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Expression of Heme Oxygenase-1 in Human Vascular Cells Is Regulated by Peroxisome Proliferator-Activated Receptors

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Objective—Activation of peroxisome proliferator-activated receptors (PPARs) by lipid-lowering fibrates and insulin-sensitizing thiazolidinediones inhibits vascular inflammation, atherosclerosis, and restenosis. Here we investigate if the vasculoprotective and anti-inflammatory enzyme heme oxygenase-1 (HO-1) is regulated by PPAR ligands in vascular cells.

Methods and Results—We show that treatment of human vascular endothelial and smooth muscle cells with PPAR ligands leads to expression of HO-1. Analysis of the human HO-1 promoter in transient transfection experiments together with mutational analysis and gel shift assays revealed a direct transcriptional regulation of HO-1 by PPAR α and PPAR γ via 2 PPAR responsive elements. We demonstrate that a clinically relevant polymorphism within the HO-1 promoter critically influences its transcriptional activation by both PPAR isoforms. Moreover, inhibition of HO-1 enzymatic activity reversed PPAR ligand-mediated inhibition of cell proliferation and expression of cyclooxygenase-2 in vascular smooth muscle cells.

Conclusion—We demonstrate that HO-1 expression is transcriptionally regulated by PPAR α and PPAR γ , indicating a mechanism of anti-inflammatory and antiproliferative action of PPAR ligands via upregulation of HO-1. Identification of HO-1 as a target gene for PPARs provides new strategies for therapy of cardiovascular diseases and a rationale for the use of PPAR ligands in the treatment of other chronic inflammatory diseases. (*Arterioscler Thromb Vasc Biol.* 2007;27:1276-1282.)

Key Words: atherosclerosis ■ heme oxygenase ■ inflammation ■ PPAR

Hyperlipidemia and diabetes type II are major causes for vascular disorders including atherosclerosis. Among the drugs that are used to treat these metabolic diseases, both the lipid-lowering fibrates and the insulin-sensitizing thiazolidinediones exert their effects via activation of peroxisome proliferator-activated receptors (PPARs).¹ PPARs are ligand-activated transcription factors that bind to specific PPAR-responsive elements (PPREs) as heterodimers together with the retinoid X receptor and govern the expression of genes involved in the regulation of lipid and glucose metabolism.^{1,2} The 3 PPAR isoforms PPAR α , PPAR β/δ , and PPAR γ show unique tissue distribution and ligand specificity. Natural PPAR ligands include various fatty acids and fatty acid-derived compounds such as eicosanoids.^{1,2} Treatment with synthetic PPAR α and PPAR γ ligands such as fibrates and thiazolidinediones,³ respectively, has been shown to potently inhibit the development of atherosclerosis³⁻⁶ and restenosis.^{7,8} Increasing evidence suggests that the beneficial effects

of PPAR ligands on the vascular wall are not only caused by changes in systemic metabolic parameters⁹ but additionally involve local anti-atherogenic effects,⁶ such as inhibition of inflammation¹⁰⁻¹² and vascular smooth muscle cell (VSMC) proliferation.⁸ The beneficial effects of synthetic PPAR agonists on cardiovascular disease outcome have been demonstrated in major clinical trials that have reported cardiovascular risk reduction in patients with dyslipidemia. However, the favorable alterations in plasma lipids can only partially explain the reduction in cardiovascular events in these studies. Therefore, many beneficial effects of PPARs have been attributed to their anti-inflammatory activity.

