial monolayers, nor did it affect trypan blue exclusion (data not shown). Furthermore, cellular integrity was maintained in the presence of 250 μ M adenosine as determined by MTT test. Because endothelial cells are a rich source of the enzyme adenosine deaminase (21, 22), which degrades adenosine into its metabolite inosine, we investigated whether the observed inhibitory effects of adenosine were limited by endogenous adenosine deaminase activity. To this end, two inhibitors of adenosine deaminase, EHNA and dCF, were used.

Figure 4.2 Effects of adenosine and receptor-specific adenosine analogues on rhIL-1 β - (A and B) and LPS-induced (C and D) cytokine release

HUVEC were incubated with rhIL-1 β (100 U/ml) or LPS (100 ng/ml) and a concentration range of either adenosine (●), 2-CA do (○), or CPA (▽) for 5 h. Control cultures were incubated with rhIL-1 β or LPS alone. The supernatants were collected and cytokine release was determined by ELISA. Control levels for rhIL-1 β -induced cytokine release were 21.4 ± 1.8 ng/ml for IL-6 and 26.5 ± 2.1 ng/ml for IL-8. Control levels for LPS-induced cytokine release were 2.6 ± 0.2 ng/ml for IL-6 and 6.4 ± 1.7 ng/ml for IL-8. Percentage inhibition was calculated as $100\% - (\text{experimental value as \% of control})$. Results are shown as mean $\pm$ SEM of five cultures. Error bars are omitted if they fall within the symbol. * indicates a statistically significant difference compared with adenosine, # indicates a statistically significant difference compared with CPA ($p < 0.05$ by one-way ANOVA).

Table 4.1 Effects of ADA-inhibitors on cytokine release by activated HUVEC^a

Treatment	IL-6 (% Inhibition) ^b		IL-8 (% Inhibition)	
	Control	100 μ M Ado	Control	100 μ M Ado
IL-1 β	–	40.5 $\pm$ 2.6*	–	21.6 $\pm$ 6.3*
IL-1 β + EHNA	39.1 $\pm$ 2.3*	83.3 $\pm$ 1.9*#	33.8 $\pm$ 1.3*	77.4 $\pm$ 1.2*#
IL-1 β + dCF	23.6 $\pm$ 10.9	81.0 $\pm$ 1.6*#	12.3 $\pm$ 4.8	72.9 $\pm$ 2.6*#
TNF α	–	27.6 $\pm$ 4.1*	–	23.7 $\pm$ 5.3*
TNF α + EHNA	20.5 $\pm$ 4.5*	69.1 $\pm$ 12.5*#	15.8 $\pm$ 7.5	72.5 $\pm$ 2.4*#
TNF α + dCF	10.3 $\pm$ 5.1	68.2 $\pm$ 16.7*#	15.0 $\pm$ 4.6	61.9 $\pm$ 12.3*#

^aHUVEC were stimulated with rhIL-1 β (100 U/ml) or rhTNF α (100 ng/ml) in the absence (control) or presence of 100 μ M adenosine (Ado) with or without 10 μ M EHNA or 10 μ M dCF. After an incubation period of 20 h, supernatants were collected and release of IL-6 and IL-8 was determined by ELISA. Control levels for rhIL-1 β -induced cytokine release were 281.4 $\pm$ 14.4 ng/ml for IL-6 and 593.6 $\pm$ 63.4 ng/ml for IL-8. Control levels for rhTNF α -induced cytokine release were 17.6 $\pm$ 1.5 ng/ml for IL-6 and 311.0 $\pm$ 42.3 ng/ml for IL-8.

^bPercentage inhibition was calculated as 100% - (experimental value as % of control). Data are expressed as mean $\pm$ SEM of three separate experiments. * indicates a statistically significant difference compared with the respective control cultures incubated with rhIL-1 β or rhTNF α only ($p < 0.05$ by Student *t*-test), # indicates a statistically significant difference compared with stimulated cultures incubated with adenosine only ($p < 0.05$ by Student *t*-test).

As shown in Table 4.1, addition of either 10 μ M EHNA or 10 μ M dCF dramatically enhanced the inhibitory effects of exogenous adenosine on the release of both cytokines. These results strongly indicate that there is indeed a potent endogenous adenosine deaminase activity in HUVEC, resulting in inactivation of exogenously added adenosine. To verify that degradation of exogenous adenosine was actually prevented by adenosine deaminase inhibition, adenosine levels of the supernatants were determined at several time points during a 20-hour incubation period (Figure 4.3). As shown, in the presence of 10 μ M EHNA adenosine levels were maintained throughout this incubation period, whereas without EHNA adenosine was rapidly degraded into inosine and hypoxanthine. Furthermore, 10 μ M EHNA attenuated the release of IL-6 and IL-8 by activated HUVEC in the absence of exogenous adenosine (Table 4.1), suggesting that endogenous adenosine can reduce cytokine release upon stimulation. This finding also indicates that adenosine and not its metabolites, such as inosine or hypoxanthine, is responsible for the observed

inhibition of cytokine release. In line with this, coincubation of HUVEC with the adenosine metabolites inosine and hypoxanthine in concentrations up to 250 μM did not affect endotoxin or cytokine-induced cytokine release (data not shown), indicating that the inhibitory effects described above are indeed specific to adenosine.

Figure 4.3 EHNA prevents degradation of exogenous adenosine by adenosine deaminase

HUVEC were preincubated with (●) or without 10 μM EHNA (○) for 15 min, and subsequently coincubated with 100 μM adenosine and 100 U/ml rhIL-1 β during 20 hours. Control cultures were preincubated with 10 μM EHNA, and subsequently coincubated with 100 U/ml rhIL-1 β (▽). At the indicated time points, supernatants were collected as described in *Methods*, and adenosine levels were determined by HPLC.

4.3.2 EFFECTS OF ADENOSINE ON EXPRESSION OF E-SELECTIN, ICAM-1 AND VCAM-1

When HUVEC were stimulated with a concentration range of either TNF α , IL-1 β , or LPS, a dose-dependent *de novo* expression of E-selectin was observed, that was maximal after 5 h of incubation. After an incubation period of 20 h expression of ICAM-1 was maximally upregulated, while VCAM-1 was maximally expressed *de novo*. In our hands, TNF α was the most potent inducer of expression of the three adhesion molecules studied, followed in order of potency by IL-1 β and LPS (data not shown).

Figure 4.4 Effects of adenosine on rhTNF α -induced expression of adhesion molecules

HUVEC were incubated with a concentration range of rhTNF α in the absence (●) or presence of 250 μ M adenosine (○) during 5 h for expression of E-selectin (A) or during 20 h for expression of ICAM-1 (B) and VCAM-1 (C). At the end of the respective incubation periods, monolayers were fixed and cell surface expression of adhesion molecules was determined by ELISA. The results of one experiment, representative of four experiments, are shown. Data are expressed as mean $\pm$ SEM of at least four replicates. Error bars are omitted if they fall within the symbol. * indicates a statistically significant difference compared with control ($p < 0.05$ by Student t -test).

At a concentration of 1 ng/ml of $\text{TNF}\alpha$, which induced maximal expression of the adhesion molecules, adenosine up to 250 μM did not significantly inhibit expression of either of the three adhesion molecules studied (Figure 4.4). In contrast, with submaximal induction of adhesion molecules at a low $\text{TNF}\alpha$ -concentration of 10 pg/ml, 250- μM adenosine attenuated the expression of E-selectin and VCAM-1, whereas expression of ICAM-1 was not affected. Also, constitutive expression of ICAM-1 by unstimulated HUVEC was not altered by adenosine.

Figure 4.5 ADA-inhibition potentiates adenosine-mediated reduction of $\text{IL-1}\beta$ and $\text{TNF}\alpha$ -induced expression of E-selectin

HUVEC were preincubated with 10 μM EHNA for 15 min, and subsequently coincubated with (*double-hatched bars*) or without 250 μM adenosine (*hatched bars*) and either rhIL-1 β (1 U/ml) or rhTNF α (10 pg/ml) during 5 h. Control cultures without EHNA were incubated with rhIL-1 β or rhTNF α with (*open bars*) or without 250 μM adenosine (*solid bars*). At the end of the incubation period monolayers were fixed and cell surface expression of E-selectin was determined by ELISA. The results of one experiment, representative of three experiments, are shown. Data are expressed as mean $\pm$ SEM of at least six replicates. * indicates a statistically significant difference compared with controls without adenosine (* $p < 0.05$ and ** $p < 0.01$ by Student *t*-test)

De novo expression of E-selectin and VCAM-1 by HUVEC submaximally stimulated with IL-1 β (1 U/ml) or LPS (1 ng/ml) was attenuated by adenosine to a similar extent as observed during stimulation with 10 pg/ml TNF α , whereas

upregulated expression of ICAM-1 was not affected by adenosine under these conditions (data not shown). In line with the effects of adenosine deaminase inhibitors on adenosine-mediated reduction of cytokine release, EHNA potentiated the inhibitory effects of exogenous adenosine on expression of E-selectin (Figure 4.5) and VCAM-1 (not shown). However, EHNA alone did not affect expression of either E-selectin, ICAM-1 or VCAM-1, indicating that endogenous adenosine levels are not sufficient to reduce induced expression of these adhesion molecules.

In line with the earlier described lack of effect on cytokine release, inosine and hypoxanthine were also without effect on the stimulated expression of either three adhesion molecules studied (data not shown).

4.3.3 EFFECTS OF RECEPTOR-SPECIFIC ADENOSINE ANALOGUES

Next, we sought to determine whether the observed inhibitory effects on endothelial cell activation could be related to the binding of adenosine to specific adenosine receptors. To this end, the effects on cytokine release of adenosine, the A2 receptor-specific analogue 2-CAdo and the A1 receptor-specific analogue CPA were compared. As shown in Figure 4.2A, 2-CAdo was significantly more potent than adenosine in reducing IL-1 β -induced release of IL-6 and IL-8. CPA also tended to reduce cytokine release more potently than adenosine, although statistical significance was not reached at every concentration used. However, when comparing the activities of the two analogues, 2-CAdo and CPA did not differ significantly in their potency to reduce IL-1 β -induced release of IL-6 (maximum inhibition $78.8 \pm 3.8\%$ vs. $66.1 \pm 6.0\%$, respectively), whereas at concentrations of 100 μ M and 250 μ M 2-CAdo was more potent than CPA in attenuating release of IL-8 by IL-1 β -activated HUVEC (maximum inhibition $69.7 \pm 4.1\%$ vs. $55.4 \pm 1.3\%$, respectively). Furthermore, 10-100 μ M 2-CAdo reduced the release of IL-6 by HUVEC activated with LPS stronger than adenosine (Figure 4.2B), but was equipotent to adenosine in reducing LPS-induced IL-8. Finally, the effects of both analogues on LPS-induced cytokine release were practically identical to each other. HUVEC viability, as determined by MTT test, was always more than 95% during incubation with 10-250 μ M 2-CAdo or CPA.

Expression of E-selectin and VCAM-1, induced by low doses of either TNF α (10 pg/ml), IL-1 β (1 U/ml) or LPS (1 ng/ml), was slightly more reduced by 2-CAdo than it was by adenosine or CPA. As an example, Figure 4.6 shows the effects of adenosine and its receptor-specific analogues on IL-1 β -induced

expression of E-selectin. In line with the data presented above, neither analogue affected constitutive or upregulated expression of ICAM-1 (data not shown).

Figure 4.6 Effects of adenosine and receptor-specific adenosine analogues on rhIL-1 β -induced expression of E-selectin

HUVEC were incubated with rhIL-1 β (1 U/ml) and a concentration range of either adenosine (●), 2-CADO (○), or CPA (Δ) for 5 h. At the end of the incubation period monolayers were fixed and cell surface expression of E-selectin was determined by ELISA. The results of one experiment, representative of three experiments, are shown. Data are expressed as mean $\pm$ SEM of at least five replicates. Error bars are omitted if they fall within the symbol. All data presented differed significantly from controls incubated with rhIL-1 β only ($p < 0.05$ by one-way ANOVA, data not shown). * indicates a statistically significant difference compared with adenosine, # indicates a statistically significant difference compared with CPA ($p < 0.05$ by one-way ANOVA).

4.4 DISCUSSION

During ischemia, cellular ATP is metabolized into the purine nucleosides adenosine, inosine and hypoxanthine, which accumulate in the ischemic tissue. Upon reperfusion, these metabolites are rapidly washed out by the recirculating bloodstream. It has been demonstrated that agents that enhance local tissue levels of adenosine have protective effects in various animal models of reperfusion injury or septic shock (21, 23, 24). Although the mechanism of

action of adenosine under these conditions has not been fully elucidated, it is thought to be related mainly to binding to specific receptors on target cells, such as vascular smooth muscle cells and PMN. Ligation of A2 receptors on vascular smooth muscle cells induces vasodilation (25), whereas occupancy of A2 receptors on neutrophils and mononuclear phagocytes results in inhibition of their inflammatory functions (6, 7, 11, 14, 15, 26). As an illustration of these receptor-mediated beneficial effects, Marts *et al.* (27) recently demonstrated that the A2 receptor-specific adenosine analogue 2-CAdo reduced pulmonary vascular resistance as well as alveolar leukocyte influx in a rabbit model of lung ischemia reperfusion injury. While there are numerous studies in which PMN and mononuclear phagocytes have been identified as target cells for the anti-inflammatory actions of adenosine or its receptor-specific analogues, thus far a direct anti-inflammatory effect of adenosine on endothelial cells has not been clearly established. In this study however, we demonstrate that adenosine does have direct inhibitory effects on two major determinants of endothelial cell activation, that is release of cytokines and *de novo* expression or upregulation of adhesion molecules.

First, it was demonstrated that adenosine dose-dependently inhibits release of IL-6 and IL-8 by HUVEC stimulated with either IL-1 β , TNF α or LPS. Recently, we reported that adenosine inhibits release of TNF α , IL-6 and IL-8 by LPS-activated human monocytes (14). However, maximum inhibition of endotoxin-induced endothelial cytokine release ($47.6 \pm 11.5\%$ for IL-6 and $16.0 \pm 5.7\%$ for IL-8, Figure 4.2B) was less than the maximum inhibition we reported for endotoxin-induced IL-6 and IL-8 release by monocytes, using similar concentrations of adenosine. In this context it must be taken into consideration that, in contrast to monocytes, endothelial cells are a rich source of adenosine deaminase activity (21), resulting in degradation of adenosine into its metabolites inosine and hypoxanthine. Indeed, if endogenous adenosine deaminase activity was blocked, the inhibitory effects of exogenous adenosine on endothelial cytokine release were strongly enhanced, approaching the extent of inhibition of cytokine release by activated monocytes as reported by us earlier. In addition, this observation shows that inhibition of cytokine release is indeed specific to the actions of adenosine. Furthermore, our finding that enhancement of endogenous adenosine activity by preventing its degradation, in the absence of exogenously added adenosine, also results in inhibition of cytokine release by activated endothelial cells, points to a possible novel therapeutic potential of adenosine deaminase inhibitors to reduce inflammatory injury (24).

