THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 36, Issue of September 6, pp. 33518–33528, 2002
Printed in U.S.A.

Inhibition of Cytokine-induced Matrix Metalloproteinase 9 Expression by Peroxisome Proliferator-activated Receptor α Agonists Is Indirect and Due to a NO-mediated Reduction of mRNA Stability*

Received for publication, February 28, 2002, and in revised form, June 9, 2002
Published, JBC Papers in Press, July 1, 2002, DOI 10.1074/jbc.M202008200

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Rat renal mesangial cells express high levels of matrix metalloproteinase 9 (MMP-9) in response to inflammatory cytokines such as interleukin 1β (IL-1β). We tested whether ligands of the peroxisome proliferator-activated receptor (PPARα) could influence the cytokine-induced expression of MMP-9. Different PPARα agonists dose-dependently inhibited the IL-1β-triggered increase in gelatinolytic activity mainly by decreasing the MMP-9 steady-state mRNA levels. PPARα agonists on their own had no effects on MMP-9 mRNA levels and gelatinolytic activity. Surprisingly, the reduction of MMP-9 mRNA levels by PPARα activators contrasted with an amplification of cytokine-mediated MMP-9 gene promoter activity and mRNA expression. The potentiation of MMP-9 promoter activity functionally depends on an upstream peroxisome proliferator-responsive element-like binding site, which displayed an increased DNA binding of a PPARα immunopositive complex. In contrast, the IL-1β-induced DNA-binding of nuclear factor κB was significantly impaired by PPARα agonists. Most interestingly, in the presence of an inducible nitric-oxide synthase (iNOS) inhibitor, the PPARα-mediated suppression switched to a strong amplification of IL-1β-triggered MMP-9 mRNA expression. Concomitantly, activators of PPARα potentiated the cytokine-induced iNOS expression. Using actinomycin D, we found that NO, but not PPARα activators, strongly reduced the stability of MMP-9 mRNA. In contrast, the stability of MMP-9 protein was not affected by PPARα activators. In summary, our data suggest that the inhibitory effects of PPARα agonists on cytokine-induced MMP-9 expression are indirect and primarily due to a superinduction of iNOS with high levels of NO reducing the half-life of MMP-9 mRNA.

Dysregulation of extracellular matrix turnover is an important feature of glomerular inflammatory processes and may result in the loss of the mechanical and functional integrity of the glomerulus (1–3). Physiologically, the balance between synthesis and degradation of matrix proteins is guaranteed by the action of a family of zinc-dependent, neutral proteinases designated matrix metalloproteases (MMPs).[1] A tight regulation of these proteases is accomplished by different mechanisms, including the regulation of gene expression, the processing of the inactive zymogenes by other proteases, and finally, the inhibition of the active enzymes by the action of endogenous inhibitors of MMPs, the TIMPs (for review, see Ref. 4). Cultured mesangial cells (MC) respond to proinflammatory cytokines such as tumor necrosis factor or interleukin-1β (IL-1β) with the production of several MMPs, including MMP-9 (gelatinase-B), mainly due to an increase in gene transcription (5, 6). The transcriptional regulation of the rat MMP-9 gene by proinflammatory cytokines is localized to a 0.7-kb region upstream from the transcriptional start site and critically depends on the binding sites for activator protein-1 (AP-1) and nuclear factor κB (NF-κB) transcription factors, respectively (5, 7). Besides MMP-9, MC under inflammatory conditions can express a variety of genes coding for potent inflammatory mediators, such as inducible nitric-oxide synthase (iNOS), the cytosolic and secreted types of phospholipases (PLA-2), and cyclooxygenase 2, respectively (8–11). Metabolites of these enzymes themselves can have modulatory effects on MMP-9 expression, as we have previously reported a NO-mediated suppression of cytokine-induced MMP-9 expression (6). Pharmacologically, the transcriptional activation of MMP-9 is modulated by agonists of the glucocorticoid receptor mainly through interference with NF-κB and members of the Ets/PEA transcription factor family.[2] We now searched for a possible modulation of MMP-9 expression by agonists of another member of the nuclear receptor superfamily, the peroxisome proliferator-activated receptors (PPARs) (for review, see Ref. 12). Three distinct PPAR subtypes have been identified, PPARα, -β/δ, and -γ, with each subtype showing a specific distribution, physiologic function, and a specific set of endogenous as well as synthetic ligands. Physiologically, PPARs are key players in lipid and glucose metabolism, and therefore, disturbances of

* This work was supported by the Deutsche Forschungsgemeinschaft Grants SFB 553 and PF 361/1-1 and the Stiftung Verum für Gesundheit und Umwelt. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by a grant from the Ministry of Education of the Arab Republic of Egypt.

[1] The abbreviations used are: MMP, matrix metalloproteinase; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; IL-1β, interleukin 1β; NOS, nitric-oxide synthase; iNOS, inducible NOS; MC, mesangial cells; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; kb, kilobase(s); L-NMMA, N^G-monomethyl L-arginine; PIPES, 1,4-piperazinediethanesulfonic acid.

[2] W. Eberhardt, unpublished observations.

PPAR function have been implicated in a variety of metabolic disorders such as dyslipidemia and diabetes (13). In addition, PPARα, similar to PPARγ, has been reported to exert potent anti-inflammatory activities in various cell types by inhibiting the expression of inflammatory genes such as COX-2 (14), IL-6 (15), endothelin-1 (16), and various acute-phase proteins (17) just to name a few of them. In accordance with these in *vitro* data, PPARα-deficient mice display an exacerbated response to inflammatory stimuli (14, 15). Moreover, PPARα null mice exhibit a higher degree of kidney damage in response to ischemia/reperfusion injury when compared with wild type controls, thus indicating that PPARα, in addition to its antiinflammatory actions, exhibits also cytoprotective properties in the kidney (18, 19). Interestingly, recent observations demonstrate a role of glucocorticoids in PPARα gene expression in the development of rat kidney, suggesting a cross-talk between both nuclear hormone receptor pathways (20).

Although many studies have addressed the question of how PPARs directly activate gene expression via PPREs, less information exists about negative mechanisms that mostly involve interference with transcriptional activators. In this study we provide an additional mechanism by which PPARα agonists, independent of direct transcriptional effects, indirectly, via amplification of nitric oxide production, can efficiently regulate the level of cytokine-induced MMP-9 expression.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant IL-1β was from Cell Concept (Umkirch, Germany). The PPARα activators were WY-14,643 and LY-171883, and the NO donors were DETA-NONOate and *S*-nitroso-D-penicillamine. The NOS inhibitor was N^G-monomethyl L-arginine (L-NMMA), Actinomycin-D (from *Streptomyces* species) and cycloheximide were purchased from Alexis Biochemicals (Grünberg, Germany). Ribonucleotides and restriction enzymes as well as modifying enzymes were purchased from Roche Diagnostics.

Cell Culture—Rat glomerular MC were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 5 ng/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Serum-free preincubations were performed in Dulbecco's modified Eagle's medium supplemented with 0.1 mg/ml fatty acid-free bovine serum albumin for 24 h before cytokine treatment. For experiments, $3.0–5.0 \times 10^6$ of MC/10-cm culture dish were used between passages 8 and 19. All supplements were purchased from Invitrogen. The amount of dead cells was determined by trypan blue exclusion, and the amount of cells was determined by use of a Neubauer chamber. Cell cytotoxicity was measured by the use of a cytotoxicity detection kit (Roche Diagnostics).

cDNA Clones and Plasmids—cDNA inserts for rat MMP-9 and TIMP-1 were generated as described recently (6). A cDNA for rat TIMP-2 was generated using internal primers of coding sequence of rat TIMP-2 mRNA (GenBank™ accession no AJ409332). The following primers were used: 5′-CGG GAA TGA CAT CTA TGG CAA CC-3′ (sense) and 5′-AAA GCT GGA CCA GCC TCG ATG TC-3′ (antisense).

A glyceralaldehyd-3-phosphate dehydrogenase cDNA clone was generated using the internal primers of the coding sequence of rat glyceralaldehyd-3-phosphate dehydrogenase mRNA (accession number NM017008). A cDNA insert from mouse 18 S rRNA was from Ambion (Austin, TX).

Cloning of Rat MMP-9 Promoter and Transient Transfections—The 5′-flanking region of the rat MMP-9 gene was cloned utilizing the Genome Walker kit (CLONTECH Laboratories, Heidelberg, Germany) using internal (upstream) and external (downstream) primers from the rat MMP-9 cDNA (accession number U36476) as follows: MMP-9 internal primer, 5′-AGGGGCAGCAAAGCTGTAGCCTAG-3′ and MMP-9 external primer, 5′-TTTCAGGTCTCGGGGGAAGACCACATA-3′.

A 1.8-kb fragment from a *Eco*RV-cut library was isolated by PCR under stringent conditions. The fragment was subsequently subcloned into pBluescript-II KS^+ and sequenced using the automated sequence analyzer ABI 310 (PE Applied Biosystems, Weiterstadt, Germany). The sequence has been deposited in the GenBank™/EMBL databases (accession number A7438266). Subsequently, the 1.8-kb fragment of MMP-9 promoter was subcloned into pGL-III Basic vector coding for beetle luciferase (Promega, Mannheim, Germany) using *Xba*I and *Xho*I restriction sites.