Despite the large number of known target genes for PPARs, little is known about induction of expression of anti-inflammatory and antiproliferative genes by PPARs. We hypothesized that the local upregulation of anti-atherogenic genes by PPAR ligands contributes to their beneficial effects on the vascular wall. Recently, the presence of PPREs in the

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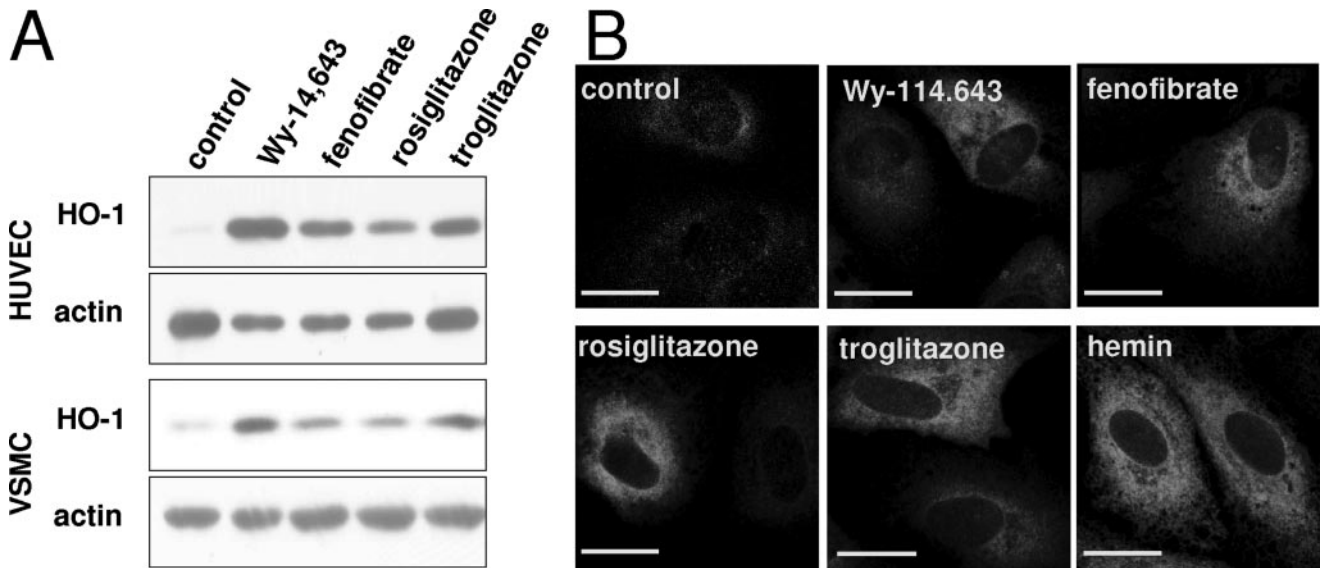


Figure 1. Induction of HO-1 protein expression by ligands for PPAR α and PPAR γ . Human umbilical venous endothelial cells and human VSMCs were treated with Wy-14,643 (250 μ mol/L), fenofibrate (200 μ mol/L), rosiglitazone (5 μ mol/L), or troglitazone (5 μ mol/L) for 20 hours. A, Western blot analysis of HO-1 expression in extracts from human umbilical venous endothelial cells and human VSMCs. B, Immunofluorescence in human umbilical venous endothelial cells.

antioxidant enzymes catalase¹³ and manganese superoxide dismutase¹⁴ have been reported.

Numerous studies demonstrated a protective role for heme oxygenase-1 (HO-1) during atherogenesis, restenosis, and other inflammatory vascular disorders.^{15–20} HO-1 is the rate-limiting enzyme of heme catabolism, catalyzing the breakdown of heme into iron, biliverdin, and carbon monoxide. Both biliverdin and carbon monoxide have been shown to act as anti-inflammatory agents and to inhibit VSMC growth.^{17,21–23} It has been shown that the length of a GT-repeat within the proximal region of the human HO-1 promoter is highly polymorphic, which seems to influence the transcription of the HO-1 gene, rendering people possessing longer (≥ 29 repeats) GT fragments more susceptible to inflammatory disorders such as coronary atherosclerosis.²⁴

Here we demonstrate induction of HO-1 expression by PPAR α and PPAR γ ligands in cultured vascular cells and we show that HO-1 enzymatic activity mediates anti-inflammatory and antiproliferative effects exerted by PPAR ligands. Moreover, we show that HO-1 is a direct PPAR target gene and that a clinically relevant (GT) n dinucleotide length polymorphism within the human HO-1 promoter significantly influences the transcriptional regulation of HO-1 by both PPAR α and PPAR γ .