For IL-1 β -induced cytokine release, a discrepancy between the actions of adenosine on endothelial cells and monocytes was observed. Adenosine at a concentration of 50 μ M and higher attenuated the release of endothelial IL-6 and IL-8, whereas the release of IL-6 and IL-8 by monocytes was enhanced (14). Interestingly, at a relatively low concentration of 10 μ M of adenosine an approximately 15% enhancement of LPS-induced, but not IL-1 β -induced, endothelial release of IL-6 was observed. Since it is known that submicromolar concentrations of adenosine can have a pro-inflammatory effect on PMN (7), it is conceivable that addition of 10 μ M exogenous adenosine resulted in an effective concentration in the submicromolar concentration range, due to the potent endogenous adenosine deaminase activity.

Second, expression of adhesion molecules was also affected by adenosine. Inhibition of E-selectin and VCAM-1 occurred only when HUVEC were submaximally activated in the presence of a relatively high concentration of 250 μ M adenosine. When endogenous adenosine deaminase activity was blocked with EHNA, the inhibitory effects of adenosine on expression of E-selectin and VCAM-1 were strongly enhanced. Moreover, in the presence of EHNA, adenosine also reduced expression of both adhesion molecules induced by higher concentrations of the inflammatory stimuli. In contrast, adenosine up to a concentration of 250 μ M was without effect on either constitutive or upregulated expression of ICAM-1 after 20 h of stimulation with IL-1 β , TNF α , or LPS. ICAM-1 expression also remained unaffected when degradation of adenosine was prevented by EHNA. Our data therefore suggest a selective inhibitory effect of adenosine on expression of E-selectin and VCAM-1. Because increased cAMP levels have been reported to selectively reduce cytokine-induced expression of both E-selectin and VCAM-1, presumably at the transcriptional level (28), it might be hypothesized that adenosine acts via a cAMP-mediated pathway to reduce expression of these adhesion molecules. Our finding that A₂ receptor ligation by 2-CA_{do} which induces enhanced intracellular levels of cAMP (7, 26), resulted in the most potent reduction of E-selectin and VCAM-1 as observed in this study, would be in support of this hypothesis. Recently, Jurgensen *et al.* (9) reported that the synthetic adenosine analogue 3-deazaadenosine inhibits biosynthesis of ICAM-1 by HUVEC stimulated with TNF α for 5 h, whereas in our study adenosine did also not inhibit expression of ICAM-1 by cytokine-activated HUVEC after a 5 h incubation period (data not shown). The lack of conformity between these data possibly reflects a different mode or site of action of adenosine and 3-deazaadenosine.

Although adenosine inhibited release of cytokines, particularly of IL-6, when HUVEC were submaximally activated, its effect on cytokine release was most pronounced if HUVEC were maximally stimulated. In contrast, adenosine only reduced expression of adhesion molecules if HUVEC were submaximally activated. These observations lead us to hypothesize that adenosine may affect both early and more advanced stages of endothelial cell activation during the development of an inflammatory response.

In this study we were unable to demonstrate a clear role for specific adenosine receptors in mediating the inhibitory actions of adenosine. The A₂ receptor-specific analogue 2-CAdo exerted significantly stronger inhibitory effects than the A₁-selective analogue CPA on IL-1 β -induced release of IL-8 and expression of E-selectin and VCAM-1, but both analogues were approximately equipotent in reducing IL-1 β -induced IL-6 release and LPS-induced cytokine release. Although these results would suggest a role of specific adenosine receptor ligation in mediating the inhibitory effects on endothelial cell activation, the lack of a strong evidence for this conclusion is in contrast with the well-documented receptor-specific action of adenosine on PMN and mononuclear phagocyte function. Furthermore, the concentration of adenosine required for inhibitory effects on endothelial cells seems to be relatively high in comparison to the concentrations of adenosine effective to inhibit leukocyte function. However, when the potent endogenous adenosine deaminase activity of endothelial cells was attenuated by adenosine deaminase inhibitors, the inhibitory effects of adenosine on cytokine release as well as on expression of adhesion molecules were strongly enhanced. Moreover, a role for endogenous adenosine in reducing cytokine release by activated HUVEC was demonstrated using adenosine deaminase inhibitors in the absence of exogenously added adenosine. Finally, it has been reported that local concentrations of adenosine and adenine nucleotides in the extracellular space may indeed reach the millimolar range during ischemia and shock (29), which emphasizes the possible relevance of the results obtained in this *in vitro* study to such clinical conditions. Taken together, our results show a clear effect of adenosine on endothelial cell function, although we were unable to define its precise mechanism of action.

The described inhibitory actions of adenosine on endothelial cell activation may be of importance in view of the role of the vascular endothelium in the pathogenesis of reperfusion injury. It has been demonstrated in various *in vitro* models that incubation of endothelial cells under anoxic conditions followed by reoxygenation induces increased adherence of PMN to the endothelium (30, 31). Several authors have linked the enhanced PMN-endothelial adhesion to *de*

novo expression and upregulation of endothelial adhesion molecules in both *in vitro* models as well as in animal models of ischemia and reperfusion (32, 33). Furthermore, it has been demonstrated in various *in vitro* models, that anoxia and reoxygenation can induce the release of the pleiotropic cytokines IL-1 α , IL-6 and the chemokine IL-8 by endothelial cells (34, 35), which are known to be involved in leukocyte chemotaxis and adhesion. Several authors have reported that agents that enhance local tissue levels of endogenous adenosine, either by preventing its release into the bloodstream (21) or by interfering with its metabolism (23, 24), provide protective effects in animal models of ischemia-reperfusion or septic shock. Based on the data of this *in vitro* study, we propose that such protective effects may, next to the well-documented inhibitory effects of adenosine on PMN (6, 7, 10) and monocyte function (11, 14, 15) also be the result of the inhibitory effects of adenosine on endothelial cell activation. In order to test this hypothesis, it remains to be determined whether substances that interfere with adenosine metabolism or release, will actually attenuate cytokine release and expression of endothelial adhesion molecules in experimental *in vitro* and *in vivo* models of ischemia and reperfusion.

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CHAPTER 5

Hypoxia/Reoxygenation of Cultured Human Endothelium: Effects on Cellular Activation and Adenosine Release

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ABSTRACT

Endothelial cells have been recognized to be actively involved in promotion as well as inhibition of inflammatory processes. Specifically during ischemia and reperfusion, the vascular endothelium is the centerstage of inflammatory events that are induced by the sequence of low ambient oxygen and oxygen reintroduction. To investigate the effects of ambient oxygen on both pro-inflammatory and anti-inflammatory characteristics of vascular endothelium, we subjected cultured human umbilical vein endothelial cells (HUVEC) to varying periods of hypoxia and reoxygenation in an *in vitro* model, as the correlate of *in vivo* ischemia and reperfusion. Neither hypoxia for 4, 8, or 16 h, nor subsequent reoxygenation for 5 or 20 h, induced pro-inflammatory endothelial activation, as determined by cell-surface expression of the adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), as well as by extracellular release of the cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8). At the same time however, 4 h hypoxia increased release of the anti-inflammatory autacoid adenosine 4-fold over normoxic control HUVEC, an event which was strongly potentiated by the adenosine kinase inhibitor GP515. We therefore conclude that hypoxia and reoxygenation *per se* do not promote the pro-inflammatory capacity of endothelium, but on the other hand provide a target for pharmacological interference in endothelial adenosine metabolism, thereby capitalizing on the anti-inflammatory potential of the endothelium.

5.1 INTRODUCTION

Ischemia followed by reperfusion is an important clinical event, occurring in various pathological situations. For instance, thrombolytic therapy after myocardial or cerebral infarction, as well as restoration of blood flow after organ transplantation or resuscitation from hypovolemic shock after trauma, are associated with the development of tissue injury and loss of organ function. There is a large body of evidence indicating that the polymorphonuclear leukocyte (PMN) is the main effector cell of the microvascular and parenchymal damage resulting from reperfusion of ischemic tissue (1). Chemotaxis and adherence of activated PMN to endothelial cells followed by transendothelial migration are crucial steps in the pathogenesis of reperfusion injury. In this respect, the endothelium is the center-stage of the inflammatory events during reperfusion.

After appropriate stimulation, the vascular endothelium promotes the inflammatory process by releasing inflammatory mediators, such as the pro-inflammatory cytokines IL-1 and IL-6, and the chemokine IL-8, as well as by expressing specific adhesion molecules on its cell surface, that bind and activate PMN. Endothelial activation, as originally defined by Pober (2), may be caused by pro-inflammatory cytokines, such as TNF α and IL-1. The involvement of TNF α and IL-1 in *in vivo* ischemia-reperfusion has been clearly demonstrated (3-5), and actual *in vivo* expression of the endothelial adhesion molecules E-selectin (6) and ICAM-1 (7) in postischemic tissues has indeed been reported. However, while there is consensus that the vascular endothelium may be activated *in vivo* secondary to cytokine-stimulation during ischemia-reperfusion, controversy exists with regard to the issue whether or not hypoxia and/or reoxygenation *per se* can directly increase the pro-inflammatory capacities of vascular endothelium. Shreeniwas *et al.* (8) and Karakurum *et al.* (9) have reported that hypoxia, but not reoxygenation, constitutes a stimulus for cultured endothelial cells to elaborate IL-1 α and IL-8. In contrast, others have reported that, following *in vitro* hypoxia, normoxic or even hyperoxic reoxygenation is required to stimulate endothelial cells to release IL-1 (10, 11), IL-6 (11) and IL-8 (12), suggesting that hypoxia/reoxygenation-induced intracellular oxidant stress rather than hypoxia alone triggers endothelial activation. Moreover, conflicting reports exist on the effects of hypoxia and/or reoxygenation on the induction of endothelial expression of adhesion molecules *in vitro*. Whereas some authors have suggested or demonstrated increased expression of E-selectin and ICAM-1 in such models (8, 10, 13, 14), others were unable to confirm this (15-19). Klein *et al.* (15) found no direct influence of hypoxia on endothelial expression of adhesion molecules, but suggested a priming effect.

While the activated endothelium promotes PMN adherence and activation, at the same time it can also downmodulate inflammatory reactions by releasing several anti-inflammatory molecules, such as nitric-oxide (NO) and adenosine (20-24). Whereas ischemia or prolonged hypoxia result in a reduced endothelial capacity to release NO (25, 26), in contrast, release of adenosine by endothelial cells under such conditions is enhanced as a result of increased ATP catabolism (27, 28) and reduced adenosine reuptake (29). During recent years, adenosine has been increasingly recognized as an important endogenous anti-inflammatory agent that protects the endothelium from reperfusion injury (20). The established anti-inflammatory features of adenosine include inhibition of neutrophil superoxide release (30) and degranulation (31), reduction of cytokine release by activated monocytes (32) and endothelial cells (33), inhibition of

neutrophil-endothelial adherence (34) and reduced endothelial expression of adhesion molecules (33). Therefore, pharmacological interference in adenosine metabolism by adenosine-regulating agents, aimed at enhancing local concentrations of endogenous adenosine, thereby capitalizing on the anti-inflammatory actions of adenosine, represents a potential target for interventional strategies in various inflammatory disorders, including ischemia-reperfusion injury.

The goals of the present study were twofold: first, we investigated whether *in vitro* hypoxia and reoxygenation directly induces human endothelial activation, as determined by release of the cytokines IL-6 and IL-8, and by expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1. Second, the potential therapeutic relevance of the adenosine-regulating agent GP515 to ischemia-reperfusion was assessed by determining its effect on endothelial release of the anti-inflammatory molecule adenosine during hypoxia.

5.2 MATERIALS AND METHODS

5.2.1 REAGENTS

Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), and cyclohexamide (CHX) were obtained from Sigma Chemical (St. Louis, MO). Recombinant human TNF α was kindly provided by BASF/Knoll (Ludwigshafen, Germany). Recombinant human IL-1 β was a kind gift of Dr. S. Gillis (Immunex, Seattle, WA). The adenosine kinase inhibitor 4-amino-1-(5-amino-5-deoxy-1- β -D-ribofuranosyl)-3-bromo-pyrazolo[3,4-d]pyrimidine (GP515) was generously provided by Gensia, Inc., San Diego, CA.

5.2.2 ENDOTHELIAL CELL CULTURE

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical cords by collagenase treatment and were seeded into fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue-culture flasks (Costar, Cambridge, MA) in complete medium consisting of RPMI 1640 (Gibco, Paisley, UK) supplemented with 50 μ g/ml heparine (Sigma Chemical Co., St. Louis, MO), 2 mmol/l glutamine, antibiotics, 30 μ g/ml Endothelial Cell Growth Supplement (ECGS; Collaborative Research Inc., Bedford, MA), 10% heat-inactivated BCS (Hyclone, Logan, UK) and 10% heat-inactivated pooled human serum, obtained

from healthy blood donors at the Red Cross Bloodbank Zuid Limburg (Maastricht, The Netherlands) after informed consent for research was given.

5.2.3 HYPOXIA/REOXYGENATION MODEL

For experiments, second or third passage HUVEC were plated onto fibronectin-coated 24-well tissue-culture plates (Costar Corp., Cambridge, MA) and used when monolayers were tightly confluent. When confluent, the monolayers were rinsed twice with Endothelial Serum Free Medium (ESFM; Gibco, Paisley, UK) supplemented with 50 $\mu\text{g/ml}$ heparin and 30 $\mu\text{g/ml}$ ECGS, and 500 μl medium was added to each well. The cultures were placed in a specially constructed sealed airtight hypoxia chamber (2000 cm^3) at 37°C, which was then flushed with a humidified 95% N_2 -5% CO_2 gas mixture at a flow rate of 3000 ml/min . for 10 min (Figure 5.1). The oxygen concentration of the atmosphere within the hypoxia chamber was measured using an oxygen analyzer (Model 570A, Servomex) and was found to drop to 0% within 10 min after initiation of gasflow. After anoxia had been achieved, the hypoxia chamber was continuously purged with the anoxic gas mixture at a flow rate of 350 ml/min for 4, 8 or 16 h. Furthermore, the oxygen concentration of the medium overlying the cells was measured on-line during anoxic incubation using a micro-oxygen electrode (Model MI-730, Microelectrodes Inc., Londonderry, NH) connected to an oxygen meter (Model OM-4, Microelectrodes Inc.). A plateau concentration of $3.5 \pm 0.1\%$ O_2 was reached in the culture medium within 15 min after initiation of gasflow, and maintained thereafter (Figure 5.2). Special care was taken to avoid inadequate humidification of the gasmixture, since it was found in preliminary experiments that insufficient humidification lead to undesirable disturbances of medium osmolarity. To ensure that the gas mixture had been adequately humidified, the weight of the total medium-volume was determined before and after anoxic incubation. In our experimental model, no volume-weight loss occurred during anoxia nor during control normoxic incubation (data not shown). Reoxygenation was achieved by replacing the cell cultures in a normoxic 95% room air-5% CO_2 atmosphere. With the use of the micro-oxygen electrode it was confirmed that the O_2 concentration of the medium returned to a basal level of $20.9 \pm 0.1\%$ within 25 min. To investigate whether hypoxia/reoxygenation would prime endothelial cells, in some experiments either recombinant human $\text{TNF}\alpha$ or recombinant human $\text{IL-}\beta$ dissolved in fresh culture medium was added at the beginning of the reoxygenation period.

