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Identification of a Functional Peroxisome Proliferator-Activated Receptor Response Element in the Rat Catalase Promoter

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Peroxisomal proliferator-activated receptor (PPAR)γ has been shown to decrease the inflammatory response via transrepression of proinflammatory transcription factors. However, the identity of PPARγ responsive genes that decrease the inflammatory response has remained elusive. Because generation of the reactive oxygen species hydrogen peroxide (H_2O_2) plays a role in the inflammatory process and activation of proinflammatory transcription factors, we wanted to determine whether the antioxidant enzyme catalase might be a PPARγ target gene. We identified a putative PPAR response element (PPRE) containing the canonical direct repeat 1 motif, AGGTGA-A-AGTTGA, in the rat catalase promoter. *In vitro* translated PPARγ and retinoic X receptor-α proteins were able to bind to the catalase PPRE. Promoter deletion analysis revealed that the PPRE was functional, and a heterologous promoter construct containing a multimerized catalase PPRE demonstrated that the PPRE was necessary and sufficient for PPARγ-mediated activation. Treatment of microvascular endothelial cells with PPARγ ligands led to increases in catalase mRNA and activity. These results demonstrate that PPARγ can alter catalase expression; this occurs via a PPRE in the rat catalase promoter. Thus, in addition to transrepression of proinflammatory transcription factors, PPARγ may also be modulating catalase expression, and hence down-regulating the inflammatory response via scavenging of reactive oxygen species. (*Molecular Endocrinology* 16: 2793–2801, 2002)

PEROXISOMAL PROLIFERATOR-ACTIVATED RECEPTORS (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that heterodimerize with the retinoid X receptor (RXR) to regulate gene expression (as reviewed in Refs. 1 and 2). PPAR/RXR heterodimers bind to a DNA recognition motif composed of a direct repeat (DR) spaced by one nucleotide (DR1) with a consensus sequence of RGGTGA-A-AGGTCA. To date, three subtypes of PPARs have been identified: PPARα (NR1C1), β (also called δ, NUC1, or FAAR) (NR1C2), and γ (NR1C3) (3). In recent years much attention has been focused on PPARγ and its role in inflammation. Studies have demonstrated a down-regulation of the inflammatory response after treatment with various PPARγ agonists in a variety of different cell types and tissues (Refs. 4 and 5; see Ref. 2 for review). This protective antiinflammatory function has been shown to reflect the ability of PPARγ ligands to repress the expression of several proinflammatory genes and cytokines. One mechanism by which PPARγ accomplishes this appears to be regulated in part by down-regulation of nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and signal transducers and activators of transcription-1 (STAT-1)-mediated transcription of proinflammatory genes via transrepression due to competition for transcriptional coactivators (5, 6). PPARγ-independent effects have also been observed with the natural PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 via inhibition of IκB kinase through covalent modification (7, 8). In addition there is the potential that as yet unidentified PPARγ-responsive antiinflammatory genes may be responsible.

Reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical are generated during normal metabolism in all aerobic cells as well as after oxidative stress (9, 10). ROS have been shown to play a role in the pathogenesis and resultant morbidity of several different inflammatory disease states including rheumatoid arthritis, ischemia/reperfusion injury, and atherosclerosis. These effects are mediated in part via activation of NF-κB, STAT, and AP-1 transcription factors by ROS leading to an up-regulation of proinflammatory genes and cytokines (11–15). Cells have developed a number of protective antioxidant enzymes to scavenge these potentially toxic molecules. Catalase, along with the superoxide dismutases and glutathione peroxidases, plays an important role in protecting cells from oxidative stress.

Catalase is an antioxidant enzyme that catalyzes the dismutation of H_2O_2 to oxygen and water (16). In most

Abbreviations: AP-1, Activator protein 1; CMV-β-gal, cytomegalovirus-β-galactosidase; DR, direct repeat; DR1, DR spaced by one nt; DTT, dithiothreitol; NF-κB, nuclear factor-κB; nt, nucleotide; PPAR, peroxisomal proliferator-activated receptor; PPRE, PPAR response elemet; RBMECs, rat brain microvascular endothelial cells; ROS, reactive oxygen species; RXR, retinoid X receptor; STAT, signal transducer and activator of transcription.

mammalian tissues catalase is contained predominantly within peroxisomes, cellular organelles responsible for several important metabolic functions, including β-oxidation of long-chain fatty acids (17). Because peroxisomal fatty acid β-oxidation generates large amounts of H_2O_2, the presence of catalase is required to protect cells against this toxic ROS. In addition, recent evidence indicates that overexpression of catalase both *in vitro* and *in vivo* can inhibit NF-κB activation and protect cells and tissues from inflammatory and oxidant insults (18, 19).

Catalase was among the first enzymes to be discovered, and its biochemical function has been extensively characterized. In contrast, little is known regarding transcriptional regulation of the catalase gene in mammalian cells (20–23). Given that PPARγ has been shown to protect against inflammatory insult, we tested the hypothesis that PPARγ is involved in the regulation of catalase. We report here that PPARγ ligands can indeed increase catalase mRNA and activity, and this is mediated via a functional PPAR response element (PPRE) in the rat catalase promoter.

RESULTS

The Rat Catalase Promoter Contains a Putative PPRE

As a first step to determining the potential regulation of catalase by PPARγ, we examined the 5′-proximal promoter region (~5000 bp) of catalase for a potential PPRE using the GCG Wisconsin package. We identified a putative PPRE in the rat catalase promoter located at nucleotide (nt) −1027 to −1015 with respect to the translation start site (Fig. 1). The putative catalase PPRE has a DR1 sequence similar to the consensus PPRE for known PPAR target genes (Table 1).