Materials and Methods

Expression of HO-1 and PPAR isotypes was studied using real-time polymerase chain reaction or Western blotting. The constructs (4.9 kb to 0.3 kb) used for human HO-1 promoter analysis were described previously.²⁵ Site-directed mutagenesis of the HO-1 promoter was performed by a polymerase chain reaction-based technique using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif). Electromobility shift assays were performed using the TNT reticulocyte lysate kit (Promega). For more details, please see the supplemental Materials available online at <http://atvb.ahajournals.org>.

Results

Ligands for PPAR γ and PPAR α Induce HO-1 Expression in Human Vascular Cells

Activation of both PPAR γ and PPAR α was shown to inhibit the inflammatory response in endothelial cells, VSMCs, and macrophages,^{3,26,27} and ligands for both receptors have been shown to reduce atherosclerosis in mice^{3–5} and humans.^{28,29} Therefore we determined the influence of ligands specific for either PPAR α (Wy-14,643 and fenofibrate) or PPAR γ (rosiglitazone and troglitazone) on HO-1 expression in vascular cells. Western blot analysis (Figure 1A) and immunofluorescence (Figure 1B) demonstrated that both PPAR α and PPAR γ ligands induced HO-1 expression in human umbilical venous endothelial cells (human umbilical venous endothelial cells) as well as in human VSMCs.

We found a maximum expression of HO-1 protein at 18 to 20 hours after incubation with PPAR ligands, and dose-response experiments revealed a concentration-dependent increase of HO-1 protein (Figure 2A) and mRNA (Figure 2B and 2C) after stimulation with the PPAR α ligand Wy-14,643 or the PPAR γ ligand rosiglitazone in both human umbilical venous endothelial cells and VSMC.

Quantification of PPAR α and PPAR γ mRNA levels showed that PPAR α was the predominant PPAR isotype in both human umbilical venous endothelial cells and VSMC (Figure 2D). Accordingly, measurement of HO-1 mRNA levels in human umbilical venous endothelial cells and VSMC by quantitative polymerase chain reaction showed a marked increase in HO-1 mRNA after activation of PPAR α by Wy-14,643, whereas treatment with the PPAR γ -specific ligand rosiglitazone induced HO-1 mRNA to a lesser extent (Figure 2B and 2C). Together, these data suggest that depending on the expression levels of the respective PPAR isotypes, PPAR α and PPAR γ ligands induce HO-1 in vascular cells.