Figure 5.1 Experimental setup of the *in vitro* hypoxia/reoxygenation model

Figure 5.2 Oxygen concentration in HUVEC culture medium during *in vitro* hypoxia/reoxygenation

The curve represents the on-line registration of the O₂ concentration in the culture medium overlying the cells during a typical hypoxia/reoxygenation experiment. At T=10 min, hypoxia (H) was induced by purging the hypoxia chamber with a 95% N₂-5% CO₂ gas mixture, reaching a plateau level of 3% O₂ within 15 min. At T=240 min, reoxygenation (R) to 21% O₂ was achieved within 25 min by replacing the culture in a 95% room air-5% CO₂ atmosphere.

5.2.4 ENDOTHELIAL VIABILITY, LACTATE RELEASE AND TOTAL PROTEIN SYNTHESIS

Viability of HUVEC subjected to hypoxia and reoxygenation was tested by trypan blue exclusion test. After trypsinization, HUVEC were resuspended in ESFM, mixed with 0.2% trypan blue, and counted in a hemacytometer. Percentage viability was calculated as $100\% \times \text{total number of viable cells per ml aliquot} / \text{total number of cells per ml aliquot}$. For determination of lactate release into the culture supernatants, 200 μl sample was vortexed with 20 μl trichloroacetic acid (TCA; Merck, Darmstadt, Germany) 50%, frozen in liquid nitrogen, and stored at -80°C until analysis. Lactate analysis was performed spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic method, using a commercially available kit as described previously by Dejong *et al.* (35).

To determine overall endothelial protein synthesis, incorporation of [^3H]-labeled phenylalanine ([^3H]-Phe) into TCA-precipitable material was measured. Upon confluency, HUVEC were rinsed twice with ESFM and 0.15 μCi [^3H]-Phe in complete ESFM (specific activity 0.5 $\mu\text{Ci/ml}$) was added to each well. HUVEC were subjected to either 8 or 16 h of hypoxia, followed by 20 h reoxygenation, whereas HUVEC incubated under normoxic conditions for the same time periods served as controls. At the end of the incubation period, monolayers were washed four times with ice-cold PBS supplemented with Ca^{2+} and Mg^{2+} , 500 μl 10% TCA at 4°C was added to each well and monolayers were placed on ice for 1 h. The supernatant was removed and 500 μl 5% TCA at 4°C was added for 5 min. Finally, after removal of the supernatant, the precipitate was dissolved in 300 μl of 0.3N NaOH, after which the samples were added to scintillation fluid (Formula 989; NEN Research Products, Boston, MA) and radioactivity was counted in an automatic Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA).

5.2.5 CELL-SURFACE EXPRESSION OF ADHESION MOLECULES

Second or third passage HUVEC were plated onto fibronectin-coated 96-well flat-bottom cell culture plates (Costar, Cambridge, MA) in complete medium two days prior to experiments. Confluent monolayers were washed twice with complete ESFM, and subjected to normoxia or hypoxia/reoxygenation as described above. Next, HUVEC were washed three times with PBS supplemented with Ca^{2+} and Mg^{2+} , fixed with 0.05% glutaraldehyde for 10 min at room temperature, and kept at 4°C until use in ELISA. Cell-surface

expression of E-selectin, ICAM-1 and VCAM-1 was determined using previously described ELISA's (33).

5.2.6 CYTOKINE ASSAYS

The concentrations of the cytokines IL-6 and IL-8 in the cell culture supernatants were determined using previously described sandwich-ELISA's (32, 36).

5.2.7 DETERMINATION OF ADENOSINE LEVELS

To examine the effects of hypoxia and the adenosine kinase inhibitor GP515 on endothelial adenosine release, HUVEC were incubated with 50 μ M of the adenosine deaminase inhibitor EHNA to prevent breakdown of endogenous adenosine by adenosine deaminase, which is abundantly present within endothelial cells (37). For determination of adenosine levels by High Performance Liquid Chromatography (HPLC), cell culture supernatants were deproteinized with 0.4 M perchloric acid on ice, centrifuged at 12,000 rpm for 10 min at 4°C, and neutralized with 0.4 M K₂HPO₄. After centrifugation at 12,000 rpm for 10 min at 4°C, supernatants were kept at -70 °C until determination of adenosine levels.

5.2.8 STATISTICAL ANALYSIS

All results are presented as mean $\pm$ SEM. Differences between normoxia and hypoxia were analyzed by Student *t*-test, and were considered statistically significant at $p < 0.05$.

5.3 RESULTS

5.3.1 CELLULAR VIABILITY, LACTATE RELEASE AND PROTEIN SYNTHESIS

Incubation of HUVEC under hypoxic conditions up to 16 h did not significantly affect trypan blue exclusion compared to normoxic controls (Table 5.1), nor did it alter the normal cobblestone appearance of the monolayers on microscopic examination. Moreover, after 4 h hypoxia, a subsequent reoxygenation period of 20 h did not significantly alter viability compared with HUVEC incubated under normoxic conditions for 24 h. In contrast, a 5-h

reoxygenation period after 16 h anoxia, resulted in a slight, but significant decrease in viability (Table 5.1). No apparent morphological changes, however, on microscopic examination were observed after a reoxygenation period of 20 h, following either 4 or 16 h anoxia.

Table 5.1 HUVEC viability during normoxia, hypoxia, and reoxygenation

<i>Culture conditions</i>	<i>% viability</i>
4 h normoxia	93.2 ± 0.8
4 h hypoxia	92.3 ± 0.9
4 h normoxia + 20 h normoxia	89.3 ± 0.2
4 h hypoxia + 20 h normoxia (=reoxygenation)	90.8 ± 1.6
16 h normoxia	88.5 ± 1.6
16 h hypoxia	87.7 ± 1.0
16 h normoxia + 5 h normoxia	88.4 ± 0.8
16 h hypoxia + 5 h normoxia (=reoxygenation)	79.2 ± 1.7*

Percentage viability was determined by trypan blue exclusion test as described in *Materials and Methods*. Results are presented as mean ± SEM of eight experiments. * indicates a statistically significant difference compared with corresponding normoxic control (16 h + 5 h normoxia).

To verify that HUVEC were subjected to hypoxia, lactate production was determined as an indicator of an ischemic insult (38, 39). Indeed, HUVEC maintained for 4 h under hypoxic conditions produced approximately 350 nM/well lactate, whereas HUVEC maintained under normoxic conditions produced 230 nM/well by 4 h (Figure 5.3). After 16 h, this difference was even more pronounced with ≈ 1500 nM/well produced during hypoxia, and ≈ 900 nM/well produced during normoxia.

To assess the functional integrity of HUVEC incubated under conditions of hypoxia and reoxygenation, overall cellular protein synthesis, as determined by [³H]-Phe incorporation into TCA-precipitable material, was measured. After either 4 h (data not shown) or 8 h of hypoxia (Figure 5.4), a subsequent reoxygenation period of 5 h did not lead to a significant decrease in overall cellular protein synthesis compared with HUVEC incubated under normoxic conditions for corresponding periods of time. In contrast however, after 16 h of hypoxia followed by 5 h of reoxygenation, a small, but significant reduction in

total protein synthesis as compared with normoxic controls was observed (21 h. normoxia: 7821 ± 516 dpm/well vs. 16 h. hypoxia/ 5 h. reoxygenation: 6410 ± 271 dpm/well, $p < 0.05$ by Student *t*-test, not shown).

Figure 5.3 Lactate production by HUVEC during normoxia and hypoxia

HUVEC were incubated for 4, 8 or 16 h under either normoxic (*closed circles*) or hypoxic conditions (*open circles*), and lactate content of the culture supernatants at the indicated timepoints was determined by HPLC. Control culture media were incubated under hypoxic conditions without the presence of HUVEC, and lactate (*open squares*) content was determined at the indicated timepoints. The results from a representative experiment with duplicate determinations are depicted.

5.3.2 EXPRESSION OF ENDOTHELIAL ADHESION MOLECULES

Unstimulated HUVEC, incubated under normoxic conditions for 4 up to 36 h, expressed low basal levels of E-selectin and VCAM-1, whereas ICAM-1 was constitutively expressed. Hypoxia during either 4, 8 or 16 h, with or without subsequent reoxygenation for either 5 or 20 h, did not induce *de novo* expression of E-selectin or VCAM-1, nor did it stimulate HUVEC to upregulate expression of ICAM-1 (Figure 5.5).

Figure 5.4 Overall protein synthesis by HUVEC during hypoxia and reoxygenation as determined by [^3H]-Phe incorporation into TCA-precipitable material

HUVEC were subjected to hypoxia (H) for either 8 or 16 h, and subsequently reoxygenated for 5 h. [^3H]-Phe incorporation by HUVEC during hypoxia/reoxygenation was compared to that by HUVEC incubated under normoxic conditions (N) for corresponding periods of time. Normoxic HUVEC cultures incubated for 8 h in the presence of 5 $\mu\text{g/ml}$ cyclohexamide (CHX) served as negative controls. Results are presented as mean + SEM of duplicate determinations of four experiments, expressed as % of normoxic controls. * indicates $p < 0.05$, ** indicates $p < 0.01$ compared with normoxic control cultures incubated without CHX.

To investigate whether hypoxia would prime HUVEC to express any of the adhesion molecules studied, HUVEC were stimulated with 0,1 ng/ml rhTNF α or 10 U/ml rhIL-1 β upon reoxygenation. In normoxic controls stimulated at the corresponding timepoints, treatment with either TNF α (data not shown) or IL-1 β (Figure 5.5) potentially induced *de novo* expression of E-selectin and VCAM-1, and strongly upregulated ICAM-1 expression. After hypoxia, cytokine-induced expression of either E-selectin, ICAM-1 and VCAM-1 was similar to controls, indicating that in our model hypoxia did not prime HUVEC for subsequent activation by pro-inflammatory cytokines.

Figure 5.5 Effects of hypoxia and reoxygenation on endothelial expression adhesion molecules

HUVEC were subjected to 4 h normoxia (*open bars*) or hypoxia (*hatched bars*). At T=4 h, fresh medium either without activator or with 0.1 ng/ml rhTNF α or 10 U/ml rhIL-1 β was added to the cultures, which were subsequently incubated under normoxic conditions for either 5 or 20 h. Cell-surface expression of E-selectin (*A*) was determined by ELISA at T=4 h and at T=9 h. Cell-surface expression of ICAM-1 (*B*) and VCAM-1 (*C*) was determined by ELISA at T=4 h and at T=24 h. Results are presented as mean + SEM of quadruplicate determinations of a representative experiment.

5.3.4 RELEASE OF CYTOKINES

In parallel, the release of the inflammatory cytokines IL-6 and IL-8 by HUVEC during hypoxia and reoxygenation was studied. While normoxic HUVEC spontaneously released small quantities of both cytokines, no significant alterations in the amounts of either cytokine released into the supernatants were observed during hypoxia for either 4, 8, or 20 h in comparison with controls at any of the timepoints studied (Figure 5.6). Similarly, after a subsequent reoxygenation period of 5 or 20 h there was no significant difference in the amount of cytokine released by hypoxic and control HUVEC when hypoxia was limited to 4 or 8 h (Figure 5.7). This would indicate that in this setting neither hypoxia nor reoxygenation acts as a stimulus for cytokine production by endothelial cells. In contrast, after 16 h of hypoxia followed by reoxygenation for 5 or 20 h, IL-6 and IL-8 levels were significantly lower than in controls (Figure 5.7), which is compatible with decreased cell viability and reduced protein synthesis capacity after such a prolonged period of hypoxia.

Analogous to the results described above for expression of adhesion molecules, hypoxia did not prime HUVEC for subsequent activation by pro-inflammatory cytokines to release IL-6 and IL-8. Stimulation of HUVEC with IL-1 β (Figure 5.8) or TNF α (data not shown) after 4 h hypoxia resulted in release of IL-6 and IL-8 to levels identical to those detected after stimulation of normoxic controls. Similar results were obtained with cytokine stimulation of HUVEC after 8 or 16 h hypoxia (data not shown).

5.3.5 RELEASE OF ADENOSINE

As mentioned earlier, during ischemia or hypoxia cellular ATP is catabolized, resulting in enhanced release of adenosine from the cell. Since adenosine release may thus serve as a marker of ischemia (29, 37) we determined the adenosine levels in the culture supernatants of HUVEC subjected to hypoxia. In addition, the effects of the adenosine kinase inhibitor GP515, designed to enhance local adenosine levels during inflammatory events (40), on adenosine release under these conditions were determined. As depicted in Figure 5.9, during 4 h hypoxia adenosine release by HUVEC increased approximately 4-fold compared with that by HUVEC incubated under normoxic conditions for the same duration (4 h normoxia: 158 ± 90 nM vs. 4 h hypoxia: 640 ± 90 nM adenosine, $p < 0.01$ by Student *t*-test), indicating that in our experimental model hypoxia was indeed achieved.

Figure 5.6 *Effects of hypoxia on endothelial release of cytokines*

HUVEC were incubated for either 4, 8 or 16 h under normoxic (*open bars*) or hypoxic conditions (*hatched bars*), and release of IL-6 (*A*) and IL-8 (*B*) into the culture supernatants was determined by ELISA. Results are presented as mean + SEM of duplicate determinations of four experiments.

Figure 5.7 *Effects of reoxygenation on endothelial release of cytokines*

HUVEC were incubated for either 4 (*open bars*), 8 (*hatched bars*), or 16 h (*closed bars*) under hypoxic conditions, and subsequently reoxygenated for either 5 or 20 h. At the end of the incubation periods, the culture supernatants were collected and release of IL-6 (*A*) and IL-8 (*B*) was determined by ELISA. Results are expressed as % of cytokine release by HUVEC incubated under normoxic conditions for corresponding periods of time, and presented as mean + SEM of duplicate determinations of at least four experiments. * indicates a statistically significant difference compared with corresponding normoxic controls.

Figure 5.8 *Effect of hypoxia/reoxygenation on rhIL-1 β -induced endothelial release of cytokines*

HUVEC were subjected to 4 h normoxia (*open bars*) or hypoxia (*hatched bars*). At T=4 h, fresh medium containing 10 U/ml rhIL-1 β was added to the cultures, which were subsequently incubated under normoxic conditions for 5 or 20 h. At the end of the incubation periods, the culture supernatants were collected and release of IL-6 (*A*) and IL-8 (*B*) was determined by ELISA. Results are expressed as mean + SEM of duplicate determinations of four experiments.