PPAR/RXR Heterodimers Bind to the Putative Catalase PPRE

To demonstrate direct binding of PPAR/RXR heterodimers to the putative catalase PPRE, we used EMSAs. A double-stranded oligonucleotide probe was end labeled with ^{32}P and incubated with *in vitro* translated PPARγ and RXRα. Neither PPARγ nor RXRα alone bound to the catalase PPRE oligonucleotide (data not shown). However, PPARγ/RXRα heterodimers bound to the catalase PPRE oligonucleotide (Fig. 2, lane 2). This binding was specific as it could be competed out with unlabeled catalase PPRE oligonucleotide (Fig. 2, lanes 3–5) or incubation with a PPRE from the ACOX promoter (Fig. 2, lanes 6–8). In contrast, an excess of a nonspecific double-stranded oligonucleotide (NF-κB) did not displace the labeled catalase PPRE oligonucleotide (Fig. 2, lane 9).

The Rat Catalase Promoter Contains a Functional PPRE

Although PPARγ/RXR heterodimers are able to bind to the putative catalase PPRE, this does not demonstrate that the catalase PPRE is functional. To determine the functionality of the putative PPRE in the rat catalase

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3361-1328TTGCTGGTCC CATGGGATTT TAGACATGAC TGTCACTGCC TTTATGGGCT TCACATTCTA
3421-1268GGGACAGTGT AGATGTAGAT TAGCAAAAGA CACCAAAACA TGGCCCAATG AATGAAATTT
3481-1208TACAATTGTG TTAAGAACAA TGTACAAAAA AATAACATTG ATTAGATCAA TTTGTGACTG
3541-1148TTTTCCCAAG GGATTGCAAA ACTTACAATT TTACCAATCT CTGTTACCAC ATCTTTGTCA
3601-1088AATCAAGAAC AAGTTTTGGA GTGTCCTCAG TCACGTGCCC ACAGCCCACA GCCCATAATC
3661-1028AAGGTGAAAG TTGAGAAGGT TTATTTTGAA AATGACAGCA TCAAGGTGTT TAAAGATAAT
3721 -968TTCTAGCAAC TGATAGAAAA TCTTTAACCT ATTGATTAAA ATGAAAAATA AGCGACAAAG
3781 -908TCACCAAATA TTTACAAACA TTAGTAAATG TTTTACTTTC TAGCTTTTTT TATTTTTTGA
3841 -848ATCGGAGTTT CATATCCCAG TCTGGTCTCA AAGGAGCCAT GAAGCTGAAG ATGACCTTTG
3901 -788ATTTCTGAAC CTTAGCTTCT ACCTCCGGAG GGCTGGGAGT AACGGCCTGC GCCACCGCAC
3961 -728AGTTTATATG GTCCTGGCGA CTAAACTCGG GGCTTTCTGC ATGCTAGACA CTCAACCTTC
4021 -668CGAGCCACAT CCTCAGACCT TCACAGATCC CCCTCTCCCC AAAGACAAAG AGCTTTCTAT
4081 -608TATTCTCCAG TGTCAGGGGA GAAAATGGAG GAGTCTGTTA CTGCAGTTAA TACAGGCCAC
4141 -548TTTGGCACAG TGGGCCAAGT GACAAGTCAG ATTTCCCCTC TCACAGAGGA GGAAATCCGT
4201 -488ACAGATCTGT AGGAAGATTA AACAGTTCCT GAGCGTTTTT ACAATAAATT ATTGGTATAC
4261 -428AGCTTGGTAA ATGATAATCT GAGTGAGAGA AGTTAGAAAA AGAACTACCC ACCAATTAGT
4321 -368ACCAAATAAA TAAGCAAAGT GAGAAAACGT TGCACAGAGG AATTGGTCCC CGAACTGTGA
4381 -308CTCTCAGAGG TCAGAGAGCA CCGCCTTTCG GTCCCCACCC ATCTCCCCAG CCTCTTCCCA
4441 -248TCCCGGGGTC CACCTCCCGG AGCCCACTGC TCGCCCCACC CTCCTTCCAA TCCTGTCCCT
4501 -188TCTAGAGTTT CAGTGGCCAA TCAGGAGGCG GCCGTCCCGA GAGGTGGGGG GTGGTGCTGA
4561 -128TTGGCAGAGC CTGAAGTCAC CACTCCAGCG GGCCTGACTG ACGCGATTGC CTACCCCGGG
4621  -68TGGAGACCGT GCTCGTCCGG CCCTCTTGCC TCACGTTCTG CAGCTCTGCA GCTCCGCAAT
4681   -8CCTACACCAT GGCGGACAGC CGGGACCCAG CCAGCGACCA GATGAAGCAG TGGAAGGAGC
4741    AGCGGGCCCC TCAGGTATCC AGTGTTCCTC AGAGCCTCAC GAGAACTTGG GAGGCGGCGA
4801    GTAA
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Fig. 1. Identification of a Putative PPRE in the Rat Catalase Promoter

The PPRE is *bold and underlined*. The translation start site is in *bold*. See Ref. 21 for other putative transcription factor binding sites.