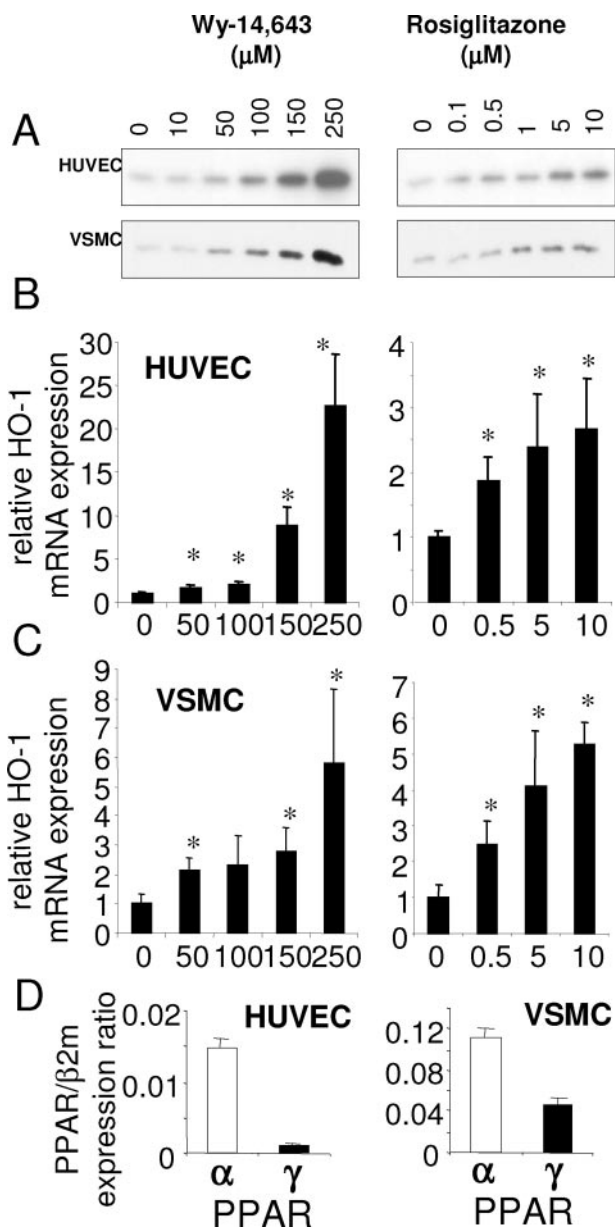


Figure 2. HO-1 expression is induced in human umbilical venous endothelial cells and SMC by ligands for PPAR α and PPAR γ in a dose-dependent manner. A, Western blot analysis in human umbilical venous endothelial cells and human VSMC that had been treated with Wy-14,643 or rosiglitazone at indicated concentrations for 20 hours. B, Measurement of HO-1 mRNA levels in human umbilical venous endothelial cells by quantitative polymerase chain reaction. C, Measurement of HO-1 mRNA levels in human VSMC by quantitative polymerase chain reaction. D, Measurement of PPAR α and PPAR γ mRNA expression levels. * $P < 0.05$ compared with the control value.

HO-1 Is a Direct PPAR Target Gene

To determine if HO-1 was a direct PPAR target gene, we analyzed the activity of a full-length (4.9 kb) human HO-1 promoter construct that had been previously cloned in our laboratory.²⁵ HO-1 promoter activity was induced by cotransfection of expression vectors for PPAR α or PPAR γ together with a plasmid encoding for retinoid X receptor- α in HEK293 cells followed by treatment with the respective PPAR ligand. Analysis of deletion mutants demonstrated that PPAR α or

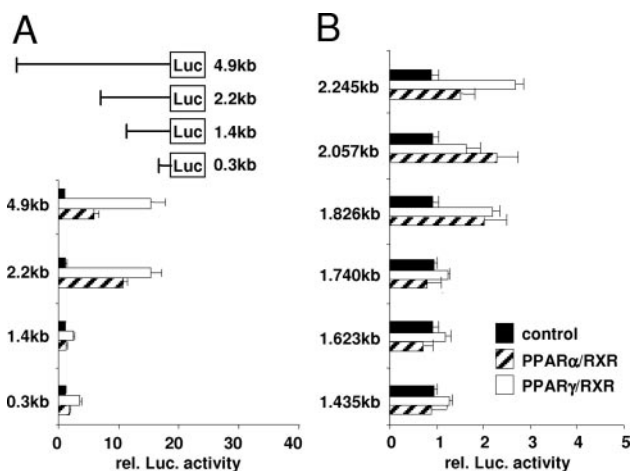


Figure 3. Regulation of the human HO-1 promoter by PPAR α and PPAR γ . A and B, HEK293 cells were transfected with HO-1 promoter luciferase reporter constructs containing promoter fragments with the indicated length or the full-length promoter and cotransfected with PPAR α or PPAR γ and retinoid X receptor- α expression plasmids.

