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PPAR γ inhibits NF- κ B-dependent transcriptional activation in skeletal muscle

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Remels AH, Langen RC, Gosker HR, Russell AP, Spaapen F, Voncken JW, Schrauwen P, Schols AM. PPAR γ inhibits NF- κ B-dependent transcriptional activation in skeletal muscle. *Am J Physiol Endocrinol Metab* 297: E174–E183, 2009. First published May 5, 2009; doi:10.1152/ajpendo.90632.2008.—Skeletal muscle pathology associated with a chronic inflammatory disease state (e.g., skeletal muscle atrophy and insulin resistance) is a potential consequence of chronic activation of NF- κ B. It has been demonstrated that peroxisome proliferator-activated receptors (PPARs) can exert anti-inflammatory effects by interfering with transcriptional regulation of inflammatory responses. The goal of the present study, therefore, was to evaluate whether PPAR activation affects cytokine-induced NF- κ B activity in skeletal muscle. Using C₂C₁₂ myotubes as an in vitro model of myofibers, we demonstrate that PPAR, and specifically PPAR γ , activation potently inhibits inflammatory mediator-induced NF- κ B transcriptional activity in a time- and dose-dependent manner. Furthermore, PPAR γ activation by rosiglitazone strongly suppresses cytokine-induced transcript levels of the NF- κ B-dependent genes intracellular adhesion molecule 1 (ICAM-1) and CXCL1 (KC), the murine homolog of IL-8, in myotubes. To verify whether muscular NF- κ B activity in human subjects is suppressed by PPAR γ activation, we examined the effect of 8 wk of rosiglitazone treatment on muscular gene expression of ICAM-1 and IL-8 in type 2 diabetes mellitus patients. In these subjects, we observed a trend toward decreased basal expression of ICAM-1 mRNA levels. Subsequent analyses in cultured myotubes revealed that the anti-inflammatory effect of PPAR γ activation is not due to decreased RelA translocation to the nucleus or reduced RelA DNA binding. These findings demonstrate that muscle-specific inhibition of NF- κ B activation may be an interesting therapeutic avenue for treatment of several inflammation-associated skeletal muscle abnormalities.

peroxisome proliferator-activated receptor- γ ; nuclear factor- κ B; rosiglitazone; inflammation; type 2 diabetes mellitus; skeletal muscle atrophy

THERE IS INCREASING EVIDENCE in the literature that chronic activation of nuclear factor- κ B (NF- κ B), which is an essential signaling pathway involved in conveying inflammatory signals, is causally related to skeletal muscle pathology. Wasting of skeletal muscle mass is commonly observed in chronic inflammatory diseases and has been associated with increased morbidity and mortality (20, 44). Several studies have shown that systemic inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), are proximal markers of cachexia (24, 30, 35). More specifically, quadriceps muscle biopsies of severely cachectic patients with chronic obstructive pulmonary disease (COPD) and chronic heart failure are characterized by activation of the NF- κ B signaling

pathway (1, 2). Moreover, forced activation of NF- κ B in skeletal muscle causes profound muscle wasting that resembles clinical cachexia, while inhibition of NF- κ B signaling has proven to be sufficient to block inflammation-induced protein degradation in cultured skeletal muscle myotubes (10, 24). In type 2 diabetes mellitus (T2DM), skeletal muscle insulin resistance is associated with decreased protein abundance of the inhibitory protein of NF- κ B I κ B α (39) and increased transcript levels of NF- κ B-dependent genes, including IL-8 in skeletal muscle (17, 27). These observations indicate that anti-inflammatory agents, capable of inhibiting NF- κ B signaling, could be useful for treatment of inflammation-associated skeletal muscle abnormalities in chronic disease states.

Interestingly, it is known that activation of peroxisome proliferator-activated receptors (PPARs) can exert anti-inflammatory effects in several cell types, such as smooth muscle cells, endothelial cells, and macrophages (40, 42). PPARs are a set of nuclear receptors implicated in a multitude of physiological processes (16). They display transcriptional activity and are tissue-selectively expressed (9). To date, three PPAR subtypes have been identified: PPAR α , PPAR δ , and PPAR γ (41). Anti-inflammatory properties of the PPARs include the potential to interfere with transcriptional pathways involved in inflammatory responses, e.g., modulation of NF- κ B signaling. Several mechanisms have been described, including inhibition of I κ B α degradation, reduction of RelA (p65) nuclear translocation, and diminished binding of RelA to the DNA (21, 46). Data on the potential of PPARs to interfere with NF- κ B signaling in skeletal muscle, however, is lacking. Therefore, in the present study we investigated whether PPAR activation in skeletal muscle is able to modulate inflammatory mediator-induced NF- κ B activity. Our results reveal that PPAR γ activation suppresses cytokine-induced NF- κ B transcriptional activity and target gene expression in skeletal muscle independently of nuclear translocation and DNA binding.

SUBJECTS AND METHODS

Cell culture. The murine skeletal muscle cell line C₂C₁₂ was obtained from the American Type Culture Collection (ATCC no. CRL1772; Manassas, VA). These cells are able to undergo differentiation into spontaneously contracting myotubes upon growth factor withdrawal (25). C₂C₁₂ cells were cultured as described previously (26). Cells were treated with a submaximal dose of TNF- α (1 ng/ml) or IL-1 β (1 ng/ml) and/or PPAR agonists at day 6 of differentiation.

Chemicals and reagents. Tetracyclithioacetic acid (TTA) (Sigma Aldrich, Zwijndrecht, the Netherlands) was dissolved in 100% ethanol to a final concentration of 100 mM and coupled to 0.4% fatty acid-free bovine serum albumin (BSA) (Sigma Aldrich) in differentiation medium (DM) by 30' incubation at 37°C before use. Rosiglitazone-maleate (Alexis Biochemicals, Lausen, Switzerland), WY-14643 (Bi-omol, Plymouth Meeting, PA, USA), GW-501516 (Alexis Biochemi-

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icals), and GW-1929 (Sigma Aldrich) were all dissolved in 100% dimethyl sulfoxide (DMSO) to a stock solution of 50, 5, 25, and 20 mM, respectively. TNF- α and IL-1 β (both from Calbiochem, San Diego, CA) were dissolved in 0.1% BSA.

Transfections and plasmids. For assessment of NF- κ B transcriptional activation, C₂C₁₂ cells were stably transfected with the 6 κ B-TK luciferase plasmid (NF- κ B reporter) as described previously (26). For assessment of PPAR transcriptional activation, C₂C₁₂ cells were stably transfected with a Human CPT IB promoter (HCBP) luciferase reporter plasmid (PPAR reporter, pSG5; Stratagene, La Jolla, CA). Cells were grown to 70% confluence and transfected using Lipofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Transfection was performed in the presence of 3.75 μ g HCBP plasmid DNA and 0.25 μ g of a plasmid containing the neomycin resistance gene (pSV2-Neo; Stratagene). For selection of positive clones, cells were cultured in GM containing 800 μ g/ml G418 (Calbiochem). Transient transfections were all performed by Lipofectamine according to the manufacturer's instructions (Invitrogen).