Introduction of a double point mutation into a putative distal PPRE-like site (GT to CA) to generate pGL-MMP-9-ΔPPRE-1 was performed using the forward primer 5′-ATG GAG ACT CAA GCA CAC CTA TGT GT-3′ (corresponding to a region from −1763 to −1738). Generation of a double transition into a second proximal-lying PPRE-like binding site to generate pGL-MMP-9 ΔPPRE-2 was done using the forward primer 5′-TCC CAT CCA GCA CAC CCC GAG GCT TA-3′ (corresponding to a region from −896 to −871). All mutant constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Transient transfections of MC were performed using Effectene reagent (Qiagen, Hilden, Germany). Transfections were performed following the manufacturer's instructions. The transfections were performed as triplicates and repeated at least three times to ensure reproducibility of the results. Transfection with pRL-CMV coding for *Renilla* luciferase was used to control for transfection efficiencies. Luciferase activities were measured with the dual reporter gene system (Promega) using an automated chemiluminescence detector (Berthold, Bad Wildbad, Germany)

Northern Blot Analysis—Total cellular RNA was extracted from MC using the Tri reagent (Sigma). Procedures for RNA hybridization were as described previously (6).

SDS-PAGE Zymography—Assessment of gelatinolytic activity of proteins from cellular supernatants was performed as described previously (6). To exclude the possibility that alterations in gelatinolytic contents were due to differences in cell numbers, we routinely determined total cell numbers under each of the experimental conditions. Proteins with gelatinolytic activity were visualized as areas of lytic activity on an otherwise blue gel. Migration properties of proteins were determined by comparison with that of prestained full range rainbow protein markers (Amersham Biosciences).

Cell-free Incubation Experiments—In these experiments we tested the effects of the PPARα agonist WY-14,643 on MMP activities in the conditioned culture medium harvested from MC. Incubations were carried out in a total volume of 100 μl. After incubation (16 h) at room temperature, samples were mixed with 2× sample buffer (4% SDS, 0.005% bromphenol blue, and 20% glycerol) and loaded directly on gels for SDS-PAGE zymography.

Electrophoretic Mobility Shift Assay (EMSA)—Preparation of crude nuclear extracts from cultured mesangial cells and subsequent EMSA was performed as described previously (21). The primers used for EMSA are as depicted in Table I. Competition experiments were done by coincubation with different dilutions of a primer stock solution corresponding to 10-, 100-, and 1000-fold excess (10, 100, 1000 pmol) of unlabeled double-stranded oligonucleotide in the DNA-protein binding reaction. Wild type and mutant consensus oligonucleotides for competition experiments were from Santa Cruz Biotechnology. The sequences for wild type and mutant (forward) PPRE oligonucleotides are depicted in Table I.

PPARα- and -γ-specific antibodies used for supershift experiments were purchased from Affinity Bio Reagents (Golden, CO). Polyclonal antibodies specific for p50 and p65 were purchased from Santa Cruz Biotechnology. For supershift analysis, 2 μl of the antibody were preincubated overnight in a cold room before the binding reaction.

Transcription Assay—For each assay nuclei from $\sim 3 \times 10^7$ cells were isolated by lysing cells in ice-cold 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40. Nuclei were isolated by spinning at $800 \times g$ and, finally, resuspended in 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol. For further procedures we combined the classical run-on protocol with that of the more sensitive RNase protection assay and proceeded as followed. Nuclei suspensions were mixed with an equal volume of 2× reaction buffer containing 100 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 300 mM KCl, 200 units of RNasin and 1 mM of each ATP, GTP, CTP, and UTP and incubated for 30 min at 30 °C. Transcription was stopped by adding 20 μg of DNase I followed by 80 μg of proteinase K. Subsequently the nuclear RNA was isolated by using the Tri reagent and by subsequent chloroform precipitation after simple ethanol precipitation. A cDNA clone coding for rat MMP-9 (pKs-MMP-9 rat) was linearized with *Pvu*II and used as a template for MMP-9 antisense RNA. The MMP-9 antisense transcript was synthesized *in vitro* using T7 RNA polymerase and [α-^{32}P]UTP (3000 Ci/mmol). Nuclear RNA and 100,000 cpm of the labeled T7-derived MMP-9 antisense transcript were co-precipitated by ethanol precipitation and hybridized at 42 °C overnight in 30 μl of FAB hybridization buffer containing 80%, 1 mM EDTA, 40 mM PIPES (pH 6.4), and 400 mM NaCl. After hybridization, samples were digested with RNase A and T1 for 1 h at 30 °C. RNA samples were again extracted with phenol/chloroform, and protected double-stranded RNA hybrids were ethanol-precipitated. The protected fragments were separated on a 5% acrylamide, 8 M urea gel and analyzed using a phosphorimaging. The amount of MMP-9 RNA

signals was normalized by hybridizing the same set of RNA samples with a radioactively labeled antisense RNA coding for rat glyceralaldehyd-3-phosphate dehydrogenase.

Western Blot Analysis—The total cellular levels of iNOS protein were analyzed by Western blot analysis using total cellular extracts (50 μg) and probed with a polyclonal antibody specific for rat N-terminal iNOS (22). Total cellular levels of MMP-9 were assessed by use of a polyclonal antibody specific for rat MMP-9 (Chemicon, Hofheim, Germany).

Nitrite Measurements in MC Supernatants—Nitrite contents of cellular supernatants were measured as a readout for NOS activity. 100 μl of supernatants were mixed with 100 μl of Griess reagent (Merck). The absorbance at 540 nm with a reference wavelength at 595 nm was measured, and nitrite concentration was determined using a calibration curve with sodium nitrite standards.

Statistical Analysis—Results are expressed as the means ± S.D. The data are presented as x-fold induction compared with control conditions or compared with IL-1β-stimulated values (#). Statistical analysis was performed using Student's *t* test and analysis of variance for significance. p values < 0.01 (** or ##) were considered significant.

RESULTS

Effect of the PPARα Activator WY-14,643 on Cytokine-induced MMP-9 Activity and mRNA Steady-state Levels of MMP-9, TIMP-1, and TIMP-2—The family of gelatinases includes the two genetically distinct 72- and 92-kDa type IV collagenases, MMP-2 and MMP-9, respectively, both of which are expressed in MC. Whereas MMP-2 shows a constitutive expression, MMP-9 expression is highly induced by proinflammatory cytokines such as IL-1β (6, 7). Because the expression of MMP-9 in MC after treatment with IL-1β reaches a maximal level after 24 h we chose incubation times of 24 h. To evaluate possible effects of PPARα activators on the proteolytic activity secreted into the conditioned media from cytokine-treated cells, MC were treated with IL-1β (2 nM) in the presence or absence of different concentrations of WY-14,643, a potent activator of PPARα (12).

The gelatinolytic content of conditioned medium of MC withdrawn after 24 h of stimulation was tested by zymography using gelatin as a substrate. WY-14,643 reduced in a dose-dependent manner the IL-1β-stimulated MMP-9 activity with a maximal inhibition seen at 100 μM. WY-14,643 alone had no effects on enzyme secretion (Fig. 1*A*). Higher concentrations used in 24-h incubations caused cytotoxic effects as monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (data not shown). The levels of the lytic activity of latent and active MMP-2, represented by the two lytic bands at 68 and 72 kDa, remained unchanged under all experimental conditions, consistent with the constitutive expression of MMP-2 in rat MC (7).

To evaluate whether the reduction of the lytic content of MMP-9 is due to a decrease in the amount of the corresponding MMP-9 mRNA, we performed Northern blot analysis using a cDNA probe from the rat MMP-9 gene (6). As shown in Fig. 1*B*, WY-14,643 dose-dependently attenuated the cytokine-induced MMP-9 mRNA level with a maximal inhibition seen at 100 μM WY. WY-14,643 given alone had no effects on the basal MMP-9 mRNA levels. These data indicate that the alterations of cytokine-induced zymogen activity by WY-14,643 predominantly result from changes in the MMP-9 expression levels. We next checked mRNA levels of TIMP-1 and TIMP-2, the endogenous inhibitors of MMP-9. Most interestingly, TIMP-1 followed a similar expression pattern as MMP-9, showing a strong increase of basal mRNA levels by IL-1β and a dose-dependent attenuation of IL-1β-induced steady-state mRNA levels by WY-14,643 (Fig. 1*B*). Similarly to MMP-9, basal TIMP-1 mRNA levels were not affected by WY-14,643. In contrast, TIMP-2 displayed high basal steady-state mRNA levels that were attenuated by IL-1β and furthermore attenuated by the highest concentration (100 μM) of WY-14,643 (Fig. 1*B*).