Table 1. Comparison of Identified PPRE Sequences[a]

Gene	Species	Element	Sequence	Protein function
Acyl-CoA oxidase	Rat	ACOA	AGGACA-A-AGGTCA	Peroxisomal oxidation
		ACOB	AGGTAC-A-AGGTCA	
Acyl-CoA synthase	Rat	ACS(Cl)	AGGGCA-T-CAGTCA	Peroxisomal oxidation
ALBP/aP2[b]	Mouse	ARE6	GGGTGA-A-ATGTGC	Fatty acid-binding protein
		ARE7	GGATCA-G-AGTTCA	
Bifunctional enzyme	Rat	BIF	AGGTCC-T-AGTTCA	Peroxisomal oxidation
Cytochrome P450 A1	Rat	CYP4A1	AGGGTA-A-AGTTCA	ω-Oxidation
Cytochrome P450 A6	Rabbit	CYP4A6	AGGGCA-A-AGTTGA	ω-Oxidation
Fatty acid transport protein	Mouse	FATP	GGGGCA-A-AGGGCA	Fatty acid transport
HMG-CoA synthase	Rat	HMG	GGGCCA-A-AGGTCT	Liver ketogenesis/sterol synthesis
Lipoprotein lipase	Rat	LPL	GGGGGA-A-AGGGCA	Triglyceride clearance
Malic enzyme	Rat	MEp	GGGTCA-A-AGTTGA	Fatty acid synthesis
Muscle-type carnitine palmitoyltransferase	Human	MCPT1	AGGGAA-A-AGGTCA	Fatty acid transport
PEPCK	Rat	PCK1	CGGCCA-A-AGGTCA	Glycerogenesis/gluconeogenesis
		PCK2	GGGTGA-A-ATGTGC	
Uncoupling protein I	Mouse	URE1	TGGTCA-A-GGGTGA	Thermogenesis
Rat catalase	**Rat**	**CTLS**	**AGGTGA-A-AGTTGA**	**H_2O_2 detoxification**
Consensus			RGGTCA-A-AGGTCA	

[a] See Refs. 1 and 49 and references therein.
[b] ALBP/aP2, Adipocyte lipid-binding protein; CoA, coenzyme A; HMG, hydroxymethylglutaryl; PEPCK, phosphoenolpyruvate carboxykinase.

promoter, we transfected a series of promoter deletion constructs (Fig. 3) into COS-1 cells in the presence of exogenous PPARγ_2 expression vector and the PPARγ ligand rosiglitazone.

The fragment containing the putative PPRE (rCTLS-1048) increased reporter gene activity by greater than 4-fold in the presence of PPARγ ligand (Fig. 4A). Indeed, deletion of the region containing the PPRE of the catalase promoter abolished the response to 5 μM rosiglitazone. To further establish the role of PPARγ, we transfected the COS-1 cells with the rCTLS-1048 in the presence and absence of exogenous PPARγ using the PPARγ_2 expression vector. Figure 4B demonstrates that the rCTLS-1048 fragment was not capable of increasing reporter activity in the absence of exogenous PPARγ_2. The addition of exogenous PPARγ_2 greatly increased reporter activity, which was even further enhanced by the addition of rosiglitazone. This indicates that a region between nt -1048 and -938 is PPARγ responsive. Within this region is the PPRE we identified at nt -1027 to -1015.

PPARγ forms heterodimers with RXRs and synergistically activates reporter genes when both receptors are activated by their respective ligands (24, 25). To determine whether the rat catalase PPRE could be similarly activated, we supplemented the transfected cells with rosiglitazone and the RXR-specific ligand LG268 either alone or together. As shown in Fig. 5, LG268 increased reporter gene activity 50% (not statistically significant). Rosiglitazone on its own increased reporter gene activity 3-fold (similar to that seen in Fig. 4A compared with rCTLS-1048 fragment without ligand). However, the two ligands together increased reporter gene activity greater than 7-fold.

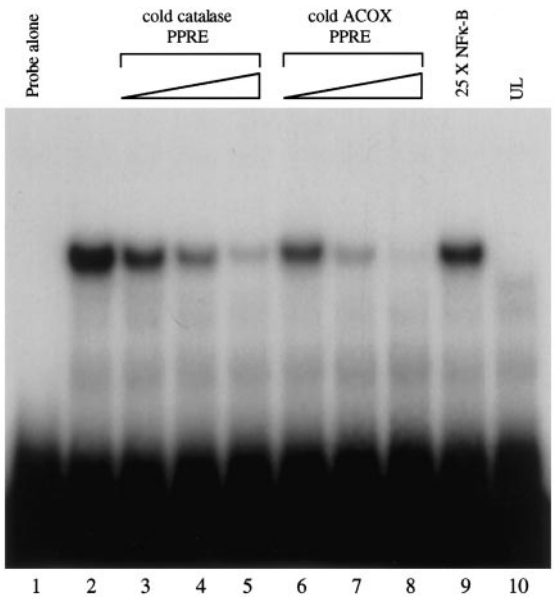

Fig. 2. PPARγ/RXRα Heterodimers Bind to the Catalase PPRE

PPARγ and RXRα *in vitro* translated proteins were incubated with ^{32}P-labeled catalase PPRE and electrophoresed on a 5% nondenaturing polyacylamide gel (lanes 2–9). Reactions were incubated with 5- to 25-fold excess cold catalase PPRE (lanes 3–5), and cold ACOX PPRE (lanes 6–8) and 25-fold excess cold NF-κB response element (lane 9) to determine affinity and specificity of binding. Gels were wrapped in plastic wrap and exposed to film at -80 C. UL, Unprogrammed lysate (lane 10).

These data provide further support for the hypothesis that PPARγ participates in the regulation of catalase expression.