Reporter assays. The NF- κ B- or the PPAR-sensitive reporter cell lines were plated in 35-mm dishes and allowed to grow to 70–80% confluence. Cells were differentiated for 6 days and then treated with the various stimuli. After the appropriate incubation times, cells were washed twice with cold phosphate-buffered saline (PBS) and subsequently lysed by adding (100 μ l) 1 \times Reporter Lysis Buffer (Promega, Madison, WI) and incubation on ice for 10 min. Cell lysates were centrifuged (13,000 g, 1 min), and supernatants were either snap-frozen and stored at -80°C for later analysis or placed on ice for immediate analysis. Luciferase activity was measured according to manufacturer's instructions (Promega) and corrected for total protein content (Bio-Rad, Hercules, CA). For assessment of the inhibitory potential of PPAR activation on NF- κ B transcriptional activation, cells from the NF- κ B-sensitive reporter cell line were incubated with PPAR activators prior to the administration of the inflammatory stimulus (TNF- α or IL-1 β).

RNA isolation, cDNA synthesis, and qPCR. Total RNA from C₂C₁₂ myotubes was extracted using the acid guanidium thiocyanate-phenol-chloroform extraction method (Ambion, Ijssel, The Netherlands). RNA concentration was determined using a spectrophotometer. One microgram of RNA per sample was reverse transcribed into cDNA according to the manufacturer's instructions (Reverse-IT-TM 1st strand synthesis; Abgene, Leusden, The Netherlands). Transcript levels of intracellular adhesion molecule 1 (ICAM-1) and the chemokine CXCL1 (KC: keratinocyte-derived chemokine) were determined in C₂C₁₂ samples using a Bio-Rad quantitative PCR apparatus. Transcript levels for the constitutive housekeeping gene product cyclophilin were quantitatively measured in each sample to control for sample-to-sample differences in RNA concentration. ICAM-1 and IL-8 mRNA levels were determined in cDNA generated from skeletal muscle from diabetic subjects treated with rosiglitazone. Gene expression was quantified and expressed as arbitrary units (AU). Total RNA was extracted from these skeletal muscle biopsies using TRIzol reagent (Invitrogen, Breda, The Netherlands). One microgram of RNA was reverse transcribed to cDNA using random hexamer primers and a Stratascript enzyme (Stratagene, Amsterdam, The Netherlands). Reverse transcription and quantitative PCR (qPCR) were performed using an MX3000p thermal cycler system and Brilliant SYBR Green QPCR Master Mix (Stratagene). To control for any variations in the efficiencies of the reverse transcription and PCR, acidic ribosomal phosphoprotein PO (36B4) was used as an internal control. The number of cycles at which the best-fit line through the log linear portion of each amplified curve intersects the noise band is inversely proportional to the log copy number. This value is referred to as the critical threshold (C_T) value. ΔC_T was calculated by subtracting the C_T for 36B4 from the C_T for the gene of interest. The relative expression of the gene of interest is calculated using the

expression $\Delta\Delta\text{C}_T$ and reported as arbitrary units. All PCR runs were performed in triplicate.

Preparation of nuclear extracts and western blot analysis. Nuclear extracts were prepared as described previously (45). For Western blot analysis, 4 \times Laemmli sample buffer [0.25 M Tris-HCl, pH 6.8, 8% (wt/vol) SDS, 40% (vol/vol) glycerol, 0.4 M DTT, and 0.04% (wt/vol) bromophenol blue] was added to nuclear extracts, followed by boiling of the samples for 5 min at 100°C and storage at -20°C . Total protein was assessed using the Bio-Rad DC protein assay kit according to the manufacturer's instructions. Fifteen (RelA) or 2 μ g (HIS-3) of protein was loaded per lane and separated on a 10% or 12% polyacrylamide gel (Mini Protean 3 system, Bio-Rad; RelA and HIS-3 respectively), followed by transfer to a 0.45- μ m nitrocellulose membrane (Bio-Rad) by electroblotting. The membrane was blocked for 1 h at room temperature in 5% (wt/vol) nonfat dry milk. Nitrocellulose blots were washed in PBS-Tween 20 (0.05%), followed by overnight incubation (4°C) with a polyclonal antibody specific for RelA (Santa Cruz Biotechnology, Santa Cruz, CA) or HIS-3 (Abcam, Cambridge, UK). After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

Electrophoretic mobility shift analysis. DNA binding activity of NF- κ B was assessed in nuclear extracts by analysis of complexes binding to an oligonucleotide containing a κ B consensus sequence (Santa Cruz Biotechnology). Two micrograms of nuclear protein was used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25 \times Tris-borate-EDTA buffer at 120 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1; Kodak, Rochester, NY). Shifted complexes were quantified by PhosphorImager analysis (Bio-Rad). To determine the subcomposition of the complexes, supershift reactions were performed by preincubation of the nuclear extracts with an antibody specific to the RelA subunit of NF- κ B (Santa Cruz Biotechnology) or a negative IgG antibody.

Chromatin immunoprecipitation assay. After removal of the medium, 1% formaldehyde (Fisher Scientific, Pittsburgh, PA) was added to the cells for 10 min at room temperature. Cross-linking was stopped by the addition of glycine (5 min) to a final concentration of 0.125 M. Cross-linked cells were washed twice with PBS, scraped, and resuspended in SDS buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.1, 5 mM EDTA, pH 8.0, 0.02% Na₃, and 0.5% SDS). Lysates were snap-frozen on dry ice. After thawing, the cells were pelleted (6 min, 500 g) at room temperature. Cells were subsequently sonicated (10 Amp, 16 cycles, 20 s on and 100 s off). Samples underwent Proteinase K and DNase treatment, and DNA was isolated using phenol-chloroform-isoamyl alcohol precipitation. Sonication efficiency was verified on a 2% agarose gel. The supernatant was precleared (20 min, 4°C) with protein A beads blocked with herring sperm. After removal of the beads by microcentrifugation (30 min, 14,000 g, room temperature), 4 μ g of RelA antibody (Abcam) was added overnight at 4°C with continuous mixing. A polyclonal antibody directed against hemagglutinin was used as a control antibody (Santa Cruz Biotechnology). Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A beads. Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent real-time PCR. Primers were designed around the RelA binding site in the ICAM-1 promoter: forward, 5'-GATGTCCTTCCGGTTGCAG-3'; reverse, 5'-CAGGGAAATTCCCGGAGTACA-3'.

Human subjects. To validate results from the in vitro setting, we investigated transcript levels of NF- κ B target genes in skeletal muscle of diabetic subjects who received rosiglitazone treatment, as reported previously (31, 37). In summary, 10 middle-aged obese men with T2DM participated in this study. Subjects had no major health problems besides their diabetes and did not use any medication known to interfere with the results of the study. The Medical Ethics Review Committee of Maastricht University approved the study protocol, and

all subjects gave their written informed consent before the start of the study. After baseline measurements, diabetic subjects received rosiglitazone (2×4 mg/day) for 8 wk. At the end of this 8-wk period, all measurements were repeated. Eight weeks of rosiglitazone treatment was effective in restoring insulin sensitivity in these subjects.