Furthermore, we tested other PPAR activators on MMP-9, TIMP-1, and TIMP-2 steady-state mRNA levels. The concentrations used were in a range known to maximally stimulate PPAR activation in cell cultures. As shown in Fig. 1*C*, the prototypic PPAR ligand LY-171883, in a way similar to WY-14,643, dose-dependently inhibited the amounts of IL-1β-induced MMP-9 and TIMP-1 steady-state mRNAs, respectively. The reduction of MMP-9 and TIMP-1 mRNA levels again was paralleled by a decrease of MMP-9 activity in the conditioned media (data not shown). In contrast, the high basal levels of TIMP-2 mRNA were reduced by IL-1β and further attenuated

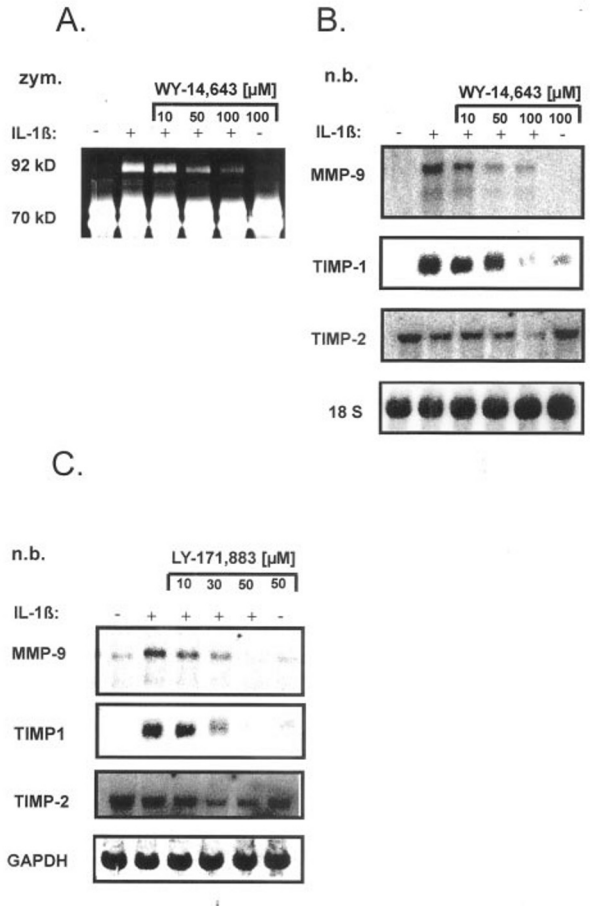

FIG. 1. **Inhibition of cytokine-induced MMP-9 and TIMP levels in MC by PPARα agonists.** *A*, dose-dependent inhibition of the IL-1β-induced gelatinase activity by WY-14,643. MC were simultaneously treated with vehicle (control) (−) or with IL-1β (2 nM) (+) in the presence of the indicated concentrations of WY-14,643. 24 h after stimulation, 10 μl of cell supernatant was subjected to SDS-PAGE zymography (*zym.*) using gelatin as a substrate. The migrating properties of lytic bands, corresponding to inactive pro-MMP-9 (92 kDa), and the inactive and active forms of MMP-2 (72 and 68 kDa) are indicated by migration properties determined with molecular mass markers. The data are representative for three independent experiments giving similar results. *B*, Northern blot analysis (*n.b.*), demonstrating a dose-dependent modulation of IL-1β-induced MMP-9, TIMP-1, and TIMP-2 mRNA steady-state levels by WY-14,643. MC were treated for 24 h as indicated and subsequently extracted for total cellular RNA. 20 μg of total RNA were hybridized to ^{32}P-labeled cDNA inserts from rat MMP-9, TIMP-1, and TIMP-2 genes. Equivalent loading of RNA was ascertained by rehybridization to an 18 S ribosomal probe. The data are representative for three independent experiments giving similar results. *C*, dose-dependent inhibition of the IL-1β-induced MMP-9 mRNA steady-state levels by LY-171,883. Quiescent MC were coincubated for 24 h with IL-1β (2 nM) and the indicated concentrations of the PPAR activator LY-171,883. Total RNA (20 μg) were hybridized to ^{32}P-labeled cDNA inserts from rat MMP-9 and TIMP-1 and TIMP-2 cDNAs, and equivalence of loading was ascertained by rehybridization to a glyceralaldehyd-3-phosphate dehydrogenase (*GADPH*) probe. The data are representative for three independent experiments giving similar results.

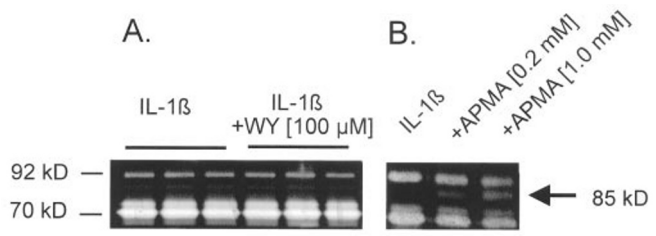

FIG. 2. *A*, WY-14,643 does not affect extracellular IL-1β-induced gelatinolytic activity of latent MMP-9. The conditioned media from MC stimulated for 24 h with IL-1β were incubated for 16 h with WY-14,643 (100 μM) at room temperature and subsequently assayed by SDS-PAGE zymography. The results are shown as triplicates. *B*, *in vitro* activation of latent MMP-9 by *p*-aminophenylmercuric acetate (*APMA*). The IL-1β-inducible band corresponds to the inactive proform of MMP-9, which is cleaved to the active 86-kDa form by treatment with *p*-aminophenylmercuric acetate. Supernatants form MC treated for 24 h with IL-1β (2 nM) were incubated for an additional 3 h with the indicated concentrations of *p*-aminophenylmercuric acetate before being subjected to SDS-PAGE zymography.

by increasing concentrations of LY-171,883 (Fig. 1*C*). These data demonstrate that the inhibition of MMP-9 levels is not paralleled by an increased expression of TIMPs.

WY-14,643 Does Not Alter Gelatinolytic Activity of Pro-MMP-9—To evaluate whether PPARα agonists are able to alter the activity of secreted MMP-9, we performed *in vitro* zymography. Conditioned media from cytokine-treated MC were incubated for 16 h with or without WY-14,643 (100 μM). The lytic band at 92 kDa, which is not detectable under control conditions but is inducible by IL-1β, corresponds to the inactive proform of MMP-9 as is demonstrated by cleavage to the active 86-kDa form by treatment with different concentrations of *p*-aminophenylmercuric acetate (*APMA*) (Fig. 2*B*). As shown in Fig. 2*A*, WY-14,643 does not alter the gelatinolytic activity of pro-MMP-9, thus indicating that the observed WY-mediated reduction of zymogen activity is due to a reduction of IL-1β-mediated expression and secretion of pro-MMP-9.

Modulation of Cytokine-induced MMP-9 Promoter Activity by PPARα Agonists—To evaluate whether the PPARα-mediated inhibition of IL-1β-induced MMP-9 steady-state mRNA levels resulted from an inhibition of MMP-9 gene transcription, we cloned a 1.8-kb promoter fragment of the rat MMP-9 gene by genome walking using MMP-9 gene-specific antisense primers as described under "Experimental Procedures." In addition to a multitude of putative elements involved in the cytokine-mediated regulation of MMP-9 expression, we found by computational analysis two regions that displayed a high homology to PPRE-like motifs and which were denoted as PPRE-1 and PPRE-2, respectively (Fig. 3*A*).

Transient transfection of MC with pGL-MMP-9 (1.8 kb), comprising the 1.8-kb promoter fragment fused to a luciferase reporter gene, was followed by a 24-h treatment with either vehicle, IL-1β (2 nM), WY-14,643 (100 μm), or both compounds in combination and assayed for luciferase activity. IL-1β significantly stimulated luciferase activity (2.67-fold, $p < 0.01$), and surprisingly, this was further amplified by the addition of WY-14,643 (4.1-fold, $p < 0.01$), which on its own had a moderate stimulatory effect on MMP-9 promoter activity (1.8-fold, $p < 0.01$, Fig. 3*B*). We recently have identified AP-1 and NF-κB response elements as being crucially involved in the IL-1β-mediated transcriptional activation of MMP-9 (7). To test for a functional role of the two putative PPREs in the amplification of cytokine-induced MMP-9 promoter activity by PPARα ligands, each PPRE was point-mutated by an exchange of two base pairs and tested for remaining promoter inducibility (Table I). Mutation of the proximal PPRE at −888/−872 (PPRE-2) had no effect on promoter inducibility independent of which stimulus was used (Fig. 3*C*). In contrast, mutation of a distallying PPRE at −1752/−1738 (PPRE-1) prevented the potentiation of cytokine-induced MMP-9 promoter activity by WY-14,643 without affecting promoter activation by IL-1β (Fig. 3*D*). These data suggest that PPRE-1, in contrast to PPRE-2, is functionally involved in the amplification of cytokine-induced MMP-9 promoter activity by PPARα without affecting cytokine-induced transcription of MMP-9. Furthermore, these data demonstrate that the modulation of cytokine-induced mRNA steady-state levels by PPARα agonists cannot be explained by regulatory events occurring in the upstream 1.8-kb MMP-9 promoter context.