Treatment with 100 μM GP515 during a 4-h incubation period dramatically enhanced endothelial adenosine release by normoxic as well as hypoxic HUVEC to $3.25 \pm 0.19 \mu\text{M}$ and $5.41 \pm 0.33 \mu\text{M}$, respectively, with GP515 producing an approximately 8.5-fold increase in adenosine release by HUVEC during hypoxia.

Figure 5.9 *Effects of hypoxia and GP515 on endothelial release of adenosine*

HUVEC were incubated for 4 h under normoxic (*open bars*) or hypoxic conditions (*hatched bars*), either without (control) or with 100 μM GP515 in the presence of 50 μM EHNA. Release of adenosine into the culture supernatants was determined by HPLC as described in *Material and Methods*. Results are presented as mean + SEM of duplicate determinations of four experiments. * indicates a statistically significant difference between normoxic and hypoxic cultures, # indicates a statistically significant difference between untreated and GP515-treated cultures.

5.4 DISCUSSION

In this study, we have used an *in vitro* hypoxia/reoxygenation model, mimicking *in vivo* ischemia-reperfusion, to investigate in parallel the effects of decreased ambient oxygen on the functional integrity, the release of pro-inflammatory cytokines and expression of specific adhesion molecules, as well as on the release of the anti-inflammatory mediator adenosine by human endothelial cells.

A prominent observation of this study is that HUVEC were quite resistant to extremely low ambient oxygen concentration for considerable periods of time. Hypoxic conditions were indeed achieved in this model, as was confirmed directly by on-line measurement of the oxygen concentration in the culture medium overlying the cells during hypoxic incubation. Actual hypoxia was also indirectly evidenced by the observation of increased lactate production and enhanced adenosine release during hypoxia as compared to normoxia. During exposure to hypoxia for a relatively long period of 16 h, HUVEC remained viable, as demonstrated by trypan blue exclusion, and remained functionally intact, as demonstrated by their ability to uphold overall protein synthesis. Moreover, careful microscopic examination revealed no disturbance of normal endothelial morphology during hypoxia. Only after reoxygenation following 16 h hypoxia, a significant decrease in cellular viability was observed, paralleled by a reduction in overall protein synthesis. In contrast, after reoxygenation following a shorter hypoxic episode of either 4 or 8 h, no significant cellular damage was found, indicating that the duration of hypoxia eventually determines the extent of injury occurring during subsequent reoxygenation, as has been suggested previously (41-43). Our observations on preservation of endothelial viability and functional integrity during hypoxia are in line with previous studies in which HUVEC were subjected to comparable periods of hypoxia without significant cellular injury (9, 11-15), and with the suggestion that tolerance to acute and chronic hypoxia may be a fundamental property of endothelial cells (44-47).

Although HUVEC remained viable and capable of protein synthesis, neither hypoxia nor reoxygenation induced *de novo* expression of E-selectin or VCAM-1 in our model, while expression of ICAM-1 also remained unaltered. Only few reports exist on the effects of hypoxia and reoxygenation on cell-surface expression of adhesion molecules by cultured HUVEC. There is sporadic evidence indicating that E-selectin (formerly ELAM-1) can be induced by hypoxia (14) or by reoxygenation following hypoxic treatment of HUVEC (8, 10, 13). In two of these studies (13, 14), involvement of E-selectin in hypoxia/reoxygenation-induced neutrophil adherence was suggested on the basis of the kinetics and protein synthesis dependency of the endothelial surface-expressed antigen responsible for adhesion, which could be blocked by an anti-E-selectin mAb in one of these studies (13). In two other studies, increased presence of E-selectin during reoxygenation, but not during hypoxia, was demonstrated by Western blot analysis of lysed cells (8) and by flow cytometry (10). However, the length of the hypoxic incubations varied considerably between these studies, making comparison difficult. In the latter

study, E-selectin was detected after 1 h hypoxia followed by 2 h reoxygenation, whereas in the former study induction of E-selectin required 16 h of hypoxia followed by 4 h reoxygenation, an experimental design which in our hands affected HUVEC viability. In contrast with these data, but in agreement with our findings, others have failed to demonstrate induction of cell-surface expression of E-selectin after hypoxia/reoxygenation of HUVEC (15-17, 19). Similarly, in the present study hypoxia nor reoxygenation induced upregulation of ICAM-1 expression by HUVEC, which is consistent with previously reported data (15-19, 48), but again in contrast with results from the above-mentioned studies of Shreeniwas *et al.* (8) and Clark *et al.* (10), who reported increases in ICAM-1 in parallel with E-selectin. To our knowledge, only Klein *et al.* (5) have previously investigated the effects of different oxygen tensions on HUVEC expression of VCAM-1, and found no significant stimulation under hypoxic conditions, which supports our present results.

Consistent with the lack of effect of hypoxia and reoxygenation in our model on endothelial activation, as determined by the expression of adhesion molecules, we were unable to demonstrate elaboration of IL-6 and IL-8 by HUVEC under these conditions. Contrary to our findings, Ala *et al.* (11) reported a 2.3-fold increase of HUVEC-derived IL-6 after 5 h hypoxia and 19 h reoxygenation, compared with 24 h normoxia. However, it must be noted that in their study HUVEC displayed an extremely low spontaneous IL-6 production of 80 ± 7 pg/ml during 24 h normoxia as detected by bioassay, whereas we detected 250 ± 42 pg/ml IL-6 by ELISA after 4 h normoxic incubation. Similarly, in contrast with our observations, Karakurum *et al.* (9) found a 5-fold increase of spontaneous release of IL-8 by HUVEC during 16 h hypoxia compared with normoxic controls, while Strieter *et al.* (12) reported an approximately 2-fold increase of HUVEC-derived IL-8, which could be detected only after hyperoxic, but not normoxic, reoxygenation following 6 h hypoxia. In the latter study, a synergistic effect of hypoxia/reoxygenation and concomitant agonist stimulation was also suggested. In comparison, we stimulated HUVEC with either TNF α or IL-1 β upon reoxygenation, but found no priming or synergistic effect of hypoxia/reoxygenation on expression of adhesion molecules or cytokine release.

Although we have currently no valid explanation for these conflicting results, they may have been caused by subtle differences in experimental designs, intrinsic HUVEC responsiveness or incubation conditions. With regard to the latter, we emphasize the importance of adequate humidification of the gas mixtures to which the cultures are exposed, since insufficient humidification causes considerable increases in culture medium osmolality due to water loss by

evaporation, resulting in cellular osmotic stress. While endothelial cells seem to tolerate hyperosmolarity well, even during anoxia and reoxygenation (49), osmotic stress is known to induce gene expression and protein synthesis of IL-8 (50).

Our results on the release of adenosine during hypoxia, and the effects of the adenosine kinase inhibitor GP515 thereon, point to the potential therapeutic relevance of this agent, and possibly other adenosine-regulating agents, to *in vivo* ischemia-reperfusion events. The endothelium plays an important role in the metabolism and release of adenosine, an ubiquitous endogenous metabolite with a broad spectrum of anti-inflammatory actions (30-34). The adenine nucleotide pool in endothelial cells is two to three times greater than that seen in other cell types (37), while HUVEC seem to contain relatively high levels of ATP in comparison with other endothelial cells (38). Adenosine is generated continuously in cultured HUVEC under normal conditions, but is immediately recycled via adenosine kinase (37). During hypoxic conditions however, release of adenosine by endothelial cells is enhanced as a result of increased ATP catabolism (27, 28) and possibly reduced adenosine reuptake (29). Indeed, 4 h hypoxia in our model resulted in an approximately 4-fold increase of adenosine production by HUVEC. Moreover, inhibition of endogenous endothelial adenosine kinase activity with GP515 strongly potentiated hypoxia-induced adenosine release. These observations lead us to suggest that, given the critical importance of the vascular endothelium as the site of the inflammatory events that are induced by ischemia and reperfusion, pharmacological interference in adenosine production at the endothelial level could be useful in the treatment of reperfusion injury. Conceivably, augmentation of endothelial adenosine production could enhance the adenosine-mediated anti-inflammatory potential of the endothelium during these events. In this regard, it is of specific interest that Gunther *et al.* (51) recently reported the inhibition of neutrophil superoxide production by adenosine released from vascular endothelial cells.

In summary, using a well-controlled *in vitro* model, we have shown that human endothelial cells display considerable tolerance to hypoxia and reoxygenation. Moreover, we conclude that hypoxia/reoxygenation in itself does not constitute a direct stimulus for human endothelial cell activation, and hypothesize therefore that activation of the vascular endothelium in the *in vivo* setting of ischemia and reperfusion may result from indirect inflammatory mechanisms, such as enhanced release of eicosanoids, elaboration of cytokines and other pro-inflammatory mediators by monocytes or macrophages, or complement activation (1, 21). At the same time, the anti-inflammatory potential of the endothelium might be enforced during such events, by

enhancing its release of the endogenous anti-inflammatory molecule adenosine, using the adenosine kinase inhibitor GP515 or other adenosine-regulating agents.

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CHAPTER 6

The Adenosine Kinase Inhibitor GP515 Attenuates Leukocyte-Endothelial Interactions in the Liver after Hemorrhagic Shock and Resuscitation in the Rat

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ABSTRACT

Adhesion of leukocytes to the vascular endothelium hallmarks a key event in neutrophil-mediated organ injury after ischemia and reperfusion. The autacoid adenosine has been shown to inhibit activated neutrophil function and to interfere with leukocyte-endothelial adherence. Its therapeutic use in ischemia-reperfusion however, has been limited by severe cardiovascular side effects. We therefore investigated the effects of the adenosine kinase inhibitor GP515 on *in vivo* hepatic leukocyte-endothelial interactions in a rat model of hemorrhagic shock and resuscitation, using intravital microscopy. Rats were pretreated with either GP515 (0.25 mg/kg) or saline in a randomized and blinded manner, and subjected to pressure-controlled hemorrhagic hypotension at a mean arterial pressure of 40 mm Hg during 60 minutes, followed by 5 hours of resuscitation. Five hours after resuscitation in saline-treated animals, firm leukocyte-sinusoidal adhesion was strongly enhanced in the periportal and midzonal sublobular regions, and also sinusoidal diameters were markedly reduced. Compared with saline-treated animals, GP515 significantly attenuated shock and resuscitation-induced leukocyte adhesion in both sublobular regions. Moreover, while GP515 did not significantly affect macrohemodynamical and hematological parameters, it also enlarged narrowed sinusoidal diameters and tended to improve sinusoidal blood flow. The anti-adhesive and microcirculatory effects of GP515 at 5 hours reperfusion were paralleled by reduced liver injury 2 days after resuscitation from shock. We propose that the adenosine-regulating agent GP515 has a therapeutic potential to attenuate ischemia-reperfusion-induced inflammation, by capitalizing on the beneficial anti-inflammatory effects of endogenous adenosine.

6.1 INTRODUCTION

Ischemia-reperfusion injury is an important pathological event, that may result from restoration of blood circulation in a variety of serious clinical conditions, such as acute arterial obstruction, major vascular surgery, and hemorrhagic shock. Hypovolemic shock, as occurs after trauma or complex surgical procedures, followed by resuscitation, essentially represents a "whole-body" ischemia-reperfusion insult, and is clinically often associated with the development of a systemic inflammatory response syndrome and multiple organ dysfunction syndrome (1, 2). Central to the pathogenesis of the inflammatory response to ischemia and reperfusion is the activation of neutrophils and their

subsequent adhesion to the vascular endothelium (3). Strategies that are aimed at attenuating the inflammatory cascades elicited during ischemia and reperfusion, include the use of anti-oxidants, PAF antagonists, anti-cytokine mAbs, and mAbs directed against vascular and leukocyte adhesion molecules. During recent years however, the anti-inflammatory potential of the endogenous metabolite adenosine has become an important focus of attention of studies seeking alternative therapeutical strategies to prevent or inhibit the detrimental inflammatory response after ischemia and reperfusion. In this context, adenosine has been termed a "retaliatory metabolite" (4). The reported anti-inflammatory effects of adenosine include inhibition of cytokine release (5-9), expression of endothelial adhesion molecules (6), leukocyte endothelial adhesion (10), and neutrophil function (11-13). Although numerous *in vitro* studies have demonstrated the anti-adhesive effects of adenosine, only few reports exist on the effects of adenosine on adhesion during postischemic conditions *in vivo* (14, 15). However, the therapeutic use of adenosine and its analogues has been limited by severe side effects, such as hypotension and bradycardia, as well as a short half life. The strategy underlying the use of adenosine-regulating agents is to enhance endogenous adenosine concentrations at local sites of inflammation, thus enhancing the beneficial anti-inflammatory effects of adenosine, while minimizing systemic toxicity. Under physiological conditions, adenosine is generated by dephosphorylation of AMP. This step is reverted by the enzyme adenosine kinase which phosphorylates adenosine to AMP. Therefore, one possibility to interfere with adenosine metabolism in order to increase endogenous adenosine levels, is to inhibit the rephosphorylation of adenosine by blocking adenosine kinase. GP515 is a novel adenosine kinase inhibitor, which anti-inflammatory actions have indeed been related to enhancement of endogenous concentrations of adenosine (10, 11, 16, 17). This compound has been reported to protect from mortality in two rodent models of septic shock without displaying systemic side effects (7). Currently however, its potential therapeutic use in ischemia-reperfusion has not been investigated. As outlined above, enhanced leukocyte-endothelial adhesion is a pivotal early step in the development of postischemic neutrophil-mediated injury of various target organs, such as the liver and the lungs (18-20). Therefore, we examined the effects of the adenosine-regulating agent GP515 on leukocyte-endothelial interactions in the liver in a rat model of hemorrhagic shock and resuscitation, using intravital microscopy as described by Marzi *et al.* (21).

6.2 MATERIALS AND METHODS

6.2.1 ANIMALS

The experiments were performed using female Sprague-Dawley rats (200-250 g body weight), obtained from Charles River, Sulzfeld, Germany. The study design was approved by the local veterinarian ethics committee. Rats had free access to standard rat chow and water until the day of the experiment. The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ using a temperature-controlled warming plate (Conrad Electronic, Hirschau, Germany).

6.2.2 SURGICAL PREPARATION

All surgical preparations were performed under aseptic conditions. Following induction of anesthesia and dermal disinfection, the rats were tracheotomized and intubated to keep the airways free and to allow spontaneous breathing. The animals were then instrumented for invasive monitoring of hemodynamical parameters as follows: the left carotid artery was cannulated with a thermistor-tipped 1.5 F catheter, which was advanced into the aortic arc for measurement of cardiac output (CO) by the transpulmonary thermodilution method (Cardiotherm 500, Columbus Instruments, Columbus, OH). The right internal jugular vein was cannulated with polyethylene tubing (0.4 x 0.8 mm, Portex, Hythe, UK) as a central venous access for infusion of GP515 and resuscitation fluids. The left femoral artery was cannulated with polyethylene tubing (0.4 x 0.8 mm) for continuous invasive monitoring of the mean arterial blood pressure (MAP) and heart rate (HR) using a pressure transducer (Hellige SMK 154-9, Freiburg, Germany). This line was used for shock induction and to take blood samples for laboratory analyses (blood gas analysis by ABL, Radiometer, Copenhagen, Denmark; hematological parameters by SYSMEX 2000, Digitana, Frankfurt, Germany). Blood samples (300 μl each) were drawn at baseline, prior to shock induction, at the end of shock, and after 1 h and 5 h of resuscitation. After shock, bloodlosses due to sampling were isovolumetrically substituted with shed blood.