Collection and processing of muscle tissue. Postabsorptive muscle biopsies of the lateral part of the quadriceps femoris were obtained under local anesthesia using the needle biopsy technique. The specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. The frozen biopsies were weighed and subsequently homogenized using a Polytron PT 1600 E (Kinematica, Littau/Luzern, Germany) for RNA extraction.

Statistical analysis. Data were analyzed according to the guidelines of Altman et al. (3), using SPSS (Statistical Package for the Social sciences; SPSS, Chicago, IL). An unpaired Student's *t*-test was used for the in vitro data, and a paired nonparametric test was used for the gene expression analysis in skeletal muscle of rosiglitazone-treated diabetic patients. Data are represented as means \pm SD or median \pm range when appropriate for in vitro and in vivo data, respectively. A two-tailed probability value of less than 0.05 was considered to be significant.

RESULTS

NF- κ B activation by inflammatory cytokines. Transcriptional activation of NF- κ B was evaluated in a stable reporter cell line. To test responsiveness to inflammatory cytokines, C₂C₁₂ myotubes were stimulated with various concentrations of TNF- α for 4 h. Stimulation with TNF- α resulted in a dose-dependent increase in NF- κ B transcriptional activity (Fig. 1A). To assess whether this cell line was also responsive to inflammatory stimuli other than TNF- α , a similar stimulation experiment was performed using various concentrations of IL-1 β for 4 h, yielding similar results (Fig. 1B). These data demonstrate that TNF- α and IL-1 β potently induce NF- κ B transcriptional activity in differentiated myotubes.

Induction of PPAR transcriptional activity by specific PPAR ligands. Responsiveness of C₂C₁₂ myocytes to PPAR agonists was verified using a PPAR-sensitive promoter reporter. Administration of the general PPAR agonist TTA for 24 h to undifferentiated C₂C₁₂ myoblasts transiently transfected with a PPAR-sensitive reporter luciferase plasmid resulted in a dose-dependent increase in PPAR-dependent transcriptional activity (Fig. 2A). Treatment of C₂C₁₂ myoblasts with specific PPAR agonists (PPAR α : WY-14643; PPAR δ : GW-501516 or PPAR γ : rosiglitazone) for 24 h also resulted in a dose-dependent increase

in PPAR transcriptional activity, with a maximal induction at 50, 1, and 75 μM , respectively (data not shown). These data show that all PPAR activators used were able to induce PPAR transcriptional activity in C₂C₁₂ myoblasts. Next, a cell line containing the PPAR-sensitive reporter construct stably integrated in the genome was developed to validate the ability of the agonists to activate PPAR-dependent transcriptional activity in differentiated C₂C₁₂ myotubes. Stimulation of C₂C₁₂ myotubes with TTA (100 μM) or specific synthetic PPAR activators (WY-14643: 50 μM ; GW-501516: 1 μM , or rosiglitazone: 75 μM) resulted in an induction of PPAR transcriptional activity in a time-dependent manner with maximal inductions after 48 h of incubation (Fig. 2B). These data demonstrate that all PPAR activators used in this study induce PPAR-dependent transcriptional activity in C₂C₁₂ myoblasts and myotubes.

PPAR γ activation inhibits NF- κ B transcriptional activation. To assess the anti-inflammatory potential of PPAR agonists in skeletal muscle, C₂C₁₂ myotubes containing the NF- κ B-sensitive reporter were treated with general or specific PPAR ligands prior to exposure to proinflammatory cytokines. Pretreatment with TTA (100 μM) resulted in a time-dependent inhibition of TNF- α -induced NF- κ B transcriptional activity, with a maximal inhibitory effect at 30-min preincubation time (Fig. 3).

To assess whether the three PPAR isoforms have a differential inhibitory capacity on TNF- α -induced NF- κ B activation, preincubation experiments were performed with specific PPAR agonists. Preincubation with a PPAR α (WY-14643, 50 μM) or a PPAR δ (GW-501516, 1 μM) activator had no significant inhibitory effect on TNF- α -induced NF- κ B transcriptional activity (data not shown), whereas PPAR γ activation (rosiglitazone, 75 μM) resulted in a time- (Fig. 4A) and dose-dependent (Fig. 4B) inhibition of TNF- α -induced NF- κ B transcriptional activity.

In line with the observed inhibitory effect of rosiglitazone on TNF- α -induced NF- κ B transcriptional activity, rosiglitazone also inhibited IL-1 β -induced NF- κ B transcriptional activity in a dose- and time-dependent manner. Here, the optimal inhibitory effect was observed at 2 h preincubation with a concentration of 200 μM rosiglitazone (Fig. 5A). To confirm that rosiglitazone-mediated inhibition of cytokine-induced NF- κ B transcriptional activity did occur through a PPAR γ -dependent mechanism, another, structurally unrelated PPAR γ agonist

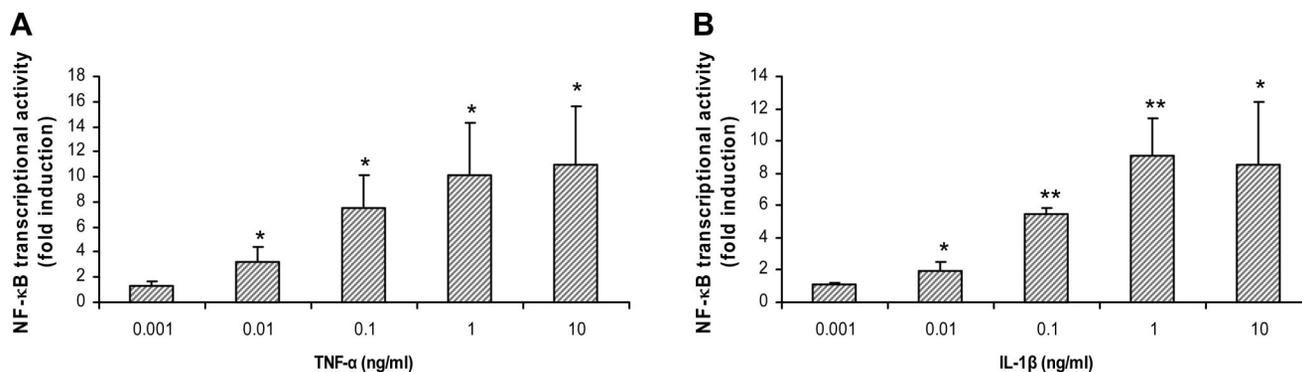


Fig. 1. NF- κ B activation by inflammatory cytokines. C₂C₁₂ myotubes from the NF- κ B-sensitive reporter stable cell line were stimulated with increasing concentrations of TNF- α (0.001, 0.01, 0.1, 1, or 10 ng/ml) or vehicle (0.1% BSA) (A) or increasing concentrations of IL-1 β (0.001, 0.01, 0.1, 1, or 10 ng/ml) or vehicle (0.1% BSA) (B) for 4 h. Cells were lysed, and luciferase activity was determined and normalized to total protein content. Significance of difference vs. control: * $P \leq 0.05$, ** $P \leq 0.01$. Each condition was performed in triplicate within an experiment. Shown are means \pm SD of 3 independent experiments.