PPARα Activators Enhance the Cytokine-induced DNA Binding to PPRE-1—To further confirm the functionality of a cognate PPRE-promoter binding site in the transcriptional amplification of MMP-9, we performed EMSA using a ^{32}P-labeled oligonucleotide comprising the critical PPRE-1 binding site. Treatment of cells with IL-1β (2 nM) or WY-14,643 (50 and 100 μM) caused binding of a single slow migrating complex (Fig. 4*A*, *left panel*). Importantly, the intensity of DNA binding was strongly increased when both reagents were given in combination (Fig. 4*A*, *left panel*). Similar results were obtained with a PPRE consensus oligonucleotide (data not shown). The identity of the DNA-bound complex was confirmed by shifting the EMSA band through the addition of a PPARα-specific antibody (Fig. 4*A*), whereas the addition of a PPARγ-specific antibody had no effects on DNA binding (Fig. 4*A*, *right panel*). Interestingly, the disappearance of the PPRE-1-positive band after the addition of the PPARα antibody was paralleled with the appearance of a strong band with faster migration properties (*arrow* in Fig. 4*A*). Probably, the binding of the antibody triggers a conformational change that allows for the binding of a further transcription factor to this promoter binding site that is normally competed by PPARα. Furthermore, the specificity of the DNA-bound complex was underlined by competition assays. The addition of different concentrations of unlabeled wild type consensus PPRE oligonucleotide (*wt PPRE*) dose-dependently impaired DNA binding, whereas the addition of cold mutant PPRE oligonucleotide only in a very high concentration (1:10) competed with the labeled PPRE-1 probe, which is most probably due to an unspecific competition by the oligonucleotide (Fig. 4*A*, *right panel*).

WY-14,643 Inhibits the IL-1-induced DNA Binding of NF-κB—PPARα agonists can inhibit the transcription of proinflammatory genes indirectly by interfering with the activation of NF-κB as it has been shown for the human IL-6 promoter (15). Previously we have demonstrated that NF-κB is critically involved in the activation of a 0.6-kb promoter portion of MMP-9 by IL-1β (7). To test whether activators of PPARα could modulate the activation of NF-κB binding in MC, we performed EMSA using a MMP-9 gene-specific oligonucleotide described in Table I. Treatment of MC with IL-1β (2 nM) induced DNA binding of a single complex and a weaker binding by WY-14,643 (Fig. 4*B*, *left panel*). Supershift analysis revealed that the cytokine-induced complex contains both p50 and p65 subunits, and the p65 antibody totally inhibited DNA binding. This indicates activation of a p50-p65 heterodimeric complex that mediates the potent transactivating activity of NF-κB (Fig. 4*B*, *right panel*). The formation of this IL-1β-induced complex was markedly attenuated in cells that were simultaneously treated with WY-14,643, indicating that PPARα-dependent signaling cascades interfere with the DNA binding of NF-κB (Fig. 4*B*, *left panel*). It should be noted that WY-14,643 alone induced a pronounced increase in NF-κB binding which, however, was significantly less than the effects observed with IL-1β. Taken together these results demonstrate

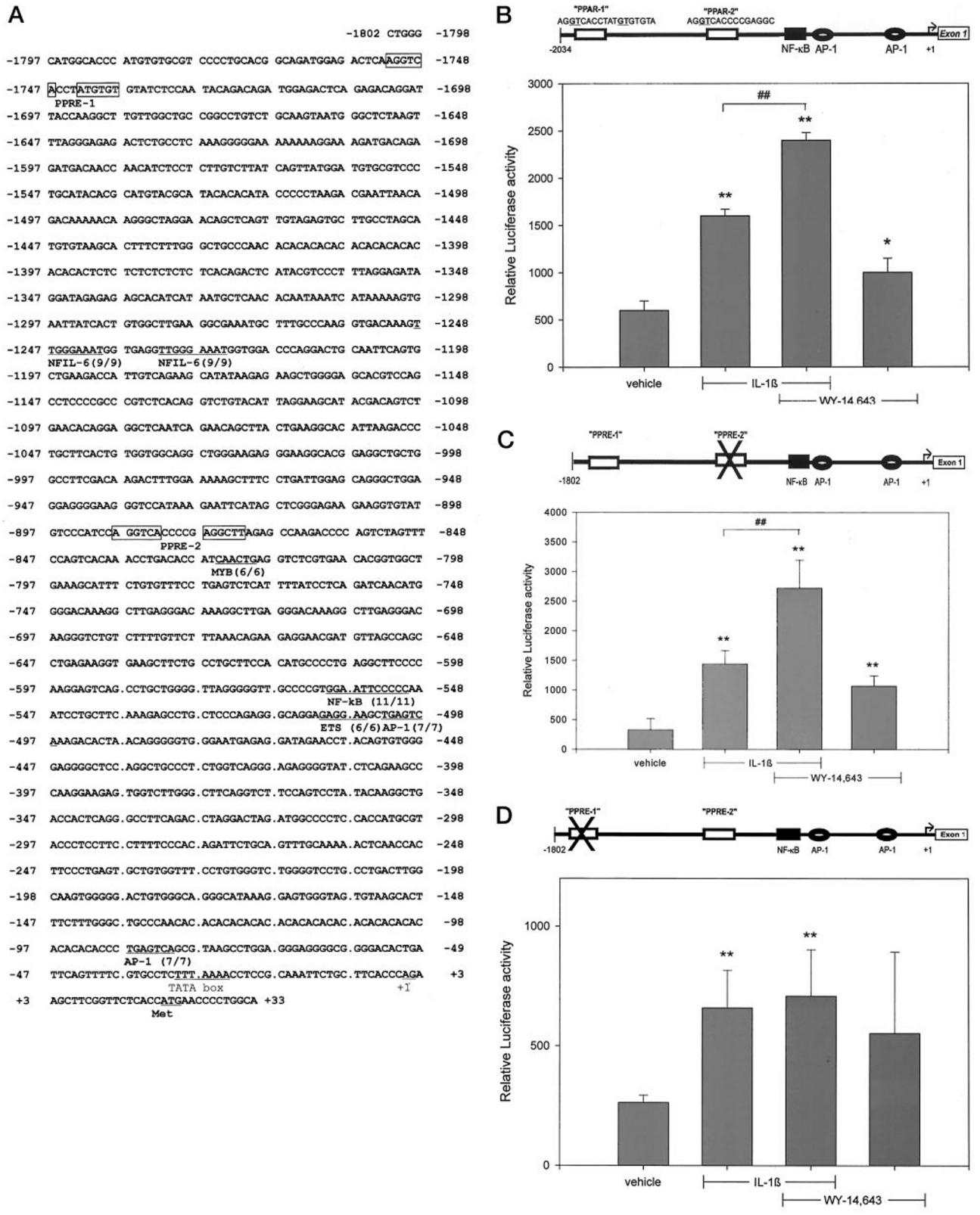

FIG. 3. *A*, sequence of the upstream 1.8 kb of the rat MMP-9 gene promoter. Potential binding sites involved in the signaling of IL-1β are *underlined*. The *numbers* in *parentheses* indicate the degree of homology to consensus sequences of transcription factor binding sites. Putative binding sites for PPARs (PPREs) are *framed*. The transcriptional start site (+1) was predicted as described previously (7). Schematic representation of a 1.8-kb wild type pGL-MMP-9 promoter construct (*B*) and PPRE-mutant analogs (*C* and *D*) and their corresponding promoter activation by IL-1β and WY-14−643 is shown. Potential binding sites for transcription factors involved in cytokine signaling of MMP-9 are indicated. Point mutations within putative PPREs are indicated. Luciferase activities of the different MMP-9 promoter constructs are shown in the *lower panels* (*B–D*). Subconfluent MC were transiently cotransfected with 0.4 μg of pGL-MMP-9 (1.8) (*B*) or with pGL-MMP-9 ΔPPRE-1 (*D*) or pGL-MMP-9 ΔPPRE-2 (*C*) and with 0.1 μg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection, MC were treated for 24 h with vehicle (control), IL-1β (2 nM), WY-14,643 (100 μM), or a combination of IL-1β and WY-14,643 as indicated. The values for beetle luciferase were related to values for *Renilla* luciferase and are depicted as relative luciferase activities. Data are the means ± S.D. ($n = 6$). *, $p \leq 0.05$; **, $p \leq 0.01$, compared with vehicle or IL-1β-stimulated conditions (##, $p \leq 0.01$).

TABLE I
Oligonucleotides used in EMSA and for point mutation generation
Consensus binding sites are underlined, and mutations are bold and italicized.