PPAR γ -induced HO-1 promoter activity was dependent on a fragment located between -1.4 and -2.2 kb upstream of the transcription start site (Figure 3A). To narrow down the responsive promoter elements we created a second series of deletion mutants, which enabled us to locate the PPAR-responsive promoter region between -1740 kb and -1826 kb (Figure 3B). Bioinformatic analysis identified 2 putative PPAR-responsive elements (PPRE1 and PPRE2 GGGA-CAAAGGTTG and AGGTGAAAGGCCG) in DR1 configuration located within the 86-bp region.

To confirm the functional relevance of the 2 identified PPREs, we introduced mutations in each of these elements, which abolished the PPAR α - or PPAR γ -induced activity of the full-length HO-1 promoter construct (Figure 4A). Electrophoretic mobility shift assays showed that both PPRE1 and PPRE2 bound to in vitro-translated PPAR α /retinoid X receptor- α and PPAR γ /retinoid X receptor- α heterodimers (Figure 4B), whereas we did not observe binding to retinoid X receptor- α homodimers (data not shown). The specificity of the bands was confirmed by competition with excess cold wild-type probes or cold probes containing point mutations. Together, these data demonstrate that HO-1 is a direct PPAR α or PPAR γ target gene regulated via 2 PPREs.

A Polymorphism Within the HO-1 Promoter Critically Affects Its Regulation by PPAR α or PPAR γ

The HO-1 promoter is highly polymorphic within the human population in that the length of a proximal (GT)_n dinucleotide repeat varies²⁴ (Figure 5A). Longer repeats have been reported to negatively influence HO-1 expression and thus are associated with a higher risk for coronary artery disease and restenosis.^{30,31} To determine if the length of the GT-repeat affects the transcriptional regulation of the human HO-1 promoter by PPARs, we generated 3 full-length (4.9 kb) HO-1 promoter constructs differing in the length of their proximal GT-repeats (11, 24, and 29) by amplification of the