6.2.3 EXPERIMENTAL GROUPS

Animals were randomly assigned to two shock groups and two non-shock control groups, receiving either the adenosine kinase inhibitor GP515 (kindly

provided by Gensia, Inc., San Diego, CA) or an equal amount of vehicle (saline) in a blinded manner. Thus, four experimental groups were formed ($n=8$ each): a *shock/saline* group (S-S), a *shock/GP515* group (S-GP), a *control/saline* group (C-S), and a *control/GP515* group (C-GP). GP515 was administered at a dose of 0.25 mg/kg diluted in 2 ml saline and infused intravenously during a 30-min period followed by an additional 30 min of steady state prior to induction of hemorrhagic hypotension. In preliminary dose-finding studies, 0.25 mg/kg infused over a 30-min period, was found to be the critical dose to produce only a slight, non-significant initial hypotensive effect, with MAP stable thereafter. The experimental group assignment is summarized in Table 6.1. Two additional groups were investigated to examine the effects of GP515 on shock and resuscitation-induced inflammation and liver injury at two days after hemorrhagic shock and resuscitation. Animals of the 2-day shock groups ($n=5$ each) were treated either with GP515 (2D-GP) or saline (2D-S). After two days, the serum activity of liver transaminases and organ edema were measured and compared with a time-matched sham-control group (2D-C).

Table 6.1 *Experimental groups*

Group	<i>n</i>	shock	treatment	reperfusion time
C-S (control/saline)	8	no	saline	5 h
C-GP (control/GP515)	8	no	GP515	5 h
S-S (shock/saline)	8	yes	saline	5 h
S-GP (shock/GP515)	8	yes	GP515	5 h
2D-C (control/saline)	5	no	saline	2 d
2D-S (shock/saline)	5	yes	saline	2 d
2D-GP (shock/GP515)	5	yes	GP515	2 d

6.2.4 HEMORRHAGIC SHOCK AND RESUSCITATION

After pretreatment with either GP515 or vehicle, hemorrhagic hypotension was induced within 5 min by blood withdrawal from the femoral arterial line, until MAP was 40 mm Hg. Shed blood was citrated to inhibit coagulation. The shock model used was pressure-controlled, *i.e.* if MAP was > 45 mm Hg, additional blood was drawn. Hypotension was maintained at 40 mm Hg for 60 min, and subsequently 60% of shed blood was retransfused. The animals were

additionally resuscitated with lactated Ringer's solution with twice the shed blood volume in the first hour, once the shed blood volume in the second and third hour, and with 10 ml/kg/h in the fourth and fifth hour. This resuscitation regimen was previously shown to sufficiently restore systemic circulation following hemorrhagic hypotension (22). Control animals were instrumented in an identical manner, but were not subjected to hypotension and subsequent resuscitation.

6.2.5 INTRAVITAL MICROSCOPY

Five hours after the start of resuscitation from shock, or at the corresponding timepoint in control animals, the hepatic microcirculation was investigated by intravital microscopy. The abdomen was opened by a midline laparotomy, and the left liver lobe was mobilized after dissection of the hepatic ligaments. The animal was positioned onto its left side on a specially designed plexiglass stage, that allowed gentle exteriorization of the left liver lobe, with its lower plane surface uppermost. The liver surface was covered with plastic foil and continuously superfused with saline at 37°C to prevent desiccation. The hepatic microcirculation was then investigated using a Nikon MM-11 epifluorescence microscope (Tokyo, Japan) with a 100 W mercury lamp, a 545 nm excitation filter, a 10x/0.30 water immersion objective, a 0.7-2.25 zoom objective, a 12x ocular, and a final magnification of 330x. The experiments were recorded with a low-light CCD camera (FK6990, Pieper, Schwerte, Germany), which was connected through a serial time-date generator (VTG 33, FOR-A Company, Tokyo, Japan) with a SVHS video recording system (Panasonic FS-1, Tokyo, Japan). Acridine orange (1 mg/ml in saline; Sigma, St. Louis, MO) as fluorescence marker of leukocytes was injected i.v. as bolus of 0.1 mg per recorded liver lobule. The microcirculation and leukocyte-endothelial interactions in five liver lobules were observed and recorded during 30 sec for off-line evaluation. Next, five central venules were brought into the center of the image, and the pericentral region was recorded for comparable determination of sinusoidal diameters at distances of 90 μ m from the central vein.

Upon completion of the experiments, the livers were harvested, and wet weight was determined. Next, the organs were dried at 60°C for 24 h, and weighed again, to determine wet/dry ratio as a marker of organ edema.

6.2.6 EVALUATION

The recorded sequences of intravital liver microscopy were evaluated off-line by an independent observer blinded to the experimental group assignment.

Leukocyte adhesion was determined as the number of leukocytes per mm² of liver surface for each of the three different sublobular fields of the liver acinus as described by Rappaport (23). According to recent previous studies (21, 22), two distinct types of leukocyte adhesion were differentiated: reversible, temporary adhesion (adhesion time: <20 sec; mean adhesion time: 1-3 sec), and mostly irreversible, firm adhesion (adhesion time: >20 sec).

The hepatic microcirculation was quantitated by assessment of sinusoidal diameters and estimated sinusoidal blood flow, as determined by leukocyte velocity measurements using a computer-supported morphometrical image analysis system (Lobulus, Medvis, Homburg, Germany). Flow was calculated under the assumption of circular diameters of sinusoids using the following equation: $F = v_L \star \pi \star (D_s/2)^2$, where F is flow, v_L is leukocyte velocity, and D_s is sinusoidal diameter.

6.2.7 STATISTICAL ANALYSIS

Data are presented as mean $\pm$ SEM. Differences between groups were tested by analysis of variance and post-hoc Student-Newman-Keuls test. Differences between various timepoints within one group were tested by paired t -test. A p -value <0.05 was considered statistically significant.

6.3 RESULTS

6.3.1 HEMODYNAMICAL PARAMETERS

At baseline, MAP, HR, and CO were identical in all experimental groups (Figures 6.1A, 6.1B, and 6.1C). Infusion of 0.25 mg/kg GP515 over a 30-min period resulted in a moderate, non-significant reduction of MAP from 125.4 ± 5.6 mm Hg to 102.9 ± 7.8 mm Hg in the control group (C-GP), and from 128.9 ± 4.6 mm Hg to 103.3 ± 6.2 mm Hg in the shock group (S-GP). Also, after infusion of GP515, MAP in both GP515-treated groups did not differ significantly from both saline-treated control groups at the corresponding timepoint (T=30 min). Similarly, i.v. administration of GP515 did not affect HR and CO prior to shock.

Figure 6.1 Mean arterial blood pressure (A), heart rate (B), and cardiac output (C) during steady state, hemorrhagic shock, and resuscitation periods

Compared with baseline, a slight, but non-significant increase in CO was observed during the first hour of the experiment, which was however identical in all experimental groups. Hemorrhagic hypotension was induced and maintained constantly at 40 mm Hg during 60 min, resulting in a reduction of HR and CO, which was the same in the saline-treated and GP515-treated group. Resuscitation completely restored shock-induced hemodynamic changes in both shock groups, with MAP, HR, and CO being identical compared with both control groups. Moreover, no significant differences in these hemodynamical parameters were observed between saline-treated and GP515-treated animals.

6.3.2 HEMATOLOGICAL PARAMETERS

Prior to induction of shock, pH, blood gases, hemoglobin concentration (Hb), hematocrit (Hct), and white blood cell count (WBC), were comparable in all experimental groups (Table 6.2). Compared with the end of steady state ($T=60$ min), shock resulted in a significant elevation of p_aO_2 , and a reduction of p_aCO_2 as well as reduced Hb and Hct. These changes were not altered by pretreatment with GP515. At the end of shock, p_aO_2 , p_aCO_2 , Hb and Hct were significantly altered compared to non-shocked animals. During reperfusion, no significant differences with regard to these parameters were observed between any of the experimental groups. Neither in control animals nor in shock animals, did GP515 affect any of the hematological parameters determined.

6.3.3 LIVER WET/DRY RATIO AND TRANSAMINASES

Liver wet dry/weight ratio's revealed no differences between all experimental groups at 5 h after shock (data not shown). However, at 2 days after shock, a significant increase in liver wet/dry ratio was observed in the shock-control group, which could be prevented by GP515 (Figure 6.2A). Moreover, at this timepoint, the shock-induced increase of serum activity of the transaminase AST was significantly reduced in the GP515-treated group (Figure 6.2B).

6.3.4 LEUKOCYTE ADHESION

Five hours after resuscitation from shock, or at the corresponding timepoint in control animals, temporary leukocyte adhesion was identical in treated as well as untreated control and shock groups, ranging from $8.6 \pm 0.7\%$ to $9.7 \pm 0.5\%$ of the total number of sinusoidal leukocytes, and displayed no differences between the various sublobular regions (data not shown).

Table 6.2 Hematological parameters

	time (min)	C-S	C-GP	S-S	S-GP
pH	60	7.32 ± 0.02	7.34 ± 0.05	7.33 ± 0.04	7.33 ± 0.03
	120	7.37 ± 0.03	7.36 ± 0.04	7.32 ± 0.04	7.35 ± 0.03
	180	7.40 ± 0.02	7.38 ± 0.03	7.38 ± 0.02	7.39 ± 0.03
	420	7.33 ± 0.02	7.33 ± 0.01	7.36 ± 0.02	7.37 ± 0.02
p _a O ₂ (mm Hg)	60	70.5 ± 6.5	77.2 ± 13.4	65.7 ± 9.8	73.3 ± 6.4
	120	80.2 ± 11.6	74.8 ± 10.9	107.9 ± 14.2 ^{*#}	105.2 ± 16.6 ^{*#}
	180	84.1 ± 6.3	83.0 ± 18.7	68.5 ± 6.5	77.4 ± 6.7
	420	108.1 ± 8.2	101.5 ± 11.6	99.7 ± 10.5	110.1 ± 12.4
p _a CO ₂ (mm Hg)	60	32.5 ± 4.3	32.0 ± 5.1	38.2 ± 9.3	35.2 ± 6.1
	120	31.0 ± 4.3	31.1 ± 5.8	21.4 ± 5.2 ^{*#}	23.2 ± 6.5 ^{*#}
	180	28.8 ± 4.4	31.1 ± 2.9	32.3 ± 4.9	30.1 ± 3.2
	420	37.8 ± 6.9	34.3 ± 5.9	39.2 ± 1.6	33.5 ± 9.4
Hb (mmol/l)	60	6.6 ± 0.7	6.7 ± 0.6	6.4 ± 0.6	6.6 ± 0.4
	120	6.3 ± 0.5	6.5 ± 0.6	3.9 ± 0.6 ^{*#}	4.1 ± 0.4 ^{*#}
	180	6.0 ± 0.4	6.1 ± 0.6	5.0 ± 0.2	5.1 ± 0.4
	420	6.5 ± 0.6	6.4 ± 1.1	5.2 ± 0.8	5.3 ± 0.4
Hct (%)	60	29.7 ± 2.6	30.5 ± 1.9	29.6 ± 2.1	30.5 ± 1.9
	120	29.5 ± 2.0	29.4 ± 1.9	19.6 ± 2.6 ^{*#}	19.8 ± 1.8 ^{*#}
	180	27.2 ± 1.7	27.9 ± 2.2	23.9 ± 1.3	24.0 ± 1.5
	420	29.2 ± 2.6	28.3 ± 4.1	24.8 ± 3.4	24.8 ± 1.7
WBC (x 10 ⁹ /l)	60	5.2 ± 2.3	5.5 ± 1.4	4.5 ± 1.1	4.7 ± 1.9
	120	6.8 ± 3.1	5.7 ± 1.2	3.9 ± 1.1	4.0 ± 1.2
	180	6.2 ± 2.3	5.7 ± 1.3	4.5 ± 1.3	4.2 ± 1.0
	420	7.4 ± 1.5	6.5 ± 1.8	5.0 ± 1.8	5.7 ± 1.3

* indicates $p < 0.05$ versus C-S, # indicates $p < 0.05$ versus T=60 within the same experimental group.

Figure 6.2 Liver wet/dry weight ratios (A) and serum activity of transaminases (B) at 2 days after hemorrhagic shock and resuscitation

* indicates $p < 0.05$ versus 2D-C, # indicates $p < 0.05$ versus 2D-S.

The number of leukocytes firmly adhering to the endothelial wall was comparable in both control groups (Figure 6.3), with the highest adhesion numbers in the periportal region (C-S: 394 ± 16 leukocytes/mm², and C-GP: 352 ± 27 leukocytes/mm²). In the non-treated shock group (S-S), firm leukocyte adhesion was significantly enhanced in the periportal (1032 ± 95 leukocytes/mm²) and midzonal (387 ± 35 leukocytes/mm²) regions of the liver acinus compared with both control groups. Also, in the pericentral field, the

number of adhering leukocytes was $311 \pm 77 / \text{mm}^2$ in the S-S group, compared with $126 \pm 54 / \text{mm}^2$ in the C-S group ($p < 0.05$), and $172 \pm 62 / \text{mm}^2$ in the C-GP group ($p > 0.05$). Pretreatment with GP515 markedly attenuated leukocyte adhesion after shock, with a significant reduction in the periportal as well as the midzonal field to 562 ± 22 leukocytes/ mm^2 and 286 ± 37 leukocytes/ mm^2 , respectively. Moreover, GP515 reduced shock-induced firm leukocyte adhesion in these two fields to a level comparable with saline-treated controls. GP515 also reduced shock-induced adhesion in the pericentral region to 224 ± 89 leukocytes/ mm^2 , however without reaching statistical significance compared with saline-treated shock animals.

Figure 6.3 GP515 reduces firm leukocyte adhesion in the liver 5 h after hemorrhagic shock and resuscitation, as determined by intravital microscopy

* indicates $p < 0.05$ versus C-S, # indicates $p < 0.05$ versus S-S.

6.3.5 HEPATIC MICROCIRCULATION

Mean sinusoidal diameter in the saline-treated control group was $11.6 \pm 0.3 \mu\text{m}$, and was non-significantly increased by approximately 7% in the GP515-treated control group (Figure 6.4A). Following shock and resuscitation, sinusoids were markedly narrowed to a mean diameter of $7.8 \pm 0.2 \mu\text{m}$. In comparison, pretreatment with GP515 partly prevented this sinusoidal narrowing, resulting in a mean sinusoidal diameter of $9.8 \pm 0.1 \mu\text{m}$, a significant increase of

approximately 26%. In parallel, GP515 tended to enhance reduced sinusoidal blood flow after shock (Figure 6.4B), almost reaching statistical significance (S-S: 37.5 ± 1.1 $\mu\text{l}/\text{sec}$ vs. S-GP: 50.8 ± 3.5 $\mu\text{l}/\text{sec}$, $p=0.06$). In control animals however, GP515 did not affect sinusoidal blood flow.