Oligonucleotides	Primer sequence (sense)
PPRE-1	5′-ATG GAG ACT CAA GGT CAC CTA TGT GT-3′
Δ-PPRE-1	5′-ATG GAG ACT CAA G**CA** CAC CTA TGT GT-3′
PPRE-2	5′-TCC CAT CCA GGT CAC CCC GAG GCT TA-3′
Δ-PPRE-2	5′-TCC CAT CCA G**CA** CAC CCC GAG GCT TA-3′
wt PPRE	5′-CAA AAC TAG GTC AAA GGT CA-3′
mut PPRE	5′-CAA AAC TAG **CA**C AAA G**CA** CA-3′
NF-κB (MMP-9)	5′-TTG CCC CGT GGA ATT CCC CCA AAT-3′

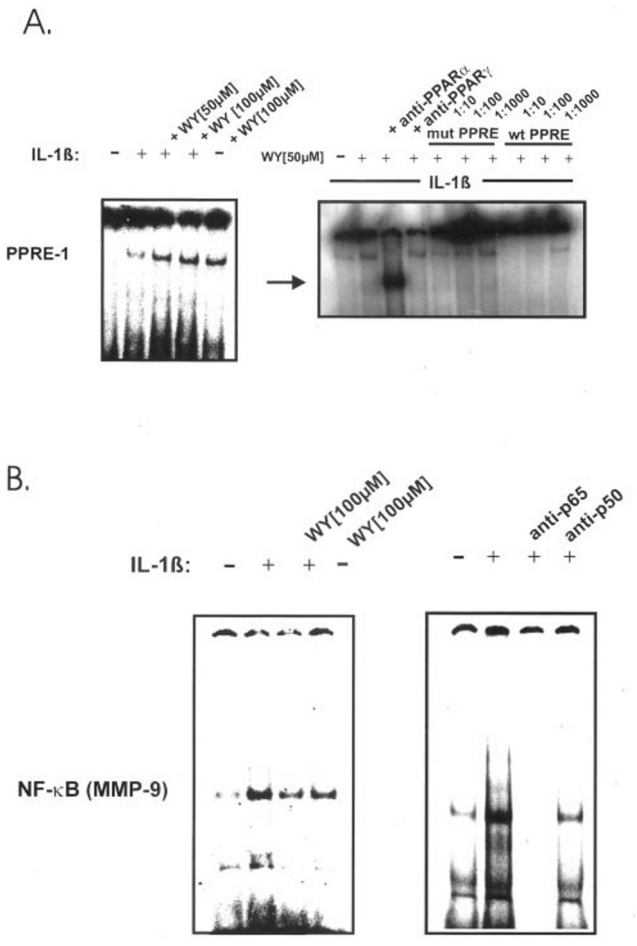

FIG. 4. **Differential modulation of IL-1β-induced binding to MMP-9-specific PPRE and NF-κB binding sites by WY-14,643.** DNA binding was analyzed by EMSA using gene specific oligonucleotides (Table I). The conditions for binding were as described under "Experimental Procedures." *A*, cytokine-induced PPAR binding is enhanced by WY-14,643. For EMSA, serum-starved MC were stimulated with either vehicle (−), IL-1β (+) (2 nM), WY-14,643 (50 and 100 μM), or IL-1β plus WY (100 μM) as indicated for 5 h before being harvested for nuclear extract preparations. The experiments were performed three times, and representative experiments are shown. Supershift analysis is shown identifying PPARα but not PPARγ as IL-1β-/WY-inducible complex in MC (*right panel*). For supershift analysis the antibodies were preincubated overnight at 4 °C before the addition of the labeled oligonucleotide. The altered migration property of the supershifted complex is indicated by an *arrow*. The same gel shows a representative competition study by using different molar excess (depicted as different dilutions of an oligonucleotide stock solution) of unlabeled wild-type (*wt PPRE*) or mutated PPRE (*mut PPRE*) consensus oligonucleotides (sequences are shown in Table I). *B*, cytokine-induced NF-κB binding is inhibited by WY-14,643. Supershift analysis is shown identifying an IL-1β-inducible p50 and p65-containing complex (*right panel*).

that activators of PPARα show mixed effects on NF-κB activity depending on the presence or absence of a simultaneous cytokine stimulus. However, their positive effects through PPRE dominate and result in the potentiation of MMP-9 gene expression.

Inhibition of Cytokine-induced MMP-9 Expression by PPARα Agonists Is Switched to a Potentiation in the Presence of the NOS Inhibitor, L-NMMA—Because the data of the promoter studies do not reflect the reduction of the cytokine-induced MMP-9 mRNA steady-state levels by PPARα agonists, we hypothesized that PPARα activators may alter posttranscriptional events regulating MMP-9 mRNA stability. Previously, we have described the inhibitory effects of NO on the IL-1β-induced MMP-9 steady-state mRNA levels in rat MC. Correspondingly, we found that inhibition of iNOS leads to a marked potentiation of cytokine-induced MMP-9 mRNA levels, thus proving a potent inhibitory action of endogenously produced NO (6). To test whether the inhibitory effects of the PPARα activators on the IL-1β-induced MMP-9 mRNA levels depends on NO production, MC were treated with IL-1β and different PPARα agonists in the presence or absence of the NOS inhibitor L-NMMA. We first measured by zymography the gelatinolytic content of conditioned medium of MC withdrawn 24 h after stimulation. Strikingly, the reduction of cytokine-induced MMP-9 content, most prominently seen with the highest concentration of the PPARα agonists WY-14,643 (100 μM) or bezafibrate (500 μM), was not only reversed but even potentiated in the presence of L-NMMA (Fig. 5*A*). The amplification of cytokine-mediated zymogen contents by L-NMMA was ~3-fold, demonstrating that PPARα activators in the absence of cytokine-triggered NO generation potently augment the cytokine-induced levels of MMP-9 (Fig. 5*A*). Similar to the amplification of gelatinolytic contents, the addition of PPARα agonists plus L-NMMA results in a strong amplification of the IL-1β-mediated MMP-9 steady-state mRNA levels (Fig. 5*B*), whereas L-NMMA by its own had no effects on the basal MMP-9 mRNA levels (data not shown). Similarly to MMP-9, cytokine-induced TIMP-1 mRNA levels were inhibited by both PPARα agonists but were strongly amplified in the presence of L-NMMA (Fig. 5*B*). In contrast, the high level of basal TIMP-2 mRNA was weakly reduced by IL-1β, and the reduction by IL-1β was weakly enhanced by the addition of WY-14,643, whereas bezafibrate had no further modulatory effect on this attenuation of TIMP-2 steady-state mRNA levels. Furthermore, the presence of L-NMMA had no effects on the TIMP-2 mRNA levels, thus demonstrating that TIMP-1, but not TIMP-2 expression, similarly to MMP-9, is negatively affected by PPARα agonists in a NO-dependent manner.

To further confirm that the modulation of cytokine-induced MMP-9 mRNA levels by PPARα activators negatively correlates with endogenous NO production, we stimulated MC with increasing concentrations of IL-1β to gradually increase iNOS expression (23, 24) in the presence of a fixed concentration of WY-14,643 (100 μM) (Fig. 6). Whereas the low dose of IL-1β (0.1 nM) was not able to induce detectable levels of MMP-9 mRNA, concentrations between 0.5 and 2 nM IL-1β caused a strong induction of MMP-9 mRNA that was paralleled by a dose-dependent increase of nitrite accumulation in the cell culture

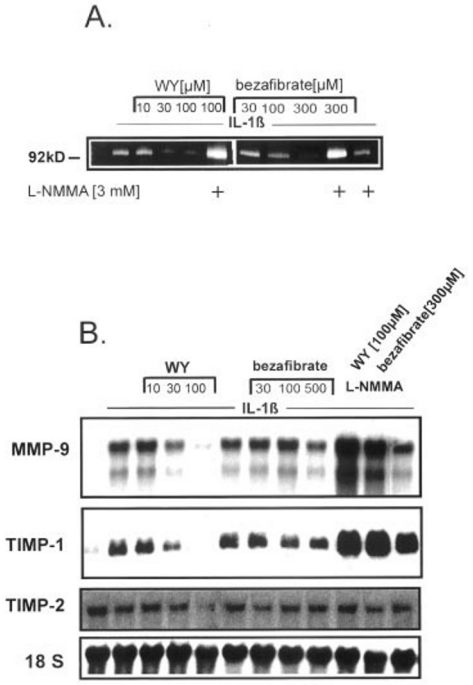

FIG. 5. **Dose-dependent effects of WY-14,643 and bezafibrate on IL-1β-induced MMP-9 secretion (*A*) and MMP-9, TIMP 1, and TIMP-2 mRNA steady-state levels in the presence or absence of the iNOS inhibitor, L-NMMA.** MC were incubated for 24 h with vehicle (control), IL-1β (2 nM), IL-1β plus the indicated concentrations of WY-14,643 or bezafibrate. In the presence of IL-1β and the highest concentration of PPARα activator, cells were additionally treated with (+) the NOS inhibitor L-NMMA (3 mM). The data shown in *A* and *B* are representative for three independent experiments giving similar results. *A*, 10 μl of aliquots from cell supernatants were assessed by SDS-PAGE zymography, and the migration property of the lytic band was determined using standard molecular mass markers. *B*, Northern blot analysis was performed using 20 μg of total RNA from the very same cells assayed for lytic activity that were successively hybridized with specific DNA probes for rat MMP-9, TIMP-1, and TIMP-2. Equivalent loading of RNA was ascertained by a final rehybridization to an 18 S ribosomal probe.