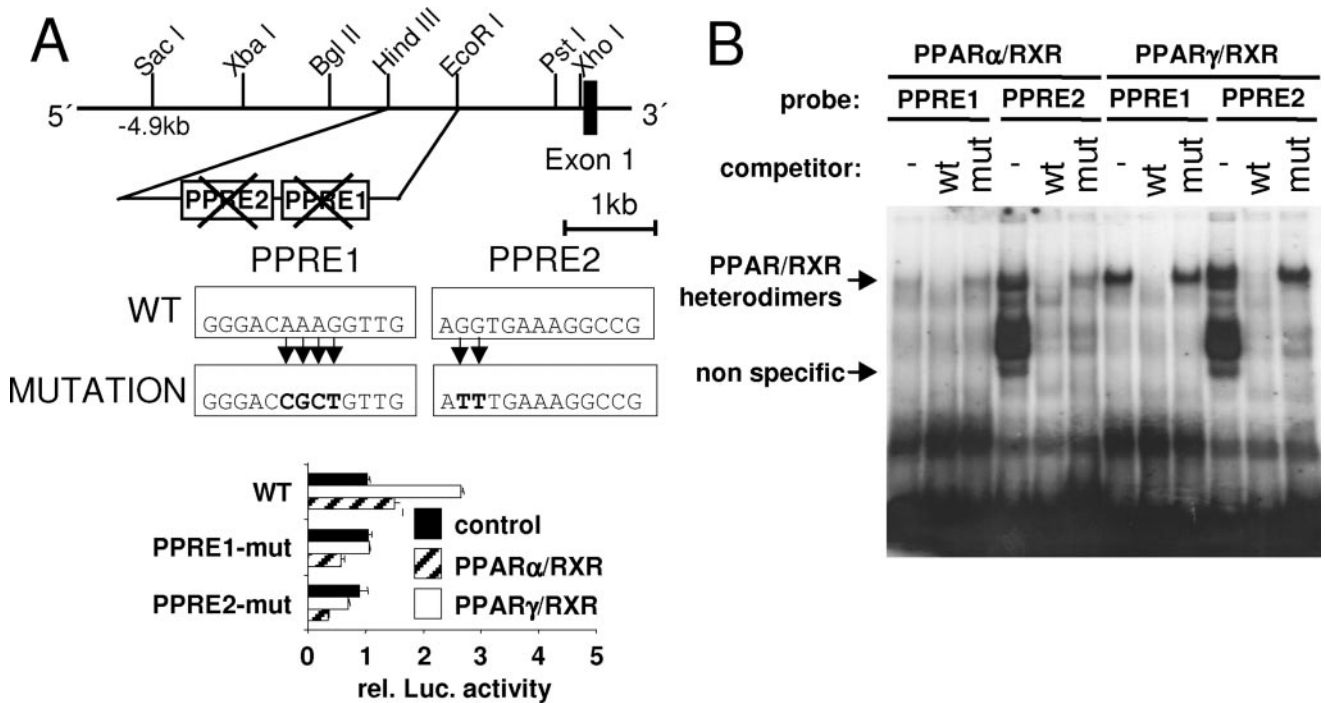


Figure 4. Functional properties of identified PPREs. A, HEK293 cells were transfected with either the wild-type (wt) HO-1 promoter constructs or constructs with the indicated mutations of PPRE1 or PPRE2 and cotransfected with PPAR α or PPAR γ and retinoid X receptor- α expression plasmids. HO-1 promoter activity was analyzed measuring firefly luciferase activity normalized to renilla luciferase activity. Activity in the absence of PPAR expression vectors was set to 1. B, Binding of in vitro-translated PPAR/retinoid X receptor heterodimers to the identified PPREs of the human HO-1 promoter was determined by electrophoretic mobility shift assay. Competition was performed using a 100-fold excess of nonlabeled oligonucleotides.

respective DNA fragments in samples isolated from genotyped individuals. Subsequent analysis revealed a strong negative correlation between the PPAR α - or PPAR γ -induced promoter activity and the increasing length of the GT-repeat within the HO-1 promoter (Figure 5B).

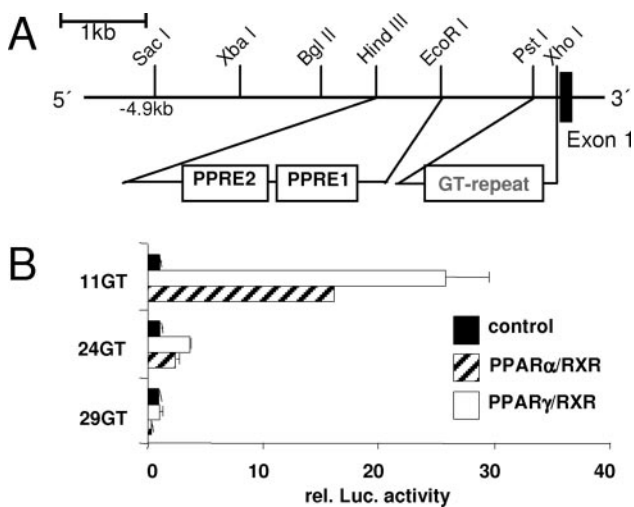


Figure 5. Influence of an HO-1 promoter polymorphism on PPAR-induced HO-1 promoter activity. A, Structure of the human HO-1 promoter. B, Promoter activity of full-length (4.9 kb) HO-1 promoter constructs carrying 11, 24, or 29 GT-repeats after cotransfection with PPAR α , PPAR γ , and retinoid X receptor- α expression plasmids and PPAR ligand treatment (24 hours). HO-1 promoter activity was analyzed measuring firefly luciferase activity normalized to renilla luciferase activity. Activity in the absence of PPAR expression vectors was set to 1.