Figure 6.4 GP515 attenuates narrowing of sinusoidal diameters (A) and enhances sinusoidal blood flow (B) 5 h after hemorrhagic shock and resuscitation

* indicates $p < 0.05$ versus C-S, # indicates $p < 0.05$ versus S-S.

6.4 DISCUSSION

In the present study we have determined the effects of pretreatment with the adenosine kinase inhibitor GP515 on firm leukocyte-endothelial adhesion in the liver after 1 hour of hemorrhagic shock followed by 5 hours of reperfusion. Previous studies using this animal model have revealed two different patterns of leukocyte adhesion in the liver after hemorrhagic shock. First, temporary (short-term) adhesion is maximally elevated in the first hour of resuscitation, and declines thereafter (21). This type of reversible leukocyte-endothelial interaction may be the correlate of postcapillary leukocyte rolling, and represents an early step in neutrophil recruitment to inflammatory sites. Second, firm (long-term) adhesion, also referred to as leukocyte sticking, increases gradually during the reperfusion period, and is significantly elevated 5 hours after resuscitation from shock (22). Adherence of neutrophils to the vascular endothelium is an important step in neutrophil-mediated injury of endothelial cells, a key pathological event in ischemia reperfusion (3). While initial rolling is considered a prerequisite for firm adherence (24), it is the strong adhesive neutrophil-endothelial interaction that is thought to create a sequestered micro-environment, in which activated neutrophils release proteolytic enzymes and reactive oxygen metabolites that cause the endothelial damage, eventually resulting in increased microvascular permeability and organ edema (3, 25).

Therefore, in order to study firm adhesion of leukocytes to the liver sinusoids, we selected the 5-hour reperfusion time frame. Indeed, our present results confirm these earlier observed adhesion patterns, with firm adhesion highly elevated in both shock groups compared with both control groups, and temporary adhesion being identical in all experimental groups. Also, the sublobular adhesion pattern with increasing numbers of firmly adhering leukocytes in the pericentral, midzonal, and periportal regions, respectively, was consistent with previous results in this model (21). Firm leukocyte adhesion to sinusoidal endothelium as observed in this study was previously shown not to be due to mechanical trapping of leukocytes in narrowed sinusoids, but was suggested to be a receptor-specific event (22). The results of the study cited clearly indicate the involvement of adhesion receptors such as ICAM-1 and the corresponding CD11b/CD18 complex in the process of firm leukocyte adhesion.

The most prominent finding of this study is that GP515 strongly attenuated shock-induced firm adhesion of leukocytes to the sinusoidal endothelium. As reported by Vedder *et al.* (19, 20) and Mileski *et al.* (26), inhibition of CD18-dependent firm neutrophil adherence effectively reduces neutrophil-mediated

injury to lungs, liver and the gastrointestinal tract after hemorrhagic shock in rodents as well as in subhuman primates. Similarly, in isolated hepatic ischemia-reperfusion models, it has been established that massive neutrophil infiltration contributes to postischemic liver injury (18, 27). While in the aforementioned hemorrhagic shock models gross organ injury was evident after long-term resuscitation, in the present study no gross liver edema, as determined by liver wet/dry ratio's, was present after 5 h of resuscitation. However, at 2 days after shock, a significant increase in liver wet/dry ratio was observed, which might be due to tissue injury by activated and transmigrated leukocytes. GP515 had protective effects at this timepoint, as indicated by reduced liver wet/dry ratio and significantly reduced serum activity of AST.

The observed anti-adhesive effect of the adenosine-regulating agent GP515 is consistent with reported effects of adenosine and adenosine A2 receptor agonists on neutrophil-endothelial interactions *in vitro* (10, 28-30) and *in vivo* (14, 15, 17, 31). Although we did not measure *in vivo* adenosine levels in this study, GP515 has been demonstrated to enhance local endogenous adenosine concentrations *in vitro* (10), and *ex vivo* (11) as well as *in vivo* (16). While activation of neutrophil A2 receptors has been implicated in the anti-adhesive effect of adenosine, currently it has not been established which specific types of leukocyte adhesion molecules are affected by adenosine. Firestein *et al.* (10) have observed that adenosine interferes with L-selectin-mediated adhesion, and does not affect CD18-dependent adhesion in a static adhesion assay. Others have demonstrated that *in vitro* integrin-mediated adhesion can also be modulated by adenosine (32, 33). Interestingly, adenosine has been shown to inhibit stimulated upregulation of the CD11/CD18 integrin on human neutrophils *in vitro* (33). Since GP515 attenuated the firm adhesive leukocyte-endothelial interactions, our results would be in support of altered integrin-mediated adhesion, although we can not rule out an early effect on selectin-mediated rolling. The present results are paralleled by previous similar findings in this same model obtained with the natural glycoprotein neutrophil inhibitory factor, that inhibits CD11b/CD18-mediated adhesion (34).

Adenosine A2 receptors are thought to couple unifocally to adenylate cyclase, with activation resulting in enhanced generation of intracellular cAMP (35). Elevated neutrophil intracellular cAMP has indeed been related to inhibition of CD11b/CD18 surface expression *in vitro* (32), thus supporting a role for A2 receptors in down-modulating postischemic integrin-mediated adhesion. In line with this, the cAMP-raising agent pentoxifylline attenuated hemorrhagic shock-induced leukocyte-sinusoidal adhesion in this animal model in an identical fashion as described here for GP515 (36).

Whereas neutrophils have clearly been identified as targets for the anti-inflammatory actions of adenosine, endothelial cells have not been ruled out as potential targets for adenosine. On the contrary, adenosine has been shown to inhibit expression of E-selectin and VCAM-1, by activated human endothelial cells *in vitro* (6). Also, the adenosine analogue 3-deazaadenosine inhibits ICAM-1 biosynthesis in TNF α -stimulated cultured human endothelium (37). It remains therefore to be determined whether interference of adenosine with endothelial expression of adhesion molecules is involved in the inhibition of neutrophil-endothelial interactions *in vivo*.

A second interesting observation in this study is that GP515 displayed a tendency to improve local microcirculation in the liver after resuscitation from shock. Whereas sinusoidal diameters in non-treated shock-animals were significantly reduced compared with sham-operated animals, GP515 significantly enhanced sinusoidal diameters after shock. Although GP515 also slightly enlarged sinusoidal diameters in control animals, it had a relatively stronger effect on sinusoidal diameter after shock. In parallel, while sinusoidal blood flow was significantly reduced after shock, GP515 tended to improve sinusoidal flow after shock, almost reaching statistical significance ($p=0.06$), but was without effect on blood flow in control animals. While adenosine is known to exert negative chronotropic and dromotropic effects (38), and to cause hypotension (39), GP515 did not affect the cardiovascular parameters MAP, HR and CO as compared with saline-treated animals in this study. Moreover, systemic hematological parameters were not altered by GP515. Together, these data suggest that GP515 acts to enhance endogenous adenosine at its local site of formation, thereby limiting systemic side effects, and at the same time capitalizing on well-recognized beneficial microcirculatory effects of adenosine during ischemia and reperfusion (40, 41).

In summary, using an experimental model that allowed direct visualization of hepatic leukocyte-endothelial interactions and microcirculation, we have demonstrated that systemic administration of the adenosine-regulating agent GP515 has significant anti-adhesive and mild beneficial microcirculatory effects in the early inflammatory response to hemorrhagic shock and resuscitation, which are paralleled by reduced liver injury at the long term. Our results point to the therapeutic potential of GP515 and possibly other adenosine-regulating agents (42-44) in the treatment of ischemia-reperfusion injury.

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CHAPTER 7

General Discussion

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The anti-inflammatory potential of adenosine in ischemia-reperfusion injury:
established and putative beneficial actions of a retaliatory metabolite

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7.1 A NEW DIMENSION TO THE RETALIATORY METABOLITE CONCEPT?

As mentioned in *Chapter 1*, adenosine has been termed a retaliatory metabolite in the setting of ischemia-reperfusion, mainly by virtue of its favorable metabolic and cardiovascular actions (Table 7.1). In addition, its well-documented specific inhibitory effects on neutrophil superoxide anion generation *in vitro* have been assumed to underlie its anti-inflammatory, cytoprotective role in reperfusion injury.

In this thesis, we have presented results from several *in vitro* studies, describing novel anti-inflammatory actions of adenosine on different immunocompetent cell types, which are known to be involved in the inflammatory response to ischemia-reperfusion, as well as in other inflammatory conditions. In addition, new data have recently emerged from experimental studies by others, that have considerably broadened the scope of adenosine's anti-inflammatory actions (Table 7.1). Although most of these new findings originate from *in vitro* studies, and have thus far not all been confirmed in *in vivo* ischemia-reperfusion, in our view they may have potential relevance to this pathological entity, and are therefore discussed here. Together, the data presented in this thesis suggest that the endogenous metabolite adenosine may be effective at the effector, the mediator, as well as the tissue level of reperfusion injury (Figure 7.1). Moreover, some of these newly recognized anti-inflammatory features of adenosine have indeed been proven effective in other inflammatory conditions, such as sepsis, where disturbances in organ microcirculation and tissue hypoxia appear to play an important role as well.

7.1.1 ANTI-INFLAMMATORY ACTIONS AT THE EFFECTOR LEVEL

In *Chapter 2* we have reported that adenosine inhibits *ex vivo* release of neutrophil granule constituents in human whole blood stimulated with either LPS or $\text{TNF}\alpha$, and that this inhibition occurs through involvement of both A2 and A3 adenosine receptors. A modulatory effect of adenosine on neutrophil degranulation had been suggested previously in few *in vitro* studies using isolated neutrophil suspensions (1, 2), but had thus far remained controversial (3-5). Particularly the inhibition of elastase release by adenosine could provide a possible target for therapeutic intervention in inflammatory conditions that are characterized by neutrophil-mediated proteolytic tissue injury. In this respect, it is of significance that Inauen *et al.* (6) have emphasized the importance of neutrophil elastase in hypoxia-reoxygenation-induced endothelial cell injury. Moreover, there is progressive and compelling evidence that the contribution of

elastase and other neutrophil proteases to postischemic injury *in vivo* may be as prominent as the oxidant injury (7-12), and that neutrophil proteolytic enzymes act in synergism with OFR to produce reperfusion injury (13). Therefore, besides its well-characterized effects on neutrophil oxidative burst, adenosine might also contribute considerably to limitation of microvascular reperfusion injury by inhibiting neutrophil degranulation.

Figure 7.1 Schematic diagram of the established and putative actions of adenosine on neutrophil chemotaxis and activation, and subsequent endothelial adherence and transendothelial emigration in ischemia-reperfusion

EC = endothelial cell; MΦ = macrophage/monocyte; SLe^x = sialyl-Lewis X tetrasaccharide; XD = xanthine dehydrogenase; XO = xanthine oxidase. For other abbreviations, see text and List of Abbreviations.

Activation of cellular anti-oxidant defense systems has recently been proposed as a novel mechanism whereby adenosine may provide protection against OFR-mediated reperfusion injury (14, 15). In these studies, evidence was presented that endogenous adenosine increases the activity of the anti-oxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase in a variety of cell types, including rat basophilic leukocytes and human endothelial cells. These effects were found to be dependent on A3 receptor activation and were accompanied by a decrease in the degree of lipid peroxidation in these cells. Thus, complementary to inhibition of oxidant production, stimulation of anti-oxidant activity by adenosine also seems to be involved in its cytoprotective effect. In view of this, the results described in *Chapter 5*, where pharmacological interference in adenosine metabolism potentially enhanced adenosine release from endothelial cells subjected to hypoxia, could be of interest.

Table 7.1 *Established and putative beneficial effects of adenosine in ischemia-reperfusion.*

	References
<i>Metabolic effects</i>	
purine salvaging	
inhibition of lipolysis	
enhancement of glucose transport	
stimulation of gluconeogenesis	
<i>Cardiovascular effects</i>	
vasodilation	
inhibition of platelet aggregation	
inhibition of vasoconstrictive mediators	
negative inotropy and dromotropy	
preconditioning	
<i>Anti-inflammatory effects</i>	
inhibition of neutrophil superoxide	
inhibition of neutrophil degranulation	1, 2, Chapter 2
activation of anti-oxidants	14, 15
inhibition of cytokines	18-22, Chapters 3 and 4
inhibition of eicosanoids	25, 26
inhibition of complement	27
inhibition of adhesion molecule expression or adhesion	29-32, Chapters 4 and 6

7.1.2 ANTI-INFLAMMATORY ACTIONS AT THE MEDIATOR LEVEL

In *Chapter 3* we have described the potent inhibitory actions of adenosine on the release of the pro-inflammatory cytokines $\text{TNF}\alpha$, IL-6 and IL-8 by LPS- or IL-1 β -stimulated human monocytes. Moreover, in *Chapter 4* we demonstrate that adenosine also negatively affects release of IL-6 and IL-8 by activated cultured human endothelial cells. Recently, several other groups have independently presented evidence that adenosine can reduce cytokine release. Specifically, release of $\text{TNF}\alpha$ by activated human monocytes (16, 17) and macrophages of human (18) as well as rodent origin (19-21) is potently attenuated by adenosine. While we and others have demonstrated the participation of A2 receptors in adenosine-mediated inhibition of $\text{TNF}\alpha$ (17, 18, 21), recently a major role for A3 receptors has been implicated in this event (18). Given the involvement of $\text{TNF}\alpha$ as a proximal mediator in the pathogenesis of sepsis and ischemia-reperfusion, this anti-inflammatory feature of adenosine may prove to be useful in the treatment of these conditions. Indeed, adenosine and adenosine-regulating agents reduce systemic levels of $\text{TNF}\alpha$ and protect from mortality in various murine models of septic shock (19, 20). Moreover, we have shown that adenosine is also capable of attenuating release of other pro-inflammatory cytokines, such as IL-6 and IL-8 by cultured human monocytes as well as endothelial cells. Likewise, its structural analogue 3-deazaadenosine has been demonstrated to inhibit the production and biological activity of IL-1 derived from human monocytes *in vitro* (22), but a similar inhibitory effect of adenosine itself on IL-1 release *in vivo* could thus far not be confirmed (19, 20). In addition to its inhibitory actions on the production of pro-inflammatory cytokines, adenosine has recently been shown by LeMoine *et al.* (23) to enhance the secretion of the anti-inflammatory cytokine IL-10.