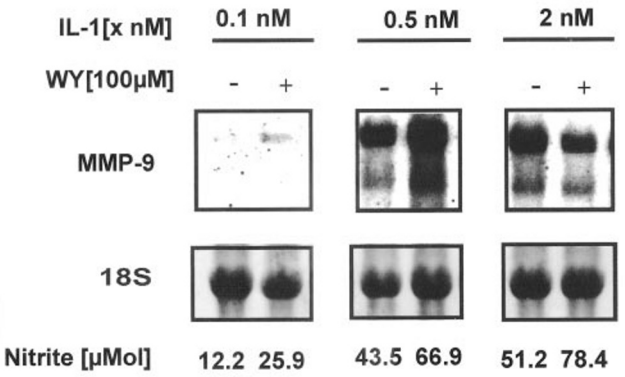

FIG. 6. **IL-1β-induced MMP-9 steady-state mRNA levels are differentially modulated by WY-14,643, depending on the concentration of cytokine used for stimulation.** Quiescent MC were coincubated for 24 h with the indicated concentrations of IL-1β in the presence (+) or absence (−) of a constant concentration of WY-14,643 (100 μM). Northern blot analysis demonstrates a dose-dependent modulation of cytokine-induced MMP-9 mRNA steady-state levels. 20 μg of total RNA were hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to an 18 S ribosomal probe. The stable end product of NO was measured by the Griess assay, and the amount of total nitrite release by MC is given at the bottom of the figure. The experiment was performed three times, and one representative experiment is shown.

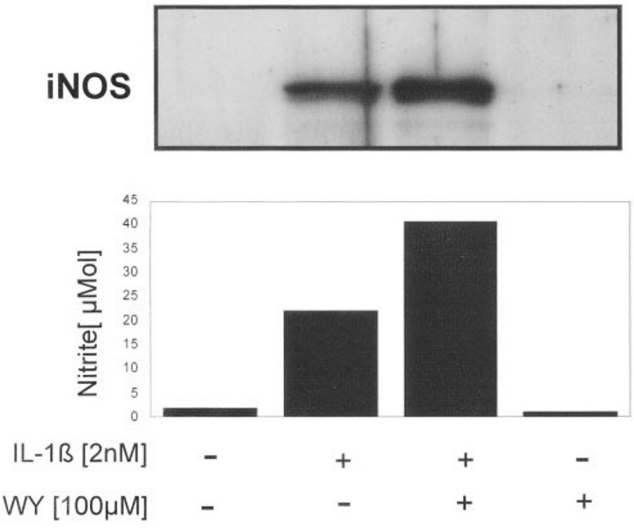

FIG. 7. **Cytokine-induced expression of inducible NO synthase is potentiated by WY-14,643 and results in an amplification of nitrite release.** Quiescent MC were treated for 24 h with vehicle (−), IL-1β (2 nM), WY-14,643 (100 μM), or IL-1β plus WY-14,643. Total cellular protein lysates (50 μg) were subjected to SDS-PAGE and immunoblotted using an anti-iNOS N-terminal antibody (22) (*upper panel*). The *lower panel* shows the nitrite levels in the corresponding cell supernatants as measured by the Griess assay. Equal loading of protein was ascertained by Ponceau S staining. Similar results were obtained in two independent experiments.

supernatants (Fig. 6). Interestingly, although the addition of WY-14,643 to all IL-1β concentrations caused a further increase in nitrite levels, WY differentially affected the IL-1β-induced MMP-9 mRNA levels depending on the concentration of IL-1β applied (Fig. 6). Whereas at low concentrations of IL-1β (0.1 and 0.5 nM) WY-14,643 clearly enhanced the cytokine-induced MMP-9 mRNA levels in the presence of higher concentrations of IL-1β (2 nM), WY-14,643 caused a significant reduction of cytokine-induced MMP-9 mRNA levels.

In summary, these data indicate that WY-14,643 principally potentiates the level of cytokine-induced MMP-9 mRNA, but this effect is antagonized by the negative effect of NO produced endogenously in large amounts upon stimulation with high concentrations of IL-1β.

The Cytokine-induced Increase in Nitrite Production Is Amplified by WY-14,643 and Is Due to Enhanced iNOS Expression—The experiments with L-NMMA clearly demonstrate a causal role of NO in the PPARα-dependent repression of MMP-9 mRNA steady-state levels. To elucidate whether activators of PPARα, similarly to MMP-9, also influence iNOS expression, we performed Western blot analysis using an N-terminal iNOS-specific antibody (22). Treatment with IL-1β for 24 h results in the appearance of an iNOS band at 130 kDa, which was not detectable in the extracts from untreated or WY-14,643-treated MC (Fig. 7, *upper panel*). When cells were simultaneously treated with IL-1β plus WY-14,643, cytokine-triggered iNOS expression was strongly enhanced and paralleled by an amplification of nitrite production (Fig. 7, *lower panel*). These results indicate that PPARα activators potentiate cytokine-mediated iNOS expression and subsequent nitrite production.

The Amplification of Cytokine-induced MMP-9 mRNA Levels by WY-14,643 in the Presence of L-NMMA Is Independent of the 1.8-kb MMP-9 Promoter Context—Next, we tested whether the modulation of MMP-9 promoter activity by WY-14,643 was affected by NO. To this end MC were transiently transfected with pGL-MMP-9 (1.8 kb) and stimulated with IL-1β to trigger endogenous NO formation or with DETA-NONOate as an exogenous source of NO. As shown before, treatment of MC with

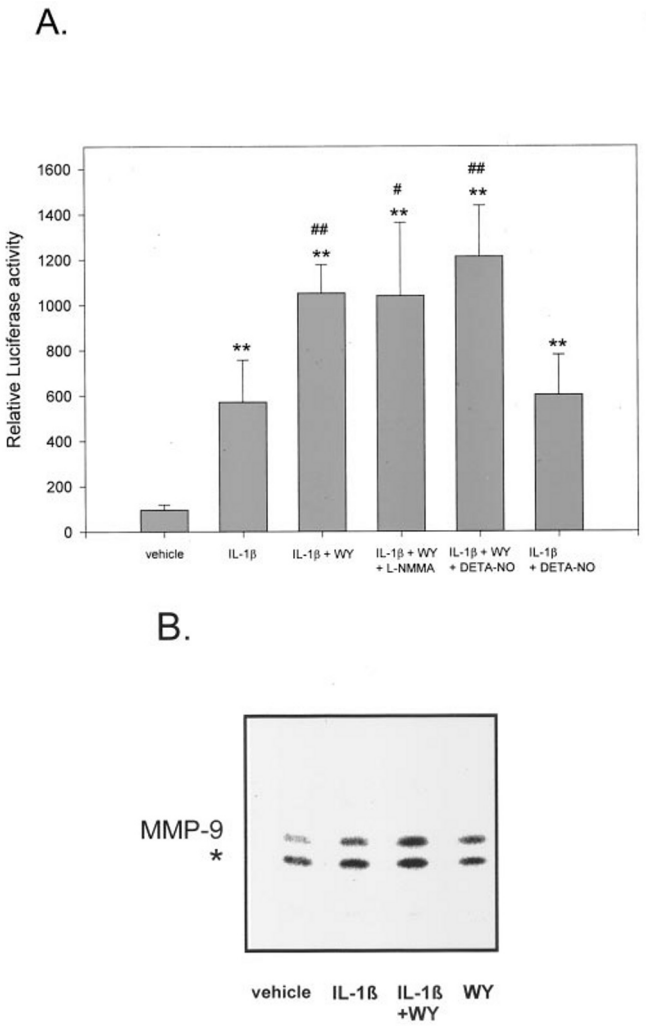

FIG. 8. **Exogenous and endogenous NO do not affect the potentiation of cytokine-induced MMP-9 promoter activity by WY-14,643.** *A*, subconfluent MC were transiently cotransfected with 0.4 μg of pGL-MMP-9 (1.8) and with 0.1 μg of pRL-CMV, coding for *Renilla* luciferase. After an overnight transfection, MC were treated for 24 h with vehicle or with IL-1β (2 nM) in the presence or absence of WY-14,643 (100 μM), L-NMMA (3 mM), or DETA-NONOate (500 μM) as indicated. The values for beetle luciferase were related to values for *Renilla* luciferase and are depicted as relative luciferase activities. Data represent the means ± S.D. ($n = 6$). *, $p \leq 0.05$; **, $p \leq 0.01$, compared with vehicle or IL-1β-stimulated conditions (#, $p \leq 0.05$; ##, $p \leq 0.01$). *B*, WY-14,643 increases the content of nuclear MMP-9 mRNA. RNase protection assay demonstrating that the cytokine-induced content of nuclear MMP-9 mRNA is further amplified by WY-14,643. MC were either treated with vehicle (control) or stimulated with IL-1β (2 nM), WY-14,643 (100 μM), or both agents in combination for 4 h. The amount of nuclear MMP-9 transcripts in isolated nuclei was determined by hybridizing the RNA transcripts with a radiolabeled antisense RNA from rat MMP-9. Equal counts of radiolabeled samples were loaded on acrylamide/urea gels and analyzed by phosphorimaging analysis. A band that possibly corresponds to hybridization of a smaller MMP-9-splicing variant is indicated by an *asterisk*. Similar results were obtained in two independent experiments.

IL-1β in the presence of WY-14,643 caused a potentiation of cytokine-dependent pGL-MMP-9 luciferase activity (Fig. 8*A*). Moreover, the addition of either L-NMMA or DETA-NONOate did not affect cytokine or cytokine plus PPARα-induced MMP-9 promoter activity (Fig. 8*A*) and also had no effects on the basal promoter activity of pGL-MMP-9 (1.8 kb) (data not shown). These data suggest that not a transcriptional but a posttranscriptional mechanism mediates NO suppression of cytokine-stimulated MMP-9 mRNA steady-state levels.