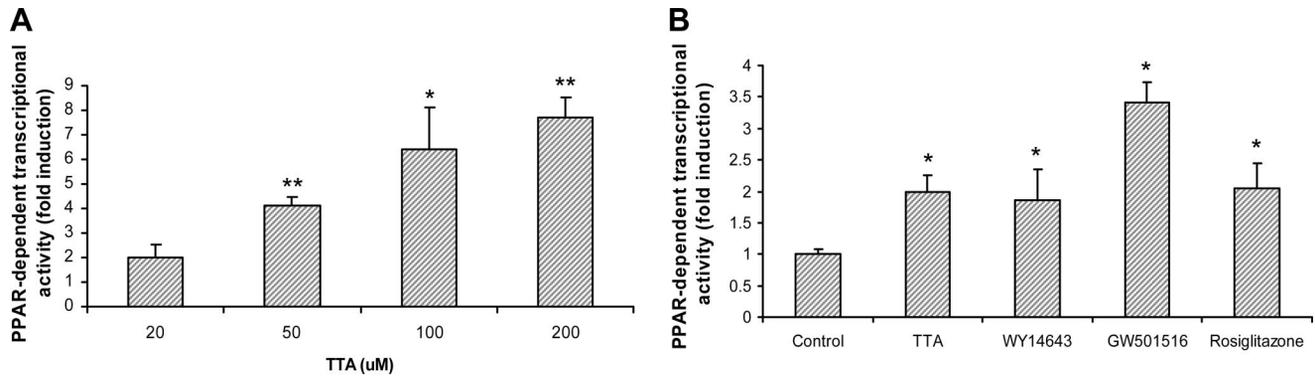


Fig. 2. Induction of PPAR transcriptional activity by specific PPAR ligands. *A*: C₂C₁₂ myoblasts were transiently transfected with the PPAR-sensitive reporter construct and treated with increasing concentrations of tetradecylthioacetic acid (TTA) or vehicle (ethanol) for 24 h. Cells were lysed and luciferase activity was determined and normalized to β -galactosidase activity to correct for differences in transfection efficiency. *B*: C₂C₁₂ myotubes stably transfected with the PPAR-sensitive reporter construct were treated with TTA (100 μ M), WY-14643 (50 μ M), GW-501516 (1 μ M), rosiglitazone (75 μ M), or vehicle (DMSO, specific agonists or ethanol, TTA) for 48 h. Cells were lysed, and luciferase activity was determined and normalized to total protein content. Significance of difference vs. control: * $P \leq 0.05$, ** $P \leq 0.01$. Each condition was performed in triplicate within an experiment. Shown are means + SD of 3 independent experiments.

(GW-1929) was used in the same experimental set-up. Preincubation of C₂C₁₂ myotubes from the NF- κ B-sensitive reporter cell line with 20 μ M GW-1929 resulted in a time-dependent inhibition of TNF- α -induced NF- κ B transcriptional activity, with a maximal inhibitory effect (~40% inhibition) at 1 h preincubation (Fig. 5B). These data show that ligand-induced PPAR γ activation potently inhibits proinflammatory cytokine-induced NF- κ B transcriptional activity in skeletal muscle cells.

PPAR γ activation reduces TNF- α -induced transcript levels of NF- κ B target genes. To investigate the functional relevance of the NF- κ B reporter data, we investigated whether TNF- α -induced expression of endogenous NF- κ B target genes was affected by PPAR γ activation in C₂C₁₂ myotubes. Expression of ICAM-1 and the expression of the chemokine KC (keratinocyte-derived chemokine, the mouse homolog of human IL-8) significantly increased in C₂C₁₂ myotubes following stimulation with TNF- α for 24 h. Pretreatment of the cells with rosiglitazone for 1 h abolished the TNF- α -induced upregulation of KC and ICAM-1 mRNA levels (Fig. 6). These obser-

vations show that PPAR γ activation can reduce NF- κ B target gene transcript levels in skeletal muscle and demonstrate that data obtained in the NF- κ B reporter are functionally relevant.

NF- κ B target genes in skeletal muscle of rosiglitazone-treated T2DM subjects. To verify whether muscular NF- κ B activity in human subjects is suppressed by PPAR γ activation, we examined the effect of 8-wk rosiglitazone treatment on muscular gene expression of ICAM-1 and IL-8 in T2DM patients. Subjects' characteristics before and after rosiglitazone treatment are summarized in table 1. No differences were observed in IL-8 mRNA expression levels (data not shown). A trend toward lower ICAM-1 mRNA expression levels was observed in skeletal muscle after rosiglitazone treatment (0.155 ± 0.0150 vs. 0.137 ± 0.006 AU, $P = 0.074$; Fig. 7).

PPAR γ activation does not reduce TNF- α -induced nuclear translocation or DNA binding of NF- κ B. To explore potential mechanisms by which PPAR γ activation can exert its anti-inflammatory effects, we investigated TNF- α -induced NF- κ B (RelA) nuclear translocation in response to preincubation with