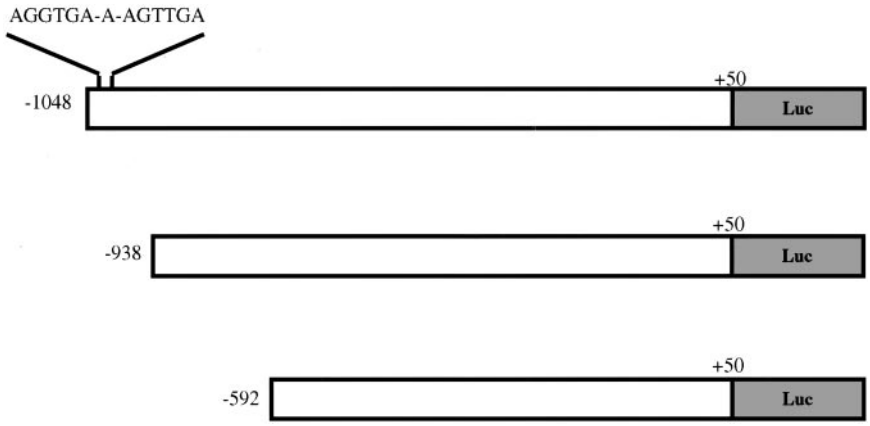

Fig. 3. Map of Promoter Deletions Cloned into the pGL3-Basic Reporter Vector
Numbers are given with reference to the translation start site.

To determine whether the catalase PPRE alone was sufficient to enhance PPARγ-stimulated promoter activity, we constructed a heterologous promoter containing a tandem tripeat of the catalase PPRE (DR1x3). COS-1 cells were transfected with the DR1x3 or empty vector, PPARγ_2, and cytomegalovirus-β-galactosidase (CMV-β-gal) expression vectors. The DR1x3 construct in the absence of ligand had significantly greater activity than the empty vector alone (Fig. 6). The addition of 0.1 or 1 μM rosiglitazone increased reporter activity more than 5- and 10-fold over the empty vector, respectively (2.5- and 4-fold compared with the DR1x3 vehicle control). However, in the absence of exogenous PPARγ, there was not a significant increase in reporter activity. This indicates that the PPRE present in the rat catalase promoter is regulated by PPARγ, and that this PPRE is necessary and sufficient for PPARγ-mediated activity.

Catalase mRNA Accumulation and Activity Are Induced by PPARγ Agonists

While the EMSAs and reporter assays demonstrate the ability of PPARγ to bind to and activate the PPRE in the catalase promoter, we wanted to determine whether PPARγ activation could alter catalase mRNA expression. For these experiments we used rat brain microvascular endothelial (RBMECs) cells, one of the cell types damaged during acute and chronic inflammatory responses as a result of ROS generation (26, 27). We performed immunoblotting for PPARγ in the RBMECs to determine whether PPARγ was present. Indeed, as shown in Fig. 7, the PPARγ_2 isoform appears to be the main isoform present. While the expression of PPARγ_2 has usually been associated with adipose tissue, studies have shown that other cell types such as endothelial cells express PPARγ_2 (28, 29). RBMECs were then supplemented with either of two different PPARγ agonists: ciglitazone or rosiglitazone. Treatment of cells with 5–20 μM of the low-affinity PPARγ agonist, ciglitazone, caused a dose-dependent increase in catalase mRNA (Fig. 8A). The high-affinity PPARγ agonist, rosiglitazone, increased catalase mRNA to a greater extent than the highest dose of ciglitazone. Catalase steady state mRNA levels increased 3-fold after 5 μM rosiglitazone, whereas 20 μM ciglitazone produced a 2-fold induction of catalase. Thus, two different PPARγ agonists can increase catalase mRNA expression. To determine the functionality of the catalase PPRE in this cell type, we transfected the RBMECs with the rCTLS-1048 fragment, without adding exogenous PPARγ expression vector and performed reporter assays. We observed a 2-fold increase in reporter activity in the absence of exogenously added RXR, once again demonstrating the functionality of the catalase PPRE and the presence of endogenous PPARγ (Fig. 8B).

The data presented demonstrate that PPARγ agonists are capable of increasing catalase promoter activity and mRNA levels. To determine whether this also leads to increased catalase function, catalase enzymatic activity assays were performed on RBMECs after rosiglitazone treatment. Catalase activity in control RBMECs was 110 $\pm$ 10.5 K/g protein. After treatment with 10 and 20 μM rosiglitazone for 48 h, activities increased significantly to 219 $\pm$ 7.1 and 277 $\pm$ 9.3 K/g protein, respectively (Fig. 9). Thus, PPARγ ligands appear to increase not only the transcription of catalase mRNA, but also the enzymatic activity of the protein as well.

DISCUSSION

Studies in recent years have demonstrated that PPARγ-mediated down-regulation of proinflammatory genes such as inducible nitric oxide synthase and cyclooxygenase-2 is attributed to squelching of coactivators, which leads to transrepression of the transcription factors AP-1, STAT-1, and NF-κB (5, 6). These previous studies elegantly demonstrated mechanisms by which PPARγ was antiinflammatory. How-