Nuclear MMP-9 mRNA Levels Are Increased by the PPARα Agonist WY-14,643—The promoter studies indicate that PPARα agonists have a positive effect on the transcriptional activity of IL-1β-triggered MMP-9. However, the use of partial promoter fragments in reporter gene assays does not necessarily reflect the real transcriptional regulation. Therefore, in addition to promoter studies, we used a modified transcription assay with intact nuclei. MC were treated for 4 h with either vehicle (control), IL-1β (2 nM), or WY-14,643 (100 μM) or both in combination before cells were lysed for nuclear fractionation. The quality of nuclei was tested by microscopy, and only fractions containing pure and intact nuclei were used for the assay. We used a combination of classical nuclear run-on with that of RNase protection assay since the sensitivity of RNase protection assay is severalfold higher than that of conventional protocols involving hybridization of labeled RNA to blots containing immobilized cDNA probes. As shown in Fig. 8*B*, MMP-9 shows a relatively high basal expression that corresponds well with the high basal promoter activities of the MMP-9 promoter constructs and which suggests a strong posttranscriptional regulation that might explain the observed low basal MMP-9 steady-state mRNA levels. Concomitantly, IL-1β treatment caused a moderate increase of MMP-9 gene expression that was further amplified by the addition of WY-14,643. In the protection assay we observed a second band with faster migrating properties (Fig. 8*B*, *asterisk*), most probably reflecting a splicing variant of nascent MMP-9 mRNA also observed in the Northern blots (Fig. 6). In agreement with the data from reporter gene assays, WY-14,643 by its own caused an increase in nuclear MMP-9 mRNA levels. Because posttranscriptional events regulating mRNA stability mainly occur in the cytoplasm, these results clearly demonstrate that the increase in nuclear MMP-9 mRNA levels results from an increased transcription rate.

In summary, these data clearly demonstrate that the inhibitory effects by PPARα agonists on the cytokine-induced steady-state MMP-9 mRNA levels result from indirect, posttranscriptional events.

Effect of NO on MMP-9 mRNA Stability—To test whether NO could affect the stability of cytokine-induced MMP-9 mRNA, we used the transcription inhibitor actinomycin D. MC were stimulated for 20 h with IL-1β (2 nM) before transcription was blocked by actinomycin D (5 μg/ml). Subsequently, cells were treated with vehicle or different NO donors. The reduction in MMP-9 mRNA observed under cytokine-stimulated conditions occurred with a half-life of ~8 h (Fig. 9*A*, *upper panel*). Interestingly, the addition of NO by either DETA-NONOate (Fig. 9*A*) or *S*-nitroso-D-penicillamine (Fig. 9*B*) markedly decreased MMP-9 mRNA stability, with a half-life of ~4 h. This is most impressively documented by the complete loss of the MMP-9 transcripts 8 h after the addition of the NO donors. Importantly, WY-14,643, in a similar experimental setting, did not affect the mRNA half-life of MMP-9 (data not shown).

Furthermore, we addressed the question of whether endogenously produced NO was, similar to exogenously added NO donors, able to reduce the stability of MMP-9 mRNA. To this aim, MC were treated with IL-1β (IL-1β) for 20 h either in the presence or absence of the NOS inhibitor L-NMMA (3 mM) before *de novo* transcription was blocked by actinomycin D (Fig. 10). As expected from the effects observed for exogenous NO, inhibition of endogenous NO production by L-NMMA caused a significant retardation of MMP-9 mRNA degradation (Fig. 10*A*). These data clearly demonstrate that endogenous NO levels similar to exogenous applied NO are sufficient to induce inhibitory effects on MMP-9 mRNA degradation.

In a further approach we tested whether iNOS inhibition

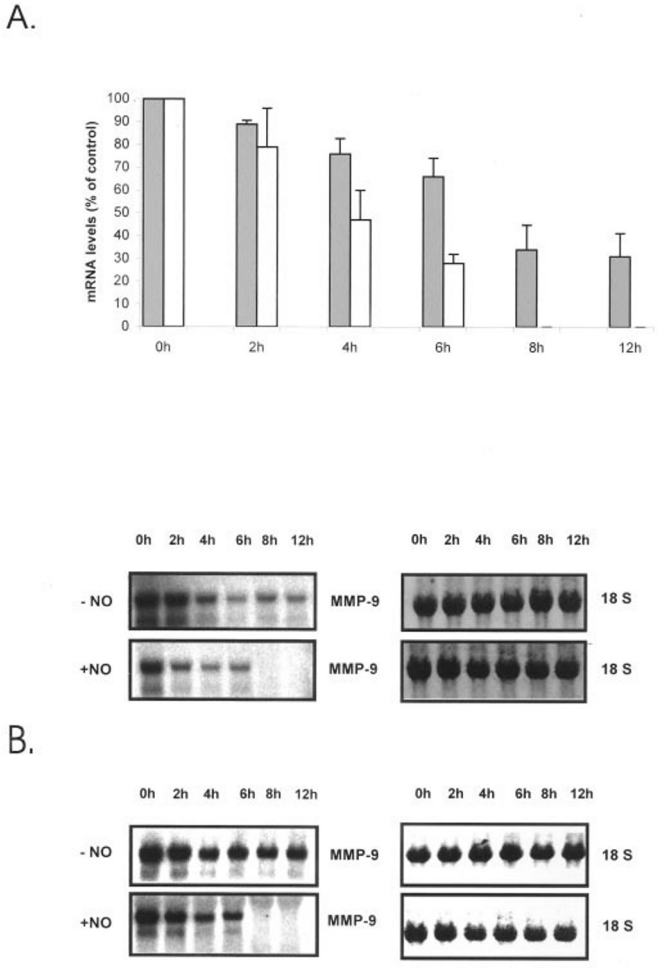

FIG. 9. **The stability of cytokine-induced MMP-9 mRNA is strongly diminished by the NO donors DETA-NONOate (*A*) and *S*-nitroso-D-penicillamine (*B*).** Quiescent MC were treated for 20 h with IL-1β (2 nM). After incubation, cells were washed twice and incubated with actinomycin D (5 μg/ml). After a short preincubation of 30 min, cells were additionally treated for the indicated time points without (*−NO*) or with (*+NO*) the different NO donors (500 μM). At the time points indicated after the start of actinomycin D treatment, cells were harvested and extracted for total cellular RNA. 20 μg of total cellular RNA were hybridized to a ^{32}P-labeled MMP-9 probe. The equivalent loading of RNA was ascertained by subsequent hybridization with an 18 S rRNA probe. The densitometric analysis of three independent experiments with DETA-NONOate is shown in the *upper panel* of Fig. 8*A*. The MMP-9 mRNA levels from MC treated with IL-1β in the absence (*filled bars*) or presence (*open bars*) of NO donor after 30 min of actinomycin-D (0 h) were set as 100%. Data in *B* are representative for two experiments giving similar results.

affects MMP-9 protein stability and/or activity. MC were treated for 20 h with IL-1β (2 nM) in the presence or absence of L-NMMA (3 mM) before protein synthesis was blocked by the addition of cycloheximide (10 μg/ml). After the indicated time points, conditioned media were collected for measurement of gelatinase activity, and protein levels were assayed by Western blot analysis using a specific MMP-9 antibody (Fig. 10*B*).

We observed that the stability of MMP-9 protein is quite high when compared with its corresponding mRNA since even a blockade of protein synthesis of 24 h did not change the total content of MMP-9 protein. Similarly to the cellular content of MMP-9, cycloheximide had no effects on the lytic content in the cell supernatants (Fig. 10*B*, *lower panels*). Most interestingly, there was no change in the MMP-9 protein contents when cells were grown in the presence of the NOS inhibitor L-NMMA, thus demonstrating that NO has no effects on the protein stability and gelatinolytic content of MMP-9.