HO-1 Enzymatic Activity Contributes to the Anti-Inflammatory and Antiproliferative Effects of PPAR α in VSMCs

Ligands for PPAR α have been shown to inhibit the cytokine-induced expression of inflammatory genes in vascular cells.^{26,27} To examine the contribution of HO-1 to anti-inflammatory effects exerted by PPAR ligands, we analyzed tumor necrosis factor-induced cyclooxygenase-2 (COX-2) expression in VSMCs, which was inhibited by the PPAR α ligand Wy14,643 in a concentration-dependent manner (Figure 6A). Addition of the specific HO-1 inhibitor tin protoporphyrin (SnPP) abrogated the inhibition of COX-2 expression by Wy-14,643 (Figure 6A), demonstrating the involvement of HO-1 as a mediator of anti-inflammatory effects of PPAR ligands in VSMCs. In endothelial cells, in contrast, expression of COX-2 was not inhibited by Wy-14,643 (Figure 6B).

Ligands for PPAR α have also been shown to inhibit smooth muscle cell (SMC) proliferation.³² Therefore, we investigated the effect of PPAR-induced HO-1 on SMC proliferation. Inhibition of HO-1 by zinc protoporphyrin dose-dependently increased SMC proliferation, induced by either platelet-derived growth factor or 15% fetal bovine serum. Moreover, treatment of SMCs with the PPAR α -ligand Wy-14,643 inhibited fetal bovine serum-induced proliferation, while concomitant inhibition of HO-1 with zinc protoporphyrin reversed this effect (supplemental Figure I, available online at <http://atvb.ahajournals.com>). Together these data demonstrate a HO-1-dependent and cell

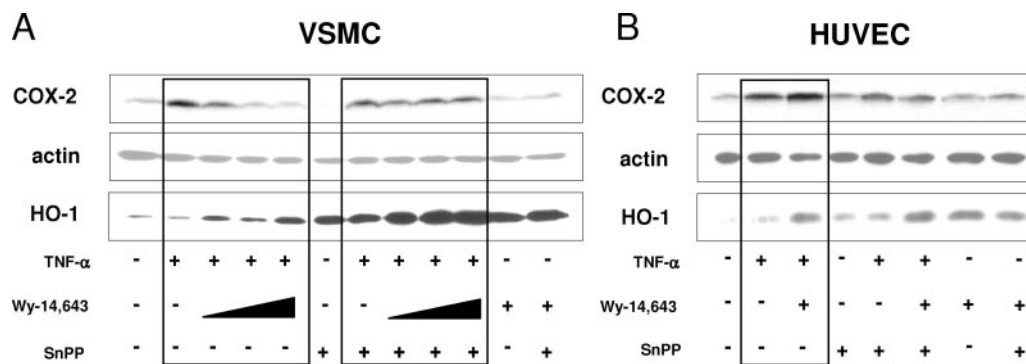


Figure 6. Inhibition of COX-2 expression by PPAR ligands is HO-1–dependent in SMCs. VSMCs (A) or human umbilical venous endothelial cells (B) were incubated with 150 $\mu\text{mol/L}$, 200 $\mu\text{mol/L}$, or 250 $\mu\text{mol/L}$ Wy-14,643 in presence or absence of SnPP (5 $\mu\text{mol/L}$) for 18 hours. Cells were then stimulated with tumor necrosis factor- α (100 U/mL) for 5 hours. COX-2 protein expression was measured in cellular extracts by Western blot analysis.

type-specific modulation of COX-2 expression and VSMC proliferation by a PPAR α ligand.

Discussion

In addition to their beneficial influence on glucose and lipid metabolism, pharmacological PPAR ligands have been shown to exert direct anti-inflammatory and antiproliferative effects on cells of the vascular wall and hence inhibit the development of atherosclerosis and reduce intimal growth.^{3–9,11} Nevertheless, the exact mechanisms behind these versatile qualities of PPARs are poorly understood. One important anti-inflammatory mechanism of PPARs is transcriptional transrepression of inflammatory genes, which involves both a direct interaction with other transcription factors and competition for cofactors.¹² Recently, it was shown that PPAR γ inhibits transcription of proinflammatory genes by SUMOylation.³³ In addition, upregulation of protective genes by PPARs may significantly contribute to their anti-inflammatory properties. With HO-1 we have identified a novel PPAR target gene that has been shown to exert multiple beneficial effects on cells of the vascular wall and to inhibit the development of atherosclerosis and restenosis *in vivo*.^{16–18,20}