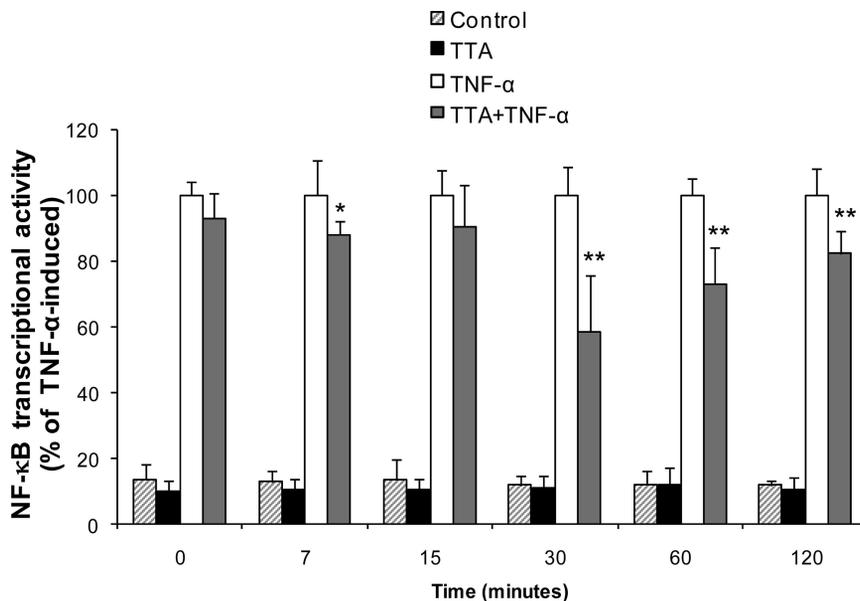
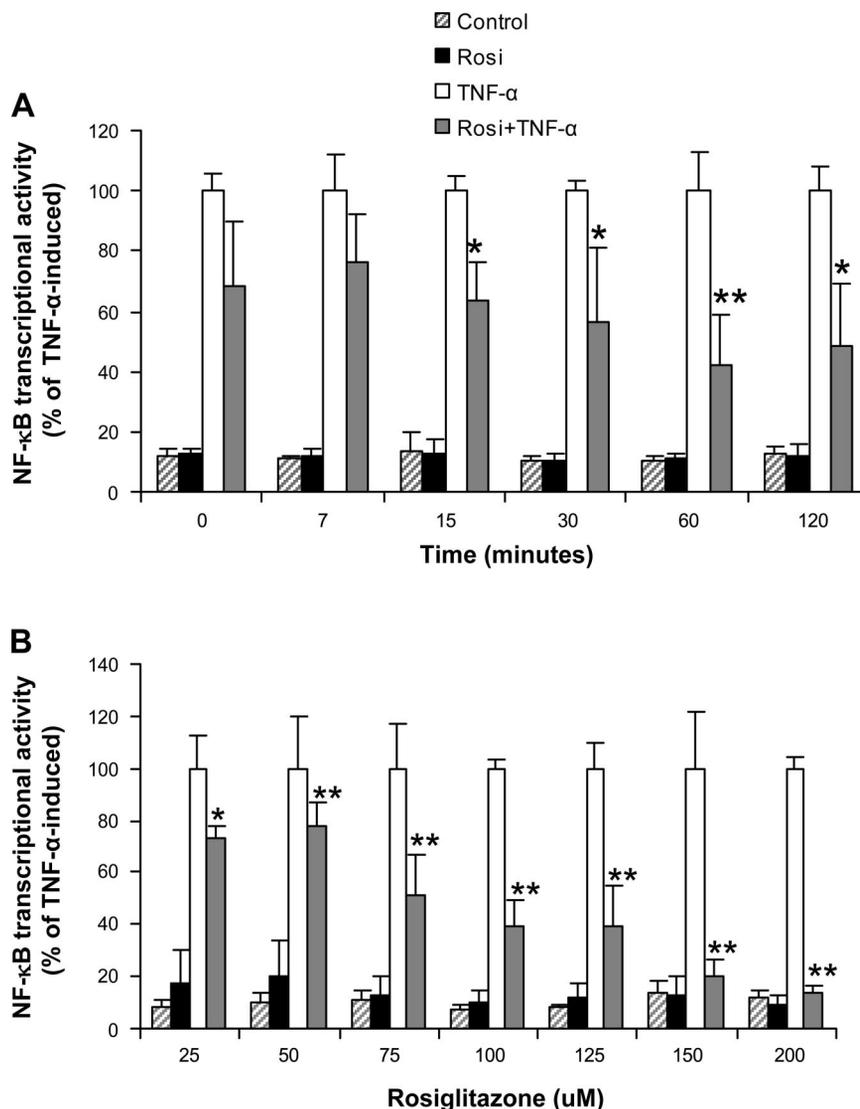


Fig. 3. PPAR activation inhibits NF- κ B transcriptional activity. C₂C₁₂ myotubes (differentiated for 6 days) from the NF- κ B-sensitive reporter stable cell line were pretreated (time in min) with TTA (100 μ M) or vehicle (0.1% ethanol final concentration) prior to 4-h incubation with TNF- α (1 ng/ml). Cells were lysed, and luciferase activity was determined and normalized to total protein content. Significance of difference vs. TNF- α -treated cells: * $P \leq 0.05$, ** $P \leq 0.01$. Each condition was performed in triplicate within an experiment. Shown are mean + SD of 3 independent experiments.

Fig. 4. PPAR γ activation by rosiglitazone inhibits TNF- α -induced NF- κ B transcriptional activity. C₂C₁₂ myotubes (differentiated for 6 days) from the NF- κ B-sensitive reporter stable cell line were preincubated (time in min) with rosiglitazone (75 μ M) or vehicle (DMSO) prior to 4-h incubation with TNF- α (1 ng/ml) (A), or with an increasing dose of rosiglitazone or vehicle (DMSO) for 1 h prior to 4-h incubation with TNF- α (1 ng/ml) (B). Cells were lysed, and luciferase activity was determined and normalized to total protein content. Significance of difference vs. TNF- α -treated cells: * P \leq 0.05, ** P \leq 0.01. Each condition was performed in triplicate within an experiment. Shown are means + SD of 3 independent experiments.



rosiglitazone. As shown in Fig. 8, a pronounced increase in nuclear translocation of RelA was observed after 1 h of TNF- α stimulation. However, no reduction in TNF- α -induced RelA nuclear translocation was observed after preincubation with rosiglitazone. In addition to nuclear translocation assays EMSA analyses were performed. Figure 9, A and C, show that RelA DNA binding upon stimulation with TNF- α increased dramatically. Pretreatment with rosiglitazone did not reduce NF- κ B DNA binding in response to TNF- α . Analysis of the subcomposition of the retarded complexes by supershift assay in Fig. 9B revealed that the major complex induced by TNF- α (*top* band) contained RelA. A negative control antibody was used in *lane 3*. CHIP assays were performed to investigate in vivo DNA binding of RelA to the ICAM-1 promoter. As indicated in Fig. 9D, stimulation of C₂C₁₂ myotubes with TNF- α potentially induced binding of RelA to its binding site in the ICAM-1 promoter. Preincubation with rosiglitazone did not reduce but rather increased TNF- α -induced binding of RelA to its binding site in the ICAM-1 promoter. Aggrecan was included as a non-target of RelA, and no differences were observed between the different experimental conditions (data not shown). These results indicate that TNF- α -induced NF- κ B

transcriptional activity is suppressed by PPAR γ activation by a mechanism downstream of RelA DNA binding.

DISCUSSION

The present study reveals that PPAR γ activation potently inhibits TNF- α - and IL-1 β -induced NF- κ B transcriptional activity in skeletal muscle cells. This anti-inflammatory effect of PPAR γ activation is not due to decreased RelA translocation to the nucleus or reduced RelA DNA binding. In addition, PPAR γ activation by rosiglitazone decreased transcript levels of known NF- κ B target genes in vitro, while a trend toward decreased basal expression of ICAM-1 was observed in skeletal muscle of rosiglitazone-treated T2DM subjects.