Interestingly, they suggested that this phenomenon participates in adenosine-mediated inhibition of $\text{TNF}\alpha$, which, in addition to the known inhibitory effects of IL-10 on OFR production (24), could be of significance in the setting of ischemia-reperfusion. Furthermore, adenosine has recently been found to inhibit synthesis of predominantly neutrophil-derived LTB_4 in $\text{TNF}\alpha$ and LPS-activated human whole blood *ex vivo* via engagement of A2 receptors (25), which could have specific significance with regard to sepsis as well as ischemia-reperfusion. Likewise, release of TxA_2 can be inhibited by adenosine in *in vivo* coronary ischemia and reperfusion (26). Adenosine may also interfere with activation of the classical complement cascade, as it has been demonstrated to inhibit the production of the second complement component (C_2) by binding to A2 receptors on human monocytes (27).

7.1.3 ANTI-INFLAMMATORY ACTIONS AT THE TISSUE LEVEL

While the anti-adhesive actions of adenosine have been attributed to its effects on neutrophil expression of integrins and selectins, endothelial cells have not been ruled out as potential targets for the anti-adhesive actions of adenosine. On the contrary, in *Chapter 4* we have presented evidence that adenosine is capable of inhibiting *de novo* expression of E-selectin and VCAM-1 by cultured human endothelial cells. Although we could not demonstrate an inhibitory effect on upregulated expression of ICAM-1, others (28) have demonstrated that the structural adenosine analogue 3-deazaadenosine inhibits TNF α -induced ICAM-1 upregulation by HUVEC. In *Chapter 6* we have presented data indicating that endogenous adenosine attenuates firm leukocyte-endothelial adhesion in liver sinusoids after hemorrhagic shock and resuscitation, suggesting an inhibitory effect on integrin-mediated adhesion. Similarly, interference of adenosine with integrin-mediated leukocyte-endothelial interactions has been suggested in other experimental *in vivo* ischemia-reperfusion models (29-31). Others have demonstrated an inhibitory effect of adenosine on selectin-mediated adhesion (32). However, actual downregulation of endothelial expression of adhesion molecules by adenosine or its analogues *in vivo* has thus far not been demonstrated, and the potential relevance of these *in vitro* findings to inhibition of ischemia-reperfusion-induced leukocyte-endothelial adhesion remains therefore to be determined.

7.2 THERAPEUTIC IMPLICATIONS

As described above, adenosine exerts metabolic, cardiovascular, and anti-inflammatory activities that may provide protection against reperfusion injury (summarized in Table 7.1), and, consequently, this has raised the possibility of therapeutic intervention to take advantage of its established and putative beneficial effects. However, the therapeutic use of adenosine is limited by its severe cardiovascular side effects, as well as its very short halflife in the systemic circulation. As stated earlier, adenosine acts on cells in an autocrine and paracrine manner. Therefore, as an alternative therapeutic strategy, different pharmacological agents have been developed that increase endogenous adenosine concentrations at its local site of formation, thereby enhancing its protective actions confined to its target site, and at the same time minimizing systemic side effects. As demonstrated in Figure 7.2 and summarized in Table 7.2, there are several potential approaches to implement this strategy. While

some of these pharmacological approaches have indeed been proven successful in limiting ischemia-reperfusion injury *in vivo*, both in animal models as well as clinically, others have been focusing mainly on the diverse anti-inflammatory effects of adenosine, and have been successfully applied in acute inflammatory conditions other than ischemia-reperfusion, such as septic shock and skin models of inflammation.

Figure 7.2 Intracellular metabolism of adenosine and potential targets for pharmacological interventions

1 = adenosine kinase, 2 = adenosine deaminase, 3 = nucleoside transport, 4 = AICAR transformylase.

Table 7.2 Potential therapeutic pharmacological interventions in adenosine metabolism in ischemia-reperfusion and other inflammatory disorders

	References
Adenosine kinase inhibitors	19, 32-35, Chapters 2, 5 and 6
Adenosine deaminase inhibitors	35, 38-45, 49, Chapters 3 and 4
Nucleoside transport inhibitors	2, 35, 39, 41, 46, 48, 49, 51, 70
Acadesine	52, 54-66

7.2.1 ADENOSINE KINASE INHIBITION

In this thesis, we have used the adenosine kinase inhibitor GP515 in several studies to support the significance of endogenous adenosine in the observed inhibitory effects on inflammatory cell function. In *Chapter 2* it was demonstrated that GP515 significantly enhances endogenous adenosine levels in whole blood *ex vivo*, thereby attenuating neutrophil degranulation. Likewise, potentiating effects of GP515 on endothelial adenosine release were reported in *Chapter 5*. These findings are supported by previous studies demonstrating that inhibition of adenosine kinase by GP515 or other adenosine kinase inhibitors enhances endogenous extracellular adenosine concentrations (32-35). In these studies GP515 reduced selectin-mediated neutrophil adhesion *in vitro* (32), as well as neutrophil emigration and vascular leakage in carrageenan-induced inflammation *in vivo* (33, 34). The observation that GP515 enhances endothelial adenosine release, particularly during hypoxic conditions as was demonstrated in *Chapter 5*, leads us to hypothesize that pharmacological interference in endothelial adenosine metabolism may be relevant to the treatment of reperfusion injury. In support of this hypothesis, it is of interest that adenosine released from endothelial cells has indeed been demonstrated to inhibit neutrophil activation and subsequent endothelial injury (36, 37). Moreover, consistent with the reported effects of adenosine *in vitro*, GP515 was recently shown to reduce systemic release of TNF α and pulmonary neutrophil accumulation, and concomitant mortality, in different animal models of septic shock (19). In *Chapter 6* we have also demonstrated its efficacy in the setting of ischemia-reperfusion, by potently attenuating hemorrhagic shock and resuscitation-induced leukocyte-endothelial adhesion and sinusoidal narrowing in the rat liver, and reducing liver injury.

7.2.2 ADENOSINE DEAMINASE INHIBITION

Pharmacological inhibition of adenosine deaminase, to enhance endogenous adenosine levels, has been applied mainly in the setting of myocardial and cerebral ischemia and reperfusion (35, 38, 39). The adenosine-mediated cardioprotective and neuroprotective actions of adenosine deaminase inhibitors include enhanced recovery of high-energy phosphates (40-42), improved blood flow (35, 40), and scavenging of OFR (43, 44). Analogous to the inhibitory effects of the adenosine kinase inhibitor GP515 on neutrophil degranulation *in vitro*, as described in *Chapter 2*, we have demonstrated that adenosine deaminase inhibition by EHNA or deoxycytosine reduces release of TNF α by monocytes (*Chapter 3*), and expression of adhesion molecules by endothelial cells *in vitro* (*Chapter 4*), presumably by enhancing endogenous levels of adenosine. Similarly, Eigler *et al.* (45) have recently emphasized the significance of endogenous adenosine in inhibition of monocyte-derived TNF α production.

7.2.3 NUCLEOSIDE TRANSPORT INHIBITION

The rationale for the use of nucleoside transport inhibitors is to prevent the washout of adenosine from the ischemic interstitial space to the vascular lumen upon reperfusion, thus prolonging the accumulation of adenosine in the ischemic and reperfused area (46). Specifically in the myocardium, where the nucleoside transport protein has been localized mainly in the endothelium lining the coronary vessels, inhibition of the functional transporter prevents degradation of adenosine within the endothelial cell by adenosine deaminase, and delays subsequent clearance into the circulation (46). The presence of an endothelial nucleoside transporter has also been demonstrated in microvessels of the brain and other organs (47). Hence, nucleoside transport inhibitors have been proven protective from reperfusion injury in the heart (39, 48, 49), and brain (41, 50). Recently, a protective effect of nucleoside transport blockade on post-transplant renal function and survival was demonstrated in a canine model of kidney autotransplantation (51).

7.2.4 ACADESINE

The purine nucleoside analogue acadesine (AICA riboside (=AICAR), 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide) has been termed the prototype adenosine-regulating agent for reducing myocardial ischemic injury (52, 53).

After cellular uptake, it enters the *de novo* purine biosynthetic pathway by phosphorylation to AICA-ribotide (ZMP), and is subsequently further metabolized to inosine monophosphate (IMP). The combined weak inhibitory effects of acadesine on adenosine deaminase and adenosine kinase, and of ZMP on enzymes that are reciprocally involved in IMP-AMP conversion (Figure 7.2), are thought to account for the adenosine-enhancing activity of acadesine (52, 53). Its functional benefits have been extensively studied in various models of myocardial ischemia and reperfusion, and may include many of the cardiovascular, metabolic and anti-inflammatory effects described above (54-58). While the protective effects of acadesine have yet been evaluated in human myocardial ischemia, its beneficial potential has also been demonstrated in animal models of intestinal ischemia-reperfusion (59) and hemorrhagic shock and resuscitation (60, 61). Moreover, the potential relevance of AICAR to treatment of inflammatory conditions other than ischemia-reperfusion has recently become evident, as it was established that the anti-inflammatory mechanism of methotrexate and sulfasalazine, two antiphlogistic agents widely used in the treatment of rheumatoid arthritis and inflammatory bowel diseases, is in fact due to their capacity to induce accumulation of AICAR with subsequent increased adenosine release at inflamed sites (62-66).

7.3 CONCLUSION AND PERSPECTIVES

In this thesis we have depicted the anti-inflammatory potential of adenosine. The concept of adenosine as an immunoregulatory agent, as originally introduced by Cronstein, was elaborated further by demonstrating the suppressive actions of adenosine on several cell types of human origin, that are involved in inflammatory reactions. While we have applied adenosine agonists and antagonists to investigate the effects of adenosine in the experimental studies described in this thesis, we have also provided evidence for a role of endogenous adenosine using adenosine-regulating agents that augment endogenous adenosine levels. Based on our findings, we propose that while under physiological conditions adenosine seems to be an important mediator of metabolic, cardiovascular, and immunological homeostasis, it could provide a "natural defense system" against severe metabolic and inflammatory insults. Particularly in ischemia-reperfusion, where its formation is directly linked to ATP catabolism, adenosine could provide protective effects at multiple levels in the pathogenesis of this condition. Therefore, pharmacological enhancement of this endogenous, multifactorial defense mechanism by agents that promote

adenosine release at its site of formation may have potential therapeutic relevance to protect from ischemia-reperfusion injury. Some of these adenosine-regulating agents, such as acadesine and nucleoside transport inhibitors, have already proven their therapeutic efficacy in the clinical setting, whereas others are still in an experimental or preclinical stage, but seem to hold therapeutic promise. Given the broad anti-inflammatory potential of adenosine, pharmacological interference in endogenous adenosine metabolism could form an attractive alternative strategy for the therapeutic approaches outlined in *Chapter 1*, since it may be effective at multiple inflammatory levels, and is not accompanied by the autoimmune phenomena associated with monoclonal or polyclonal antibody therapy (67, 68). Moreover, by capitalizing on the anti-inflammatory actions of endogenous adenosine, adenosine-regulating agents may also prove to be useful in the treatment of other inflammatory conditions, including sepsis, inflammatory bowel diseases, and rheumatoid arthritis.

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CHAPTER 8

Summary / Samenvatting

SUMMARY

In the first section of *Chapter 1*, the clinical relevance and the pathophysiology of the inflammatory response to ischemia-reperfusion are outlined, emphasizing the role of the polymorphonuclear leukocyte (neutrophil) as the primary effector cell of reperfusion injury. A central pathogenetic mechanism in the development of reperfusion injury is the generation of oxygen free radicals, predominantly by the capillary endothelium, which induce the production and release of several inflammatory mediators, such as arachidonic acid metabolites, cytokines and activated complement proteins. As a result, neutrophils are attracted to the inflammatory site, become activated and adhere to the vascular endothelium, and finally transmigrate through the endothelial barrier into the target tissue. The neutrophil-endothelial adhesive interactions consist of sequential stages, each involving different types of complementary adhesion molecules, expressed on neutrophils and endothelial cells, respectively. Eventually, activated neutrophils cause tissue injury by producing oxidants and proteolytic enzymes that degrade the extracellular matrix and induce cellular injury. Thus, three pathophysiological levels of ischemia-reperfusion injury can be distinguished: the effector level (neutrophils and oxidants), the mediator level (arachidonic acid metabolites, cytokines, activated complement-proteins), and the tissue level (the vascular endothelium expressing adhesion molecules). Accordingly, we describe the therapeutic approaches to ischemia-reperfusion injury, that are aimed at interfering at either the effector, the mediator or the tissue level of inflammation.

In the second section of the introduction, the role of adenosine as a retaliatory metabolite during ischemia and reperfusion is depicted. Adenosine, a metabolite of ATP degradation during ischemia, counteracts the deleterious effects of ischemia and reperfusion on the cardiovascular system and metabolism as a result of ligation of specific cell-surface adenosine receptors. By reducing metabolic demand and adjusting cellular energy supply, adenosine "retaliates" against the external ischemic stimulus that causes its formation, and has therefore been termed a "retaliatory metabolite" by Newby. Adenosine binding to A₂ receptors also effectively inhibits superoxide generation by activated neutrophils and reduces neutrophil adhesiveness to the endothelium, thus exerting anti-inflammatory effects. Based on the concept, originally introduced by Cronstein, that adenosine functions as an endogenous immunoregulatory negative feedback mechanism during inflammation, the main hypothesis underlying the experimental work described in this thesis, is that adenosine downregulates the inflammatory function of various different

immunocompetent cell types that are involved in inflammatory responses, particularly during ischemia and reperfusion. Furthermore, we hypothesized that pharmacological interference in adenosine metabolism, resulting in enhanced local endogenous adenosine levels, could represent an attractive, alternative target of therapeutic intervention in inflammatory conditions, including reperfusion injury.

In *Chapter 2* it is demonstrated that adenosine effectively inhibits the release of the granule proteins elastase, bactericidal/permeability-increasing protein, and defensins by TNF α - and LPS-stimulated neutrophils in human whole blood. Using specific adenosine receptor agonists and antagonists the involvement of A2 as well as A3 receptors in adenosine-mediated inhibition of neutrophil degranulation is evidenced, while the presence of A3 receptors in human neutrophils is demonstrated for the first time. In addition, the adenosine-regulating agent GP515 is shown to attenuate degranulation via an adenosine-mediated mechanism, pointing to its potential relevance to the treatment of neutrophil-mediated tissue injury.

In *Chapter 3*, we provide evidence that adenosine inhibits the production of TNF α , IL-6 and IL-8 by LPS-activated human monocytes *in vitro* with a differential potency. The results obtained with the adenosine analogue 2-chloroadenosine indicate that inhibition of cytokine release by adenosine is primarily an A2-mediated event, and suggest an additional 'retaliatory' action of adenosine during pathological conditions, in which cytokine production by activated mononuclear phagocytes is involved, such as sepsis and ischemia-reperfusion injury.