We show that ligands for PPAR α or PPAR γ upregulate HO-1 expression in cultured human endothelial cells and VSMCs. Although it has been shown that the vast majority of Wy-14,643–induced genes are regulated in a PPAR α –dependent manner,³⁵ Wy-14,643 can exert pan-PPAR agonistic activity.³⁴ Therefore, we confirmed PPAR α –dependent induction of HO-1 expression in endothelial cells and VSMCs after treatment with another PPAR α activator, fenofibrate. PPAR α was the predominant PPAR isotype expressed in both human umbilical venous endothelial cells and SMCs, and PPAR α ligands seemed to be more effective than PPAR γ ligands in inducing HO-1 expression.

PPAR ligands can also display receptor-independent effects,^{35,36} especially at high concentrations. Wy-14,643 is a fibric acid derivative with an EC50 of 5 $\mu\text{mol/L}$ for human PPAR α .³⁷ Although we demonstrate that Wy-14,643 at pharmacological doses significantly increases HO-1 expression, we also used higher doses (150 to 250 $\mu\text{mol/L}$), which likely have additional unspecific effects. However, the induction of

HO-1 expression by receptor-specific concentrations of ligands as well as our promoter studies indicated a transcriptional regulation of HO-1 expression by PPAR α or PPAR γ . Detailed analysis of the human HO-1 promoter showed that HO-1 is indeed a direct PPAR–target gene, whose transcription is regulated by both PPAR α and PPAR γ via 2 PPREs.

Previous studies have highlighted the importance of stress-responsive elements (StREs) in the transcriptional regulation of HO-1.³⁸ The repressor protein Bach1 constitutively binds to StREs and is displaced on activation of transcription factors such as Nrf2.³⁹ 15-deoxy- Δ 12,14 prostaglandin J₂, which exerts multiple PPAR–dependent and PPAR–independent effects,³⁶ has been shown to activate the Nrf2 regulatory pathway via Keap1⁴⁰ and to induce HO-1 expression via StREs in a PPAR–independent manner.^{41–44} However, prostaglandin J₂–induced transcription of glutathione S-transferase involves synergistic activation of Nrf2 and PPAR γ .⁴⁵ In our present study the StRE-containing enhancer region of the human HO-1 promoter^{25,38} was dispensable for the PPAR–induced transcriptional regulation of the HO-1 promoter, because the 3.8-kb and 2.2-kb HO-1 promoter constructs, which lack this enhancer, but include the PPREs, were still PPAR–responsive. Moreover, mutation of the StREs did not affect PPAR–induced HO-1 promoter activity (data not shown). With the 2 identified PPREs located between the StREs and the basic HO-1 promoter, it will be nevertheless interesting to determine a possible influence of the Bach1/Nrf2 system on the PPAR–mediated transcriptional regulation of HO-1 and to analyze the accessibility of the 2 PPREs under various conditions of cellular stress.

We demonstrate that PPAR–induced HO-1 promoter activity inversely correlates with the length of a polymorphic GT-repeat in the human HO-1 promoter. This polymorphism has been reported to affect hydrogen peroxide–induced HO-1 promoter activity as well as HO-1 mRNA expression and enzymatic activity in lymphoblastoid cells.^{46,47} It has been suggested that longer GT-repeats promote changes in DNA conformation, which in turn negatively affect transcriptional activity.²⁴ Moreover, the length of the respective GT-repeat is associated with the susceptibility for various inflammatory diseases including coronary heart disease and restenosis.^{30,31} It will be important to determine whether the vasculoprotec-

tive effects of PPAR ligands correlate with this HO-1 promoter polymorphism, which would provide