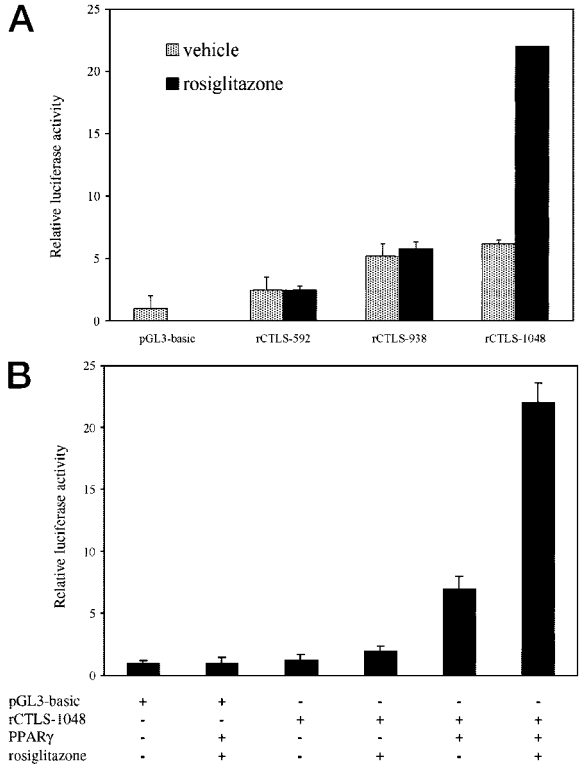

Fig. 4. A PPARγ-Responsive Element Lies Between nt −1048 and −938 of the Rat Catalase Promoter

A, COS-1 cells were transfected with a PPARγ_2 expression vector as well as the indicated reporter constructs followed by treatment with vehicle alone (*dotted bars*) or 5 μM rosiglitazone (*solid bars*). *, $P < 0.005$ *vs.* vehicle control containing the same reporter construct. B, COS-1 cells were transfected with the rCTLS-1048 construct in the presence or absence of a the PPARγ_2 expression vector, followed by treatment with vehicle alone or 5 μM rosiglitazone. A CMV-β-gal expression vector was used to control for transfection efficiency. Luciferase activity is normalized to β-gal levels, and values are normalized to the activity of the pGL3-basic vector. Data are representative of at least two independent experiments, n = 3 ± SD. *, $P < 0.01$; **, $P < 0.005$ *vs.* vehicle control containing the same reporter construct.

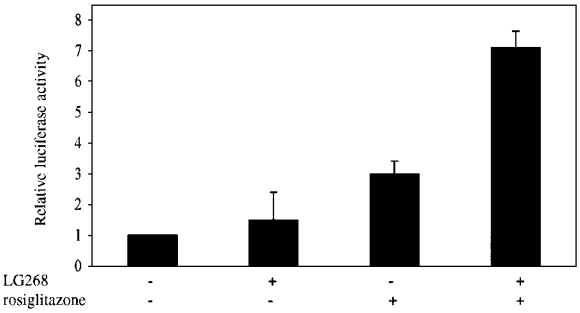

Fig. 5. PPARγ and RXR Ligands Act Synergistically to Enhance Rat Catalase Promoter Activity

COS-1 cells were transfected with a PPARγ_2 expression vector as well as the rCTLS-1048 reporter construct and treated with 5 μM rosiglitazone, 2.5 μM LG268, alone or together. CMV-β-gal was used to control for transfection efficiency. Luciferase activity is normalized to β-gal levels and to activity of the rCTLS-1048 reporter construct treated with vehicle alone. n = 3 ± SD; ANOVA; $P < 0.0001$; *, $P < 0.005$; **, $P < 0.0001$ *vs.* vehicle control.

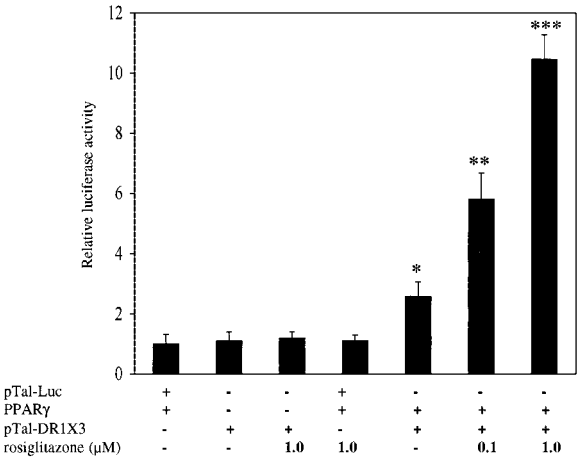

Fig. 6. The Catalase PPRE Is a Functional DR1

The pTal-Luc empty reporter construct or a tandem tripeat of the catalase PPRE was fused to a minimal promoter reporter construct and transfected into COS-1 cells with or without PPARγ_2 expression vector and CMV-β-gal as an internal control. Cells were then treated with 0.1 or 1.0 μM rosiglitazone and luciferase activity was determined. n = 3 ± SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* pTal-luc vehicle control.

ever, they failed to identify a PPARγ-responsive gene that could protect cells from inflammatory conditions. Several proinflammatory genes such as inducible nitric oxide synthase and cyclooxygenase-2 and various cytokines are up-regulated by ROS (30, 31). The mechanism by which this is thought to occur is via redox regulation of proinflammatory transcriptions factors such as AP-1, NF-κB, and STAT-1 (11, 14, 15). Indeed, H_2O_2 has been implicated as playing a major role in these processes. Furthermore, it has been demonstrated that overexpression of catalase as well as other antioxidants can protect against these inflammatory events (18, 19, 32, 33).