We used C₂C₁₂ cells as an experimental model for skeletal muscle (25), as previous reports had demonstrated that insulin resistance (29) and atrophy (18) are induced by inflammatory stimuli in myotubes of this cell line. Moreover, it has been established that all three different PPAR proteins are present in C₂C₁₂ cells (7). All synthetic agonists (rosiglitazone, GW-1929, WY-14643, and GW-501516) used in our study are highly specific activators of PPAR γ , PPAR α , and PPAR δ ,

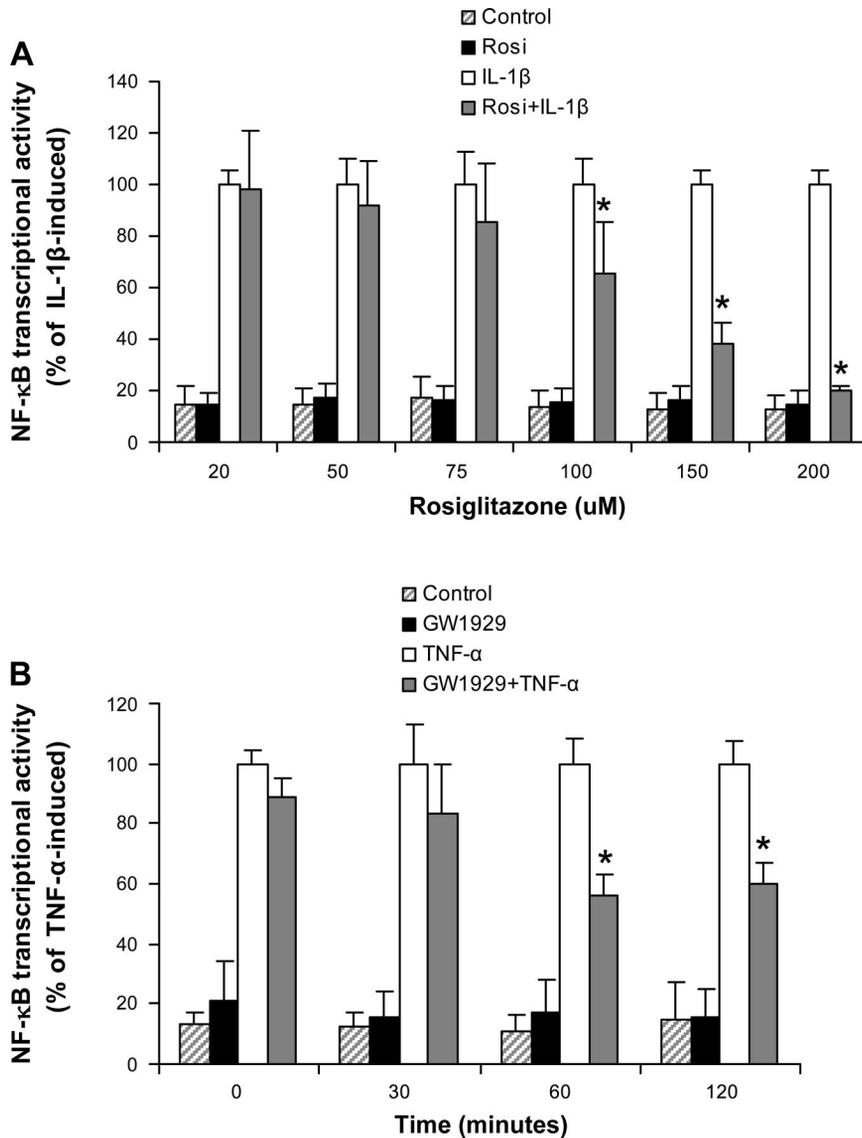


Fig. 5. PPAR γ activation inhibits TNF- α - as well as IL-1 β -induced NF- κ B transcriptional activity. C₂C₁₂ myotubes (differentiated for 6 days) from the NF- κ B-sensitive reporter stable cell line were pretreated 1 h with an increasing dose of rosiglitazone or vehicle (DMSO) prior to 4-h incubation with IL-1 β (1 ng/ml) (A), or with GW-1929 (20 μ M) or vehicle (DMSO) for an increasing amount of time prior to 4-h incubation with TNF- α (1 ng/ml) (B). Cells were lysed, and luciferase activity was determined and normalized to total protein content. Significance of difference vs. TNF- α -treated cells: * $P \leq 0.01$. Each condition was performed in triplicate within an experiment. Shown are means + SD of 3 independent experiments.

respectively (38). We demonstrated that the PPAR activators used in this study are capable of inducing PPAR transcriptional activity in skeletal muscle myoblasts. Importantly, for all activators, this response was maintained in fully differentiated C₂C₁₂ myotubes, which has only been reported for PPAR α in one previous study (48). In addition, other work demonstrated the potential of PPAR activators to induce DNA binding of the PPARs to their responsive elements in C₂C₁₂ myotubes but did not investigate the actual transcriptional activity of these proteins in response to PPAR activators (7).

All three PPAR subtypes have been implicated in the regulation of inflammatory responses (13). In skeletal muscle, however, reports on anti-inflammatory effects of PPAR activation are rather scarce. For example, anti-inflammatory properties have previously been ascribed to the fatty acid analog TTA, but so far not in muscle (15). In the present study, we demonstrate anti-inflammatory effects of TTA in skeletal muscle, based on its inhibitory actions on cytokine-induced NF- κ B transcriptional activity. The inhibitory effect of TTA on NF- κ B transcriptional activity was transient in nature, which could be

due to metabolic processing of the fatty acid analog as it can be degraded through the ω -oxidation pathway (8).

Although the PPAR α and PPAR δ activators we used did induce PPAR-dependent transcriptional activation, we did not observe any effect of PPAR δ or PPAR α activation on TNF- α -induced NF- κ B activity in skeletal muscle cells. In contrast, one recent study reported an inhibitory effect of PPAR δ activation on NF- κ B transcriptional activity in C₂C₁₂ myotubes (47). Those authors used a different activator of PPAR δ (GW-0742) compared with the PPAR δ activator used in this study (GW-501516), although both activators have been shown to be highly specific for PPAR δ (43). In addition, the duration of TNF- α treatment was fourfold longer compared that of with our study. With respect to PPAR α , anti-inflammatory potential has been investigated and demonstrated in spleen cells and smooth muscle cells but not previously in skeletal muscle (14, 34).

In contrast to PPAR α and PPAR δ , activation of PPAR γ by rosiglitazone inhibited cytokine-induced NF- κ B activity in skeletal muscle cells in the present study in a concentration range used previously in cultured skeletal muscle cells (4, 23).