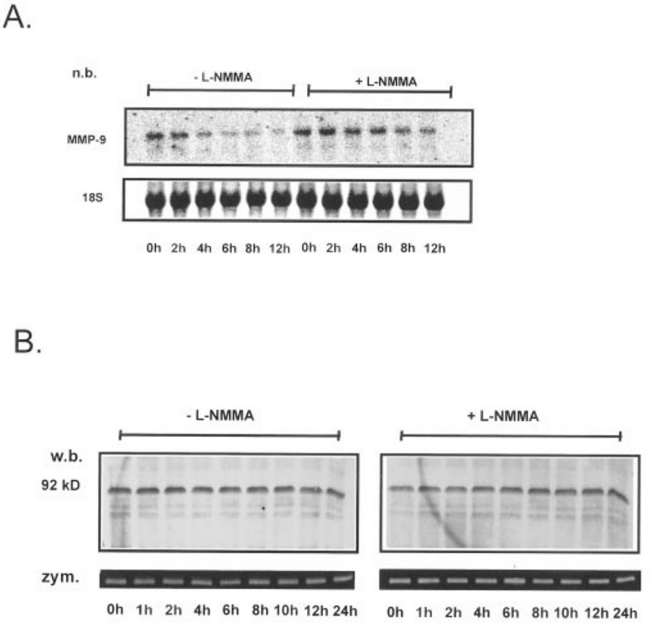

FIG. 10. *A*, the stability of cytokine-induced MMP-9 mRNA is inhibited by endogenously produced NO. Quiescent MC were treated for 20 h with IL-1β (2 nM) in the presence or absence of the NOS inhibitor L-NMMA (3 mM) as indicated. After incubation cells were washed twice, incubated with actinomycin D (5 μg/ml), and harvested for RNA extraction after the indicated time points. 20 μg of total cellular RNA were hybridized to a ^{32}P-labeled MMP-9 probe. The equivalent loading of RNA was ascertained by subsequent hybridization with an 18 S rRNA probe. Similar results were obtained in two independent experiments. *n.b.*, Northern blot analysis. *B*, the stability of MMP-9 protein and zymogen (*zym.*) activity of secreted MMP-9 is not affected by endogenously produced NO. Quiescent MC were treated for 20 h with IL-1β (2 nM) in the presence or absence of NOS inhibitor L-NMMA (3 mM) as indicated. After incubation, cells were washed twice, incubated with cycloheximide (10 μg/ml), and harvested for isolation of total protein lysates after the indicated time points. Protein lysates (100 μg) were subjected to SDS-PAGE and immunoblotted (*w.b.*) using a MMP-9-specific antibody (*upper panel*). Equal protein loading was ascertained by Ponceau S staining. The blot is representative of two independent experiments giving similar results. In parallel, 10 μl of cell culture supernatants were collected after the indicated time points and subjected to SDS-PAGE zymography (*lower panel*). The indicated band migrated at 92 kDa and corresponds to the latent form of MMP-9.

DISCUSSION

Increased expression of matrix-metabolizing enzymes is a hallmark of many inflammatory processes and may lead to irreversible alteration of tissue architecture. Particularly MMP-9 has been shown to be centrally involved in the dysregulation of extracellular matrix turnover associated with severe pathologic conditions such as rheumatoid arthritis (25) or fibrosis of lung, skin, and kidney (26, 27, 1). The inflammatory cytokines IL-1β and tumor necrosis factor α are among the most potent inducers of MMP-9 gene expression, mainly through the involvement of mitogen-activated protein kinase pathways, leading to increased activity of NF-κB and AP-1 transcription factors (5, 7). Interference with these signaling cascades, therefore, has been suggested as a promising strategy to prevent aberrant matrix turnover. Here we have focused on possible modulatory effects of PPARα agonists on cytokine-induced MMP-9 expression since the PPARα-signaling pathway exerts critical control functions in acute inflammation as shown by the exacerbated inflammatory response in PPARα null mice (18). We demonstrate that various structurally different PPARα agonists such as WY-14,643, LY-171883, and fibrates potently suppress cytokine-induced MMP-9 expression in renal MC. A down-regulation of lipopolysaccharide-induced MMP-9 secretion by the PPARα agonist fenofibrate was re-

cently documented in the human monocytic THP-1 cell line without addressing the underlying mechanisms (28). The anti-inflammatory action by PPAR transcription factors is well established and in many cases occurs by antagonizing the pro-inflammatory action of NF-κB (14, 15, 29) as well as AP-1 (15). In line with these observations, we demonstrate that in MC the cytokine-induced binding of a p50/p65-containing complex to a MMP-9-specific NF-κB binding site at −560/−550 is substantially reduced by PPARα agonists. However, the reduction of cytokine-induced NF-κB binding was not complete and somehow contrasts with the stimulatory effects on NF-κB binding by the PPARα agonist alone (Fig. 3*B*). Obviously, this reduced level of NF-κB activity is still sufficient to allow for a potentiation of the cytokine-triggered MMP-9 expression. Activation of NF-κB is crucially involved in IL-1β-mediated MMP-9 promoter activation (7). Whether the inhibition in DNA binding is caused by a physical interaction of PPARα with Rel-A transcription factors or indirectly, by the increased expression of the inhibitor of NF-κB (IκB) by PPARα activators, remains to be evaluated. Recently, Delerive *et al.* (15) have shown that both AP-1 and NF-κB activation are targeted by PPARα. In line with these considerations, the inhibition of NF-κB DNA binding by PPARα agonists in the context of a 1.8-kb portion of rat MMP-9 promoter did not influence the PPARα-dependent amplification of IL-1β-triggered MMP-9 promoter activity by the PPRE-like binding site at −1752/−1738. Interestingly, the PPRE sequence motif, which is defined as two direct AGG(T/A)CA repeats and separated by 1–5 nucleotides is shared by other nuclear receptors such as the thyroid hormone and the vitamin D receptors (12). Like PPARs these receptors bind to direct repeats in a complex with the retinoic acid receptor (RXR). It is worth mentioning that although the PPRE-like motif within the MMP-9 promoter contains only one half-site, which completely matches a PPRE sequence motif, EMSA and supershift analysis indicate PPAR binding comparable with that observed with a complete consensus motif. The observation that PPARα agonists by their own increased the nuclear content of MMP-9 transcripts and have a weak stimulatory effect on MMP-9 promoter activity paralleled by increased DNA binding to PPRE and NF-κB sites somewhat contrasts with the finding that PPARα activators do not affect the basal MMP-9 mRNA steady-state and zymogen levels. Obviously, the PPARα-triggered signals do not completely cover the cytokine-induced signaling pathways in rat MC. Moreover, in the complete promoter context additional regulatory events affecting transcriptional activators or repressors may explain the lack of MMP-9 mRNA increase in the absence of cytokine-induced signals. In line with these observations, the potentiating effects of PPARα agonists on cytokine-induced sPLA2 promoter activity in rat MC, similar to their effects on MMP-9 expression, cause activation of the sPLA2 promoter without having significant effects on the basal sPLA2 mRNA steady-state level (30).

In summary, these experiments clearly demonstrate that PPARα agonists synergistically with IL-1β amplify MMP-9 promoter activity through a PPRE-dependent DNA binding despite a partially reduced NF-κB signaling. As found by a modified nuclear run-on assay, the PPARα-mediated decrease in MMP-9 mRNA steady-state and zymogen levels is not attributable to an inhibition of MMP-9 gene expression but points to possible posttranscriptional regulatory events.

In addressing this hypothesis we most interestingly found that the reduction in cytokine-induced MMP-9 mRNA and zymogen levels by PPARα agonists is switched to strong potentiation in the presence of an inhibitor of NO synthesis. Furthermore, we demonstrate that treatment with PPARα agonists causes a strong increase of cytokine-induced iNOS expression and subsequent NO formation. These data suggest that PPARα-dependent effects on MMP-9 expression levels primarily result from alterations in NO production. This is in a line with our previous finding that NO, either given exogenously or endogenously by stimulation of iNOS expression, potently inhibits the mRNA steady-state levels of cytokine-induced MMP-9 in MC (6). The negative modulation of MMP-9 expression has been confirmed in other cell types and suggests a general mechanism of NO-triggered tissue remodeling (31–33).

When testing a 1.8-kb fragment of the promoter region of the rat MMP-9 gene by reporter gene assay we found that NO had no direct effects on cytokine-induced MMP-9 promoter activity, although the expression of many genes has been shown to be transcriptionally modulated by NO in rat MC (34, 35). However, a transcriptional control of MMP-9 gene expression seems unlikely since in our hands none of the NO-sensitive candidate transcription factors, including AP-1 and NF-κB (36–38), were significantly affected by NO (data not shown). Whether regulatory regions upstream from −1.8 kb of the MMP-9 gene may be negatively influenced by NO is the subject of ongoing investigations.

The 3′-untranslated region of the rat MMP-9 gene bears several AUUUA motifs, allowing for a posttranscriptional regulation of MMP-9 on the level of mRNA stability. In many genes AU-rich elements are specifically targeted by proteins of the ELAV-like protein family, which has been implicated in the regulation of mRNA stability (39–41). Using actinomycin D, an inhibitor of eukaryotic gene transcription, we found that exogenous NO significantly reduced the half-life of MMP-9 mRNA (Figs. 9 and 10), whereas PPARα agonist had no influence on MMP-9 mRNA stability. Similar to MMP-9, the expression of transforming growth factor β_3 is reduced by NO via destabilization of its mRNA (42). Additional experimental work is required to more precisely evaluate the molecular mechanism of posttranscriptional regulation of MMP-9 expression by NO.

In summary our study provides convincing evidence that PPARα agonists exert dominant negative effects on the steady-state levels of MMP-9 mRNA and enzyme levels in addition to their positive transcriptional effects. The action of PPARα agonists on MMP-9 mRNA is indirect and due to an NO-triggered reduction of MMP-9 mRNA half-life. The overall effects of exogenous or endogenous PPARα activators on secretion of MMP-9 will critically depend on the simultaneous production of NO by cells exposed to an inflammatory environment. High output levels of NO may account in part for the protective roles of PPARα agonists on the altered remodeling of extracellular matrix observed in many pathologies.

Acknowledgments—We thank Roswitha Müller for excellent technical assistance and Dr. Shenchu Ren for giving advice with the transcription assay.

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