In this report, we identify a functional PPRE in the rat catalase 5′-flanking region located between nt −1027 and −1015 with respect to the translation start site. This PPRE contained the canonical DR1 motif spaced by an adenine and varied from the consensus sequence by only three nucleotides. We also demonstrated the ability of two different PPARγ ligands to increase catalase mRNA expression. A PPARγ-specific effect is suggested because the induction with the higher affinity ligand, rosiglitazone, was greater than with the lower affinity ligand, ciglitazone (34). In support of our data demonstrating that *in vitro* PPARγ ligands can increase catalase expression, Way *et al.* (35) recently demonstrated *in vivo* that a nonthiazolidinedione PPARγ ligand can increase catalase ex-

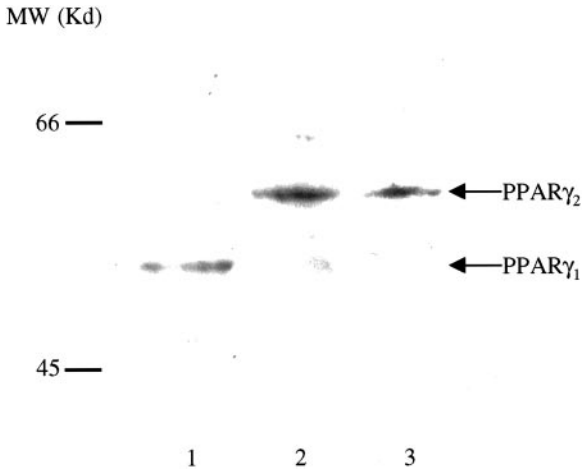

Fig. 7. Presence of PPARγ Protein in RBMECs

Western blot for PPARγ from whole-cell lysates of RBMECs (lane 3). *In vitro* translated $PPAR\gamma_1$ (lane 1) and $PPAR\gamma_2$ (lane 2) were used as a control. Western blotting was performed as described in *Materials and Methods*.

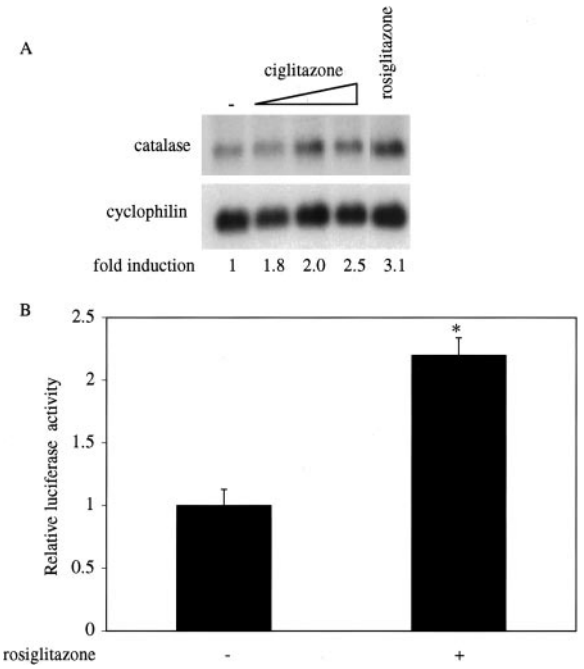

Fig. 8. Increased Catalase mRNA and Reporter Activity in RBMECs after Treatment with PPARγ Ligands

A, RBMECs were treated with vehicle, 5, 10, or 20 μM ciglitazone, or 5 μM rosiglitazone for 48 h. Total RNA (10 μg) was analyzed by Northern blot analysis for catalase and the housekeeping gene cyclophilin as described in *Materials and Methods*. Fold induction compared with vehicle control is shown *under* lanes. Representative of experiment performed at least two times. B, Endogenous PPARγ is capable of activating the CTLS-1048 reporter construct. RBMECs were transfected with the rCTLS-1048 deletion construct without additional expression vectors followed by treatment with vehicle alone or 5 μM rosiglitazone. A CMV-β-gal expression vector was used to control for transfection efficiency. Luciferase activity is normalized to β-gal levels. Data are representative of at least two independent experiments, $n = 3 \pm$ SD. *, $P < 0.005$ *vs.* vehicle control.

pression in tissues that contain PPARγ. The full significance of the induction of catalase by PPARγ is evidenced by the dose-dependent increase in catalase enzymatic activity currently observed after incubation of RBMECs with rosiglitazone. While only a 2- to 3-fold increase in catalase activity was observed, the kinetics of H_2O_2 dismutation to water and oxygen by catalase are almost diffusion rate limited (36). Thus, a relatively small change in its activity would have a profound functional effect.

Little is known regarding transcriptional control of catalase expression in mammalian cells. The rat catalase gene is a single-copy gene with 13 exons and 12 introns spanning 33 kb that possesses multiple transcription initiation sites (37). The promoter region of the gene lacks a TATA box and an initiator consensus sequence. However, characteristic of promoters lacking a TATA box, there are multiple CCAAT boxes and GC boxes (38). Studies have shown that decreased catalase activity, protein, and mRNA are due to decreased transcription (39, 40). Regulatory mechanisms may involve response elements in the 5′-flanking region. The marked decrease in catalase activity observed in tumor cell lines is thought to reflect, in part, the presence of a silencer element present in the 5′-flanking region of the catalase gene (41). In addition, other negative and/or positive regulatory elements have also been hypothesized (20, 22). However, in addition to the identification of a functional stimulating protein 1 site, little is known regarding the function of *cis*-elements within the catalase promoter that participate in its transcriptional regulation (41).

The current data demonstrate that catalase is a PPARγ target gene and suggest an additional and complimentary mechanism by which PPARγ might exert antiinflammatory effects. Thus, in addition to transrepression of AP-1, NF-κB, or STAT-1 activities as demonstrated by others, PPARγ may exert a protective effect by increasing catalase expression, resulting in decreased intracellular H_2O_2, which itself can activate these transcription factors. Although further functional studies are needed, our findings have important implications not only for the mechanism(s) by which PPARγ can protect against inflammatory related diseases, but also in understanding the regulation of mammalian catalase.