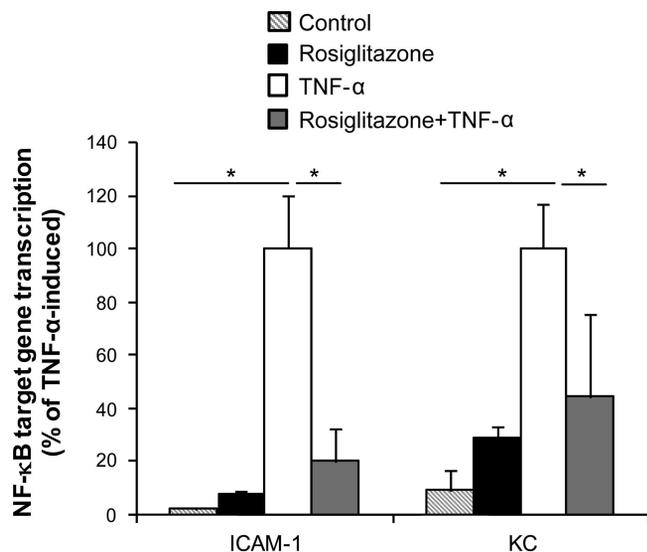


Fig. 6. PPAR γ activation reduces TNF- α -induced transcription of NF- κ B target genes. C₂C₁₂ myotubes (differentiated for 6 days) were pretreated (1 h) with rosiglitazone (150 μ M) followed by 24-h incubation with TNF- α (1 ng/ml). Intracellular adhesion molecule 1 (ICAM-1) and the chemokine CXCL1 (keratinocyte-derived chemokine; KC) mRNA levels were assessed and corrected for the housekeeping gene cyclophilin A. Expression levels are depicted as %TNF- α -induced transcript levels. Each condition was performed in triplicate within an experiment. Shown are means \pm SD of 3 independent experiments. Significance of difference: * P < 0.001.

Since nonspecific effects of rosiglitazone have been described (12), we used the structurally unrelated agonist GW-1929 to alternatively activate PPAR γ . In line with anti-inflammatory properties of rosiglitazone as a PPAR γ activator, GW-1929 also potently inhibited proinflammatory cytokine-induced NF- κ B activation, which suggested that this effect is indeed mediated by PPAR γ . In line with our study, agonist-induced PPAR γ activation was shown to repress NF- κ B activation in other cell types, e.g., macrophages and T cells, in vitro (11, 36).

To assess the functional relevance of the NF- κ B reporter data, we investigated the effect of PPAR γ activation by rosiglitazone on the transcription of known endogenous NF- κ B target genes (32). We observed that TNF- α -induced KC and, more pronounced, ICAM-1 mRNA levels were significantly attenuated by PPAR γ activation in fully differentiated C₂C₁₂ myotubes. This observation is in concordance with a recent report showing downregulated ICAM-1 mRNA levels in lung epithelial cells in response to different PPAR γ agonists (5). In

Table 1. Subject characteristics before and after rosiglitazone treatment after 2-wk cessation of any preexisting antidiabetic medication

	Before	After
Age, yr	61.8 \pm 3.7	/
Body weight, kg	90.2 \pm 9.1	90.8 \pm 10.0
Body mass index, kg/m ²	30.2 \pm 3.3	30.4 \pm 3.4
Glucose, mmol/l	9.8 \pm 2.2	8.3 \pm 1.4
HbA _{1c} , %	7.3 \pm 0.7	7.7 \pm 1.1
S _I	0.012 \pm 0.0019	0.032 \pm 0.019**
γ -GT, U/l	43.4 \pm 35	22.8 \pm 11.2*

Values are means \pm SD. S_I, insulin sensitivity; γ -GT, γ -glutamyltranspeptidase; HbA_{1c}, hemoglobin A_{1c}. * P < 0.05, ** P < 0.01 after vs. before treatment.

addition, another study demonstrated that rosiglitazone can attenuate NF- κ B-dependent ICAM-1 production in smooth muscle cells (6). To verify these in vitro observations in an appropriate and relevant clinical setting, we explored IL-8 and ICAM-1 mRNA levels in skeletal muscle of diabetic subjects who received rosiglitazone treatment as an antidiabetic therapy. ICAM-1 levels tended to be lower compared with levels prior to rosiglitazone administration but did not reach statistical significance, possibly due to a lack of statistical power. Additionally, the effective dose of rosiglitazone that reaches the muscle in the patients unfortunately was not measured; however, it is conceivable that this dose is lower than the dose made available to the cells in our in vitro experiments. Therefore, this may explain the different results obtained in patients and in vitro cultured cells. Previously, it has been shown that rosiglitazone treatment of patients with T2DM reduced circulating inflammatory markers such as C-reactive protein (CRP), metalloproteinase (MMP)-9/gelatinase B, and TNF- α (19). Our data now show that rosiglitazone treatment possibly also reduces transcript levels of NF- κ B target genes in skeletal muscle, although this observation should be confirmed in a larger patient population.

The inflammation-modulating properties of the PPARs may not depend primarily on their transcriptional activity but could rather be mediated through the ability of PPARs to interfere with inflammatory signaling cascades, including NF- κ B (28, 34). In concordance, GW-1929 did not induce PPAR transcriptional activity (data not shown) but did interfere with NF- κ B signaling, suggestive of a transrepression mechanism of NF- κ B modulation by PPAR γ in skeletal muscle, as has been reported previously in other cell types (33).

Several different transrepression mechanisms have been described to be mediated by the PPARs (14, 28, 22). These include their ability to physically associate with various transcription factors (including NF- κ B) and the ability to successfully compete for limiting amounts of coactivators. Since rosiglitazone pretreatment did not reduce TNF- α -induced RelA nuclear translocation or DNA binding, the mechanism by which PPAR γ activation inhibits NF- κ B mediated gene tran-

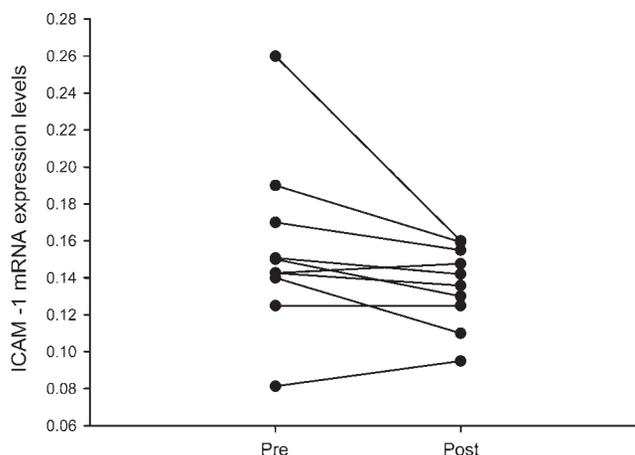


Fig. 7. ICAM-1 expression levels are reduced in skeletal muscle of rosiglitazone-treated type 2 diabetic (T2DM) subjects. Expression of ICAM-1 mRNA in skeletal muscle of T2DM patients (n = 10) before and after 8-wk rosiglitazone treatment (2 \times 4 mg/day). Phosphoprotein PO (36B4) mRNA levels were used for normalization. ICAM-1 expression levels are depicted as arbitrary units (AU) on the y-axis.

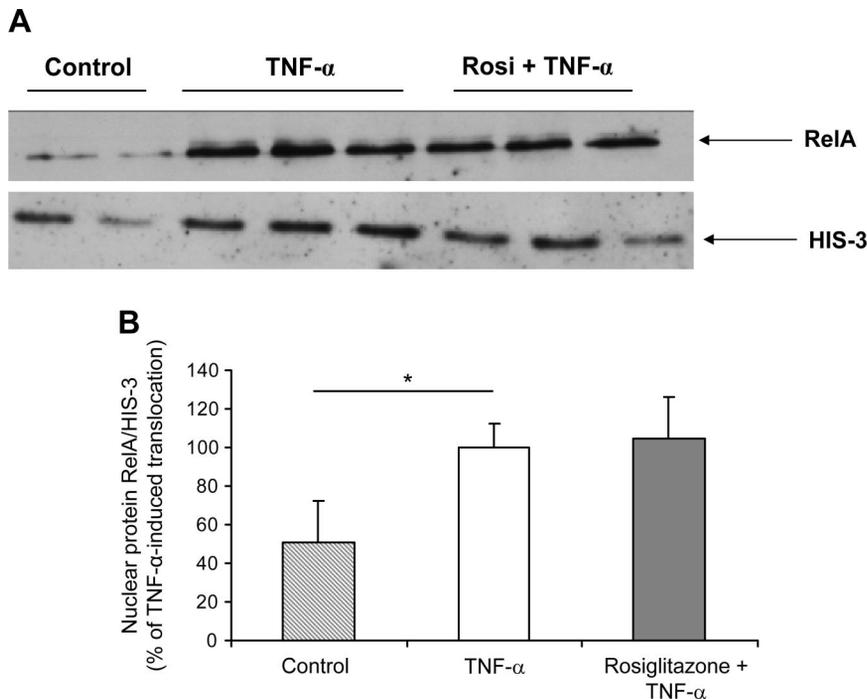


Fig. 8. PPAR γ activation by rosiglitazone does not inhibit TNF- α -induced RelA nuclear translocation. C₂C₁₂ myotubes were differentiated for 6 days and treated with vehicle (Control), TNF- α (1 ng/ml, 1 h), rosiglitazone (150 μ M, 1-h preincubation) or rosiglitazone + TNF- α . *A*: cells were lysed, and nuclear extracts were prepared to assess nuclear protein levels of RelA and His-3 (loading control) by Western blotting. *B*: RelA and His-3 bands were quantified and expressed as a ratio. Significance of difference: * P < 0.001. Each condition was performed in duplicate or triplicate within an experiment. Shown are means + SD of 3 independent experiments. A representative Western blot is depicted.

scription in skeletal muscle should be located downstream of DNA binding. This may include impaired recruitment of transcriptional cofactors to the NF- κ B-DNA complex (28). Alternatively, removal of repressor complexes of the NF- κ B tran-

scriptional machinery may be prevented by PPAR γ following its ligand-induced SUMOylation (33).

In conclusion, we show a strong inhibitory effect of agonist-mediated PPAR γ activation on inflammatory mediator-in-

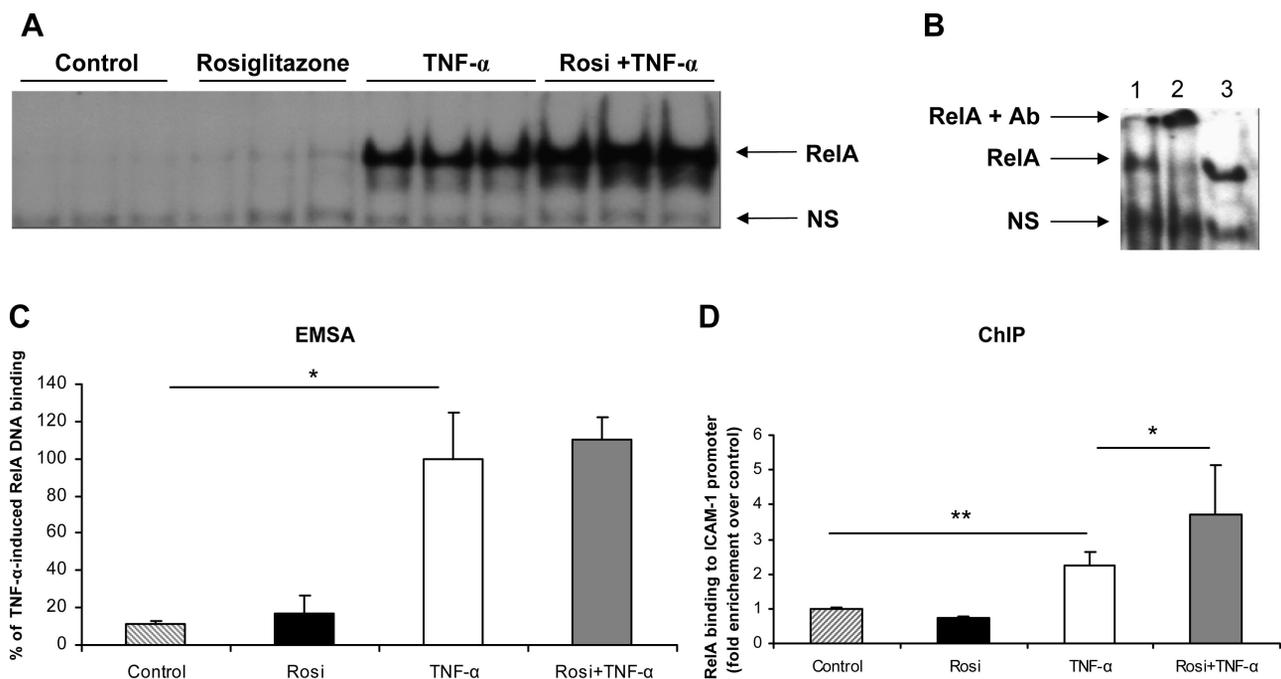


Fig. 9. PPAR γ activation by rosiglitazone does not inhibit TNF- α -induced RelA DNA binding. C₂C₁₂ myotubes were differentiated for 6 days and treated with vehicle (Control), TNF- α (1 ng/ml, 1 h), rosiglitazone (150 μ M, 1-h preincubation), or rosiglitazone + TNF- α . *A*: cells were lysed, and nuclear extracts were prepared to assess RelA DNA binding activity by EMSA; a representative EMSA is depicted. *B*: supershift analysis using no antibody (lane 1), a RelA antibody (lane 2), or an IgG control antibody (lane 3) to assess complex subcomposition. *C*: bands containing the DNA-RelA complex were quantified. *D*: cells were cross-linked and harvested, and RelA binding to the ICAM-1 promoter was quantified by ChIP. All results from ChIP assays were corrected for nonspecific precipitation using HA as a negative control antibody. Significance of difference: * P \leq 0.05, *** P \leq 0.01. Each condition was performed in triplicate within an experiment. Shown are means + SD of 3 independent experiments.

duced NF- κ B transcriptional activity in skeletal muscle, independent of RelA nuclear translocation and DNA binding. The exact mechanism by which this occurs remains obscure but warrants further investigation, as muscle-specific inhibition of NF- κ B activity may be an interesting therapeutic venue for treatment of several inflammation-associated skeletal muscle abnormalities.

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