In *Chapter 4* the effects of adenosine on two major determinants of endothelial cell activation, *i.e.* the release of pro-inflammatory cytokines and the expression of adhesion molecules, are studied. Adenosine dose-dependently inhibits the release of IL-6 and IL-8 by stimulated human umbilical vein endothelial cells (HUVEC). Expression of E-selectin and VCAM-1, but not ICAM-1, by activated HUVEC is also reduced by adenosine. Inhibition of endogenous adenosine deaminase activity by EHNA or deoxycoformycin strongly enhances the inhibitory effects of exogenous adenosine on cytokine release and expression of E-selectin and VCAM-1. However, a clear role for specific adenosine-receptors in the described inhibitory events could not be established. Taken together, these data imply that the vascular endothelium constitutes an important target for the anti-inflammatory actions of adenosine.

In Chapter 5 we use a well-controlled *in vitro* hypoxia-reoxygenation model, mimicking *in vivo* ischemia-reperfusion, to investigate in parallel the effects of hypoxia and reoxygenation on endothelial activation and adenosine release. We find that neither hypoxia nor reoxygenation for varying periods of time, induce pro-inflammatory activation of HUVEC, as determined by cell-surface expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1, as well as by extracellular release of IL-6 and IL-8, although HUVEC remain morphologically and functionally intact under these conditions. At the same time however, hypoxia significantly enhances the release of adenosine by HUVEC, an event that can be strongly potentiated by the adenosine kinase inhibitor GP515, suggesting that the anti-inflammatory potential of the endothelium can be enforced during hypoxia by pharmacological interference in endogenous adenosine metabolism.

In Chapter 6 the efficacy of the adenosine-regulating agent GP515 in *in vivo* ischemia-reperfusion is evaluated, by investigating its effects on the hepatic inflammatory and microcirculatory response to hemorrhagic shock and resuscitation in the rat. While in untreated rats subjected to hemorrhagic shock and resuscitation, firm leukocyte-sinusoidal adhesion was strongly enhanced in the periportal and midzonal sublobular regions, and sinusoidal diameters were markedly reduced, pretreatment with GP515 significantly attenuated shock and resuscitation-induced leukocyte adhesion in both regions, enlarged sinusoidal diameters, and tended to improve sinusoidal blood flow. These results were associated with reduced liver injury two days after shock and resuscitation. GP515 did not display systemic macrohemodynamical or hematological side effects. We hypothesize that the adenosine-regulating agent GP515 has a therapeutic potential to protect from reperfusion injury *in vivo* by capitalizing on the beneficial anti-inflammatory and microcirculatory actions of endogenous adenosine.

In Chapter 7 we propose that the newly discovered anti-inflammatory features of adenosine have added a new dimension to the "retaliatory metabolite" concept, as originally introduced by Newby. We have found that adenosine affects the inflammatory function of neutrophils, monocytes and endothelial cells. Based on the results from the experiments described in this thesis, as well as on data that have recently emerged from experimental studies by others, we conclude that the anti-inflammatory potential of adenosine extends to the effector, the mediator, as well as the tissue level of inflammation. Therefore, pharmacological enhancement of endogenous adenosine levels at its

local site of formation, could represent an attractive, alternative therapeutic strategy in various inflammatory conditions, including ischemia-reperfusion injury. Finally, several of these pharmacological approaches are discussed in relation to ischemia and reperfusion, as well as other inflammatory disorders.

SAMENVATTING

In het eerste gedeelte van *Hoofdstuk 1* worden de klinische relevantie en de pathofysiologie van de ontstekingsreactie op ischemie-reperfusie beschreven, waarbij de rol van de polymorf nucleaire leukocyt (de neutrofiel) als primaire effector cel in het ontstaan van reperfusieschade wordt benadrukt. Een centraal pathogenetisch mechanisme van reperfusieschade is de vorming van vrije zuurstof radicalen, overwegend door het capillair endotheel, die de productie en afgifte van verschillende ontstekingsmediatoren, zoals arachidonzuur metabolieten, cytokinen, en geactiveerde complement eiwitten induceren. Als gevolg daarvan worden neutrofielen aangetrokken naar de plaats van ontsteking en vervolgens geactiveerd, waarna ze hechten aan het endotheel van de vaatwand en tenslotte transmigreren door de endotheel barrière naar het weefsel. Adhesie van neutrofielen aan het endotheel voltrekt zich in verschillende opeenvolgende stadia, waarin steeds verschillende typen adhesiemoleculen betrokken zijn, die complementair tot expressie worden gebracht door respectievelijk neutrofielen en endotheel. Uiteindelijk veroorzaken geactiveerde neutrofielen weefselschade door de productie van oxidantia en proteolytische enzymen, die de extracellulaire matrix afbreken en celschade induceren. Er kunnen aldus drie niveaus in de pathofysiologie van ischemie-reperfusieschade worden onderscheiden: het effector niveau (vrije zuurstof radicalen en neutrofielen), het mediatoren niveau (arachidonzuur metabolieten, cytokinen, geactiveerde complement-eiwitten), en het weefsel niveau (het vasculaire endotheel dat adhesiemoleculen tot expressie brengt). We beschrijven dienovereenkomstig de therapeutische benaderingen die interfereren op het effector, het mediatoren, dan wel het weefsel niveau van de ontstekingsreactie na ischemie-reperfusie.

In het tweede gedeelte van de inleiding wordt de rol van adenosine als "vergeldings-metaboliet" in ischemie en reperfusie beschreven. Adenosine ontstaat als afbraakprodukt van ATP tijdens ischemie, en gaat de schadelijke gevolgen van ischemie en reperfusie op het cardiovasculaire systeem en het metabolisme tegen, doordat het aan specifieke adenosine receptoren op de celmembraan bindt. Doordat het de metabole vraag reduceert en de energievoorziening van de cel corrigeert, "vergelt" adenosine de externe ischemische stimulus die zijn vorming veroorzaakt, en is daarom door Newby een "vergeldings-metaboliet" genoemd. Door binding aan A₂ receptoren remt adenosine bovendien de vorming van superoxide door geactiveerde neutrofielen en vermindert het de adhesie van neutrofielen aan endotheel, en oefent daarmee ontstekingsremmende effecten uit. Gebaseerd op het idee, oorspronkelijk

geïntroduceerd door Cronstein, dat adenosine functioneert als een endogeen immuunregulerend negatief terugkoppelingsmechanisme tijdens ontsteking, is de belangrijkste hypothese die ten grondslag ligt aan het experimentele werk, zoals dat in dit proefschrift is beschreven, dat adenosine de inflammatoire activiteit van verschillende immunocompetente celtypen die betrokken zijn bij ontstekingsreacties, in het bijzonder tijdens ischemie en reperfusie, remt. Een tweede hypothese is dat farmacologische interventie in het metabolisme van adenosine, resulterend in verhoogde lokale spiegels van endogeen adenosine, een aantrekkelijk alternatief doelwit van therapeutische interventie zou kunnen vormen in ontstekingsprocessen, zoals reperfusieschade.

In *Hoofdstuk 2* wordt aangetoond dat adenosine op effectieve wijze de afgifte van de granule eiwitten elastase, bactericidal/permeability-increasing protein en defensins door $\text{TNF}\alpha$ - en LPS-gestimuleerde neutrofielen in humaan volbloed remt. Door gebruik te maken van specifieke adenosine receptor agonisten en antagonisten, wordt het bewijs geleverd voor de betrokkenheid van zowel A2 als A3 receptoren in de inhibitie van neutrofiel degranulatie door adenosine. Bovendien wordt voor het eerst de aanwezigheid van A3 receptoren op humane neutrofielen aangetoond. Verder wordt beschreven dat de adenosine-regulerende stof GP515 degranulatie remt via een adenosine-gemedieerd mechanisme, hetgeen wijst op de mogelijke therapeutische relevantie van deze stof ten aanzien van weefselschade die wordt veroorzaakt door neutrofielen.

In *Hoofdstuk 3* tonen we aan dat adenosine de productie van $\text{TNF}\alpha$, IL-6 en IL-8 door LPS-geactiveerde humane monocytten remt met een verschillende potentie. Resultaten verkregen met het adenosine-analoon 2-chloroadenosine wijzen erop dat de remming van cytokine afgifte door adenosine vooral door A2 receptoren wordt gemedieerd, en suggereren het bestaan van een additioneel "vergeldings-effect" van adenosine tijdens situaties die worden gekenmerkt door verhoogde cytokine productie door geactiveerde mononucleaire fagocyten, zoals sepsis en ischemie-reperfusieschade.

In *Hoofdstuk 4* worden de effecten van adenosine op twee determinanten van endotheelcel activatie, namelijk de afgifte van pro-inflammatoire cytokinen en de expressie van adhesiemoleculen, bestudeerd. Adenosine remt op dosisafhankelijke wijze de afgifte van IL-6 en IL-8 door gestimuleerde humane navelstrengvene endotheelcellen (HUVEC). De expressie van E-selectin en VCAM-1, maar niet van ICAM-1, door geactiveerd HUVEC wordt eveneens

geremd door adenosine. Inhibitie van endogene adenosine deaminase activiteit door EHNA of deoxycoformycine versterkt de remmende effecten van exogene adenosine op de afgifte van cytokinen en de expressie van E-selectin en VCAM-1 op krachtige wijze. Een duidelijke rol van specifieke adenosine receptoren in de beschreven remmende effecten van adenosine kon echter niet worden vastgesteld. Tesamen impliceren deze gegevens dat het vasculaire endotheel een belangrijk doelwit vormt van de anti-inflammatoire activiteit van adenosine.

In *Hoofdstuk 5* gebruiken we een goed gecontroleerd *in vitro* hypoxie-reoxygenatie model, waarin *in vivo* ischemie-reperfusie wordt nagebootst, om de effecten van hypoxie en reoxygenatie op endotheel activatie en afgifte van adenosine tegelijkertijd te kunnen onderzoeken. We stellen vast dat, hoewel HUVEC morfologisch en functioneel intact blijven onder deze omstandigheden, noch hypoxie noch reoxygenatie van verschillende tijdsduur, pro-inflammatoire activatie van HUVEC induceren, gemeten aan zowel de expressie van de adhesiemoleculen E-selectin, ICAM-1 en VCAM-1, als aan de extracellulaire afgifte van IL-6 en IL-8. Tegelijkertijd echter verhoogt hypoxie op significante wijze de afgifte van adenosine door HUVEC. De afgifte van adenosine door HUVEC tijdens hypoxie kan in hoge mate worden versterkt door de adenosine kinase remmer GP515, hetgeen suggereert dat de potentiële ontstekingsremmende activiteit van het endotheel tijdens hypoxie kan worden versterkt door farmacologisch ingrijpen in het endogene adenosine metabolisme.

In *Hoofdstuk 6* wordt de effectiviteit van de adenosine-regulerende stof GP515 gedurende *in vivo* ischemie-reperfusie bestudeerd aan de hand van de ontstekingsreactie en de microcirculatie in de lever na hemorrhagische shock en resuscitatie in de rat. In onbehandelde ratten die werden onderworpen aan hemorrhagische shock en resuscitatie, was de adhesie van leukocyten aan de sinusoiden sterk verhoogd in de periportale en midzonale gedeelten van de leverlobuli, en waren de sinusoidale diameters aanzienlijk gereduceerd. Behandeling met GP515 voorafgaand aan shock en resuscitatie verminderde daarentegen de leukocyten-adhesie in beide sublobulaire regio's op significante wijze, vergrootte de sinusoidale diameters, en verbeterde de bloeddoorstroming van de sinusoiden enigszins. Deze bevindingen werden vergezeld door een afname van de leverschade twee dagen na shock en resuscitatie. Behandeling met GP515 had geen systemische hemodynamische of hematologische bijwerkingen. We formuleren de hypothese dat de adenosine-regulerende stof

GP515 therapeutische potentie tot bescherming tegen *in vivo* reperfusieschade heeft, doordat het de gunstige ontstekingsremmende en microcirculatoire activiteiten van endogeen adenosine bevordert.

In *Hoofdstuk 7* stellen we dat de recente nieuwe ontdekkingen van ontstekingsremmende eigenschappen van adenosine een nieuwe dimensie hebben toegevoegd aan het begrip "vergeldings-metaboliet", zoals dat oorspronkelijk door Newby werd geïntroduceerd. We hebben vastgesteld dat adenosine de inflammatoire functie van neutrofielen, monocytten en endotheelcellen beïnvloedt. Op grond van zowel de resultaten van de experimenten zoals die beschreven zijn in dit proefschrift, als de gegevens die recentelijk naar voren zijn gekomen uit experimentele studies die door anderen werden verricht, concluderen we dat adenosine remmende activiteit kan hebben op zowel het effector niveau, als het mediators niveau, als het weefsel niveau van ontsteking. Derhalve zou farmacologische verhoging van lokale, endogene adenosine spiegels een aantrekkelijke alternatieve strategie kunnen zijn in de behandeling van ontstekingsreacties, zoals ischemie-reperfusieschade. Tenslotte worden enkele van dergelijke farmacologische interventies in ischemie en reperfusie, en andere inflammatoire aandoeningen, besproken.

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LIST OF PUBLICATIONS

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CURRICULUM VITAE

Maarten Bouma werd op 21 juni 1963 geboren in Groningen. Na het behalen van het diploma Gymnasium- β aan het Willem Lodewijk Gymnasium (1975-1981) in zijn geboortestad, startte hij er in 1981 met de studie Geneeskunde aan de Rijksuniversiteit Groningen. De wachttijd voor de co-schappen werd deels doorgebracht met het volgen van een klinische stage op de afdeling Chirurgie van het Mubarak Al-Kabeer Hospital te Koeweit. Op 26 augustus 1988 legde hij het artsexamen af. Nadien was hij gedurende enkele maanden werkzaam als ambulance-begeleider bij de Zieken Transport Maatschappij B.V. te Groningen en vervolgens van 1989 tot 1991 als assistent-geneeskundige op de afdeling Chirurgie van het Academisch Ziekenhuis Groningen (*Hoofd*: Prof. Dr. R. van Schilfgaarde). Na opnieuw enige maanden bij de ambulancedienst Z.T.M. te hebben gewerkt, was hij van juni 1991 tot maart 1995 als research-assistent Traumatologie verbonden aan de afdeling Chirurgie van het Academisch Ziekenhuis Maastricht (*Hoofd*: Prof. Dr. G. Kootstra) en in die hoedanigheid werkzaam op het Laboratorium Algemene Heelkunde van de Universiteit Maastricht. Daar heeft hij onder leiding van Dr. W.A. Buurman, immunoloog, en Dr. F.A.J.M. van den Wildenberg, ongevalschirurg, ondermeer het in dit proefschrift beschreven onderzoek verricht. Van 1995 tot 1996 was hij klinisch werkzaam als assistent-geneeskundige op de afdeling Chirurgie van het Academisch Ziekenhuis Maastricht. Sinds 1 december 1996 is hij in het kader van de opleiding tot orthopedisch chirurg werkzaam als arts-assistent in vooropleiding bij de Maatschap Chirurgie van het Sint Joseph Ziekenhuis te Veldhoven (*Opleider*: Dr. F.A.A.M. Croiset van Uchelen). Begin 1999 zal hij zijn opleiding voortzetten op de afdeling Orthopedie van het Academisch Ziekenhuis Maastricht.