MATERIALS AND METHODS

Cell Culture

RBMECs were isolated from newborn Sprague Dawley rat pups (Harlan Sprague Dawley, Inc., Indianapolis, IN) as previously described (42). Cells were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) with 1% L-glutamine,

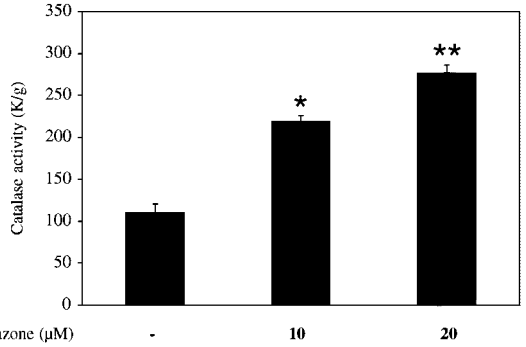

Fig. 9. PPARγ Activation Increases Catalase Enzymatic Activity

RBMECs were treated with 10 or 20 μM rosiglitazone or vehicle control for 48 h. Total cell lysates were isolated and catalase enzymatic activity was performed as described in *Materials and Methods*. n = 3 ± SD. *, $P < 0.0005$; **, $P < 0.00005$ *vs.* vehicle control.

10% fetal bovine serum, and 6 g/liter glucose at 37 C. Cells were passaged once per week and used at passage 10–17. Cos-1 cells were maintained in DMEM with 1% L-glutamine and 10% fetal bovine serum.

RXR Expression Vector Generation

RT-PCR methodology was used to generate a full-length mouse RXRα expression vector from mouse liver RNA. The upstream primer, 5′-GGGCATGAGTTAGTCGCAG-3′, and downstream primer, 5′-ACACTGCACCCCAAAGATC-3′ (GenBank accession no. X66223), were used. PCR conditions were as follows: 94 C for 4 min, then 35 cycles of 94 C for 1 min, 62.5 C for 1 min, and 72 C for 1.5 min, and a final elongation step of 7 min at 72 C. The PCR products were analyzed on a 1% low-melting point agarose gel, and a single band corresponding to the expected size of the RXRα PCR product (1534 bp) was isolated using a QIAGEN (Chatsworth, CA) gel extraction kit. The RXRα cDNA was captured into the pTargeT mammalian expression vector (Promega Corp., Madison, WI) according to manufacturer's directions, and the identity of the cDNA was verified by sequencing. The PPARγ$_2$ cDNA was generously provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA).

Promoter Reporter Construction

Total genomic DNA was isolated from the RBMECs using DNAzol reagent (Life Technologies, Inc.) according to manufacturer's directions, and restricted with *Eco*RI, and the DNA was isolated using the QIAGEN Qiamp Tissue Kit according to the manufacturer's directions. For PCR the following primers were used: rCTLS-1048, ACAGCCCACA GCCCATAATC (3641–3660); rCTLS-938, ATTGATTAAAATGAAAAATAAGCGAC (3751–3776); rCTLS-592, CTGATGCTAGACACTCAACC (3997–4017). The *number after rCTLS* indicates length of the construct with respect to the translation start site, and the *numbers in parentheses* indicate location of primers designating the first nucleotide of the published sequence (GenBank accession no. M25669) as bp 1. A common downstream primer, CAGATGAAGCAGTGGAAGGA (4719–4738), was used with all the upstream primers. The translation start site is located at base 4689 (Fig. 1).

PCR was performed using the following conditions: 95 C for 4 min, and then 35 cycles of 94 C for 1 min, 60 C for 1 min, and 72 C for 1.5 min with a final elongation step of 72 C for 7 min. PCR products were ligated into a pCR2.1 TA-TOPO cloning vector according to the manufacturer's directions (Invitrogen, San Diego, CA). The identities of the fragments were confirmed by sequencing. Sequences were identical to the published sequence (22, 37). The promoter fragments in the pCR2.1 vector were then directionally subcloned into the *Kpn*I and *Xho*I sites of the pGL3-basic reporter constructs and then resequenced.

Construction of Heterologous Catalase DR1 Reporter Construct

An oligonucleotide containing three direct tandem repeats of TAATCAAGGTGAAAGTTGAGAAG (DR1x3) was synthesized with *Kpn*I and *Xho*I restriction sites on its 5′- and 3′-ends, respectively, with 6-bp extensions beyond the restriction sites. The upstream and downstream DR1x3 was annealed and then restricted with *Kpn*I and *Xho*I, gel isolated, and ligated into the *Kpn/Xho* sites of the pTal-luc minimal promoter reporter construct (CLONTECH Laboratories, Inc., Palo Alto, CA). Fidelity was confirmed by sequencing.

Western Blot Analysis

Total protein was isolated, separated on a 10% polyacrylamide gel, and transferred to nitrocellulose as previously described (43). Three microliters of *in vitro* translated PPARγ$_1$ or PPARγ$_2$ were used as a control. Membranes were blotted for PPARγ using a mouse monoclonal antibody for PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that recognizes both PPARγ1 and PPARγ2 and visualized using ECL reagent (Amersham Pharmacia Biotech, Arlington Heights, IL).

Northern Blot Analysis

RBMECs were grown in 100-mm dishes until 70% confluent and then supplemented with ciglitazone (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) or rosiglitazone (a kind gift from Dr. Richard Heyman, Ligand Pharmaceuticals, Inc., San Diego, CA). Total RNA was isolated using RNA STAT-60 isolation reagent (Tel-Test "B") according to manufacturer's directions (Tel-Test, Friendswood, TX). A rat catalase partial cDNA was constructed by RT-PCR of total RNA from RBMECs using the sense primer 5′-AAACCCGATGTCCTGACCAG-3′ and antisense primer 5′-CCTTTGCCTTGGAGTATCTGG-3′ with the Expand HiFi PCR System (Roche Molecular Biochemicals, Indianapolis, IN). The following PCR conditions were used: 94 C for 5 min, and then 35 cycles of 94 C for 1 min, 64 C for 30 sec, and 72 C for 1 min, followed by a final extension step at 72 C for 5 min. The resulting 228-bp fragment was ligated into a pCR2.1 TA cloning vector (Invitrogen) according to the manufacturer's directions. The identity of the PCR fragment was confirmed by sequencing (DNA Core Facility, University of Iowa). ^{32}P-labeled catalase cDNA probe was made by random-primer labeling of 50 ng of rat catalase cDNA *Eco*RI fragment. Total RNA (10 μg) was subjected to Northern blotting as previously described (43). Membranes were stripped and reprobed with the housekeeping gene cyclophilin as a control for loading and transfer. Blots were quantified by densitometry performed at the Image Analysis Facility at the University of Iowa.

Nuclear Protein Isolation and EMSA

Nuclear extracts were isolated by a modified method of Dignam and colleagues (44, 45). Cells were rinsed in cold 1× PBS and then scraped into ice-cold buffer A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT)] and incubated on ice for 20 min. Cells were then Dounce homogenized and checked microscopically for cell lysis. Nuclei were then iso-

lated by centrifugation at 5000 rpm for 30 sec at 4 C and supernatant was removed (performed twice). The nuclear pellets were resuspended in ice-cold buffer C [20 mM HEPES, 25% glycerol (vol/vol), 0.42 NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM DTT] and incubated on ice for 15 min. The suspensions were centrifuged at 10,000 $\times$ *g* for 5 min and supernatants were removed and diluted into ice-cold buffer D (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM DTT). Nuclear extracts were then stored at −80 C.

In vitro translation of PPARγ2 and RXRα were carried out with the Promega Corp. TNT-coupled system according to manufacturer's directions. The reaction was also carried out in the presence of ^{35}S-methionine, separated by SDS-PAGE, gel dried, and exposed to film to verify translation of the correct size protein (data not shown). For EMSA the following upstream and downstream oligonucleotides were used: acyl-coenzyme A oxidase PPRE (ACOX-PPRE) (46): 5′-AGCTGGGACCAGGACAAAGGTCACGTT-3′, 5′-GATCAACGTGACCTTTGTCCTGGTCCC-3′; rat catalase putative DR-1: 5′-AGCTTAATCAAGGTGAAAGTTGAGAAG-3′, GATCCTTCTCAACTTTCACCTTGATTA, and an NFκB response element: 5′-AGCTAACTCGGGGCTTTCTGC-3′, 5′-GATCGCAGAAAGCCCCGAGTT-3′. The imperfect PPREs are *underlined* as well as the NF-κB site. Probes for EMSA were made as previously described (45). Nuclear extracts (1–10 μg) or 1 μl of *in vitro* transcribed/translated protein were incubated with 1 μg poly(deoxyinosine-deoxycytidine) (Pharmacia Biotech, Piscataway, NJ), gel shift buffer (10 mM Tris, pH 7.5; 4% glycerol; 50 mM NaCl; 1 mM $MgCl_2$; 0.5 mM EDTA; 0.5 mM DTT) and ^{32}P-labeled probes. Bound DNA complexes were separated from free probe by PAGE on a 5% native gel at 15 mA for 1 h in 1$\times$ Tris-buffered EDTA. Gels were wrapped in Saran wrap and exposed to x-ray film at −80 C.

Transfections and Reporter Assays

COS-1 cells were seeded into six-well culture plates (Corning, Inc., Corning, NY). Cells were then transfected with 200 ng of the various catalase promoter deletions or empty pGL3 vector using Superfect according to manufacturer's directions (QIAGEN). PCMV-β-gal plasmid (100 ng) was included in each transfection to control for transfection efficiency. Except where noted, 100 ng of PPARγ_2 were also cotransfected. The cells were treated for 36 h with the rosiglitazone and/or LG268 (a kind gift from Dr. Richard Heyman, Ligand Pharmaceuticals, Inc.). Luciferase activity was measured on a luminometer (Lumat LB 9507, EG&G Berthold, Oak Ridge, TN) and β-galactosidase (β-gal) activity was determined according to the manufacturer's specifications (Promega Corp.). Luciferase activity was normalized to β-gal activity.

COS-1 cells were also transfected with PPARγ_2 expression vector and CMV-β-gal along with pTal-luc empty vector or vector containing the catalase DR1x3, as described above. Cells were treated with vehicle control, 0.1 μM or 1.0 μM rosiglitazone, and assayed after 24 h on a luminometer. Luciferase activity was normalized to β-gal activity.

Catalase Activity Analysis

RBMECs were supplemented with 10 or 20 μM rosiglitazone for 48 h. Whole-cell extracts were isolated as previously described (47). Catalase activity was performed as described by Clairborne (48). In brief, 200 μg of protein were added to a cuvette containing 50 mM phosphate buffer (pH 7.8), and H_2O_2 was added to a final concentration of 10 mM. The disappearance of H_2O_2, as determined by its absorbance at 240 nM, was measured immediately at 30-sec intervals for 1 min. Activity was expressed as units per gram of protein (K/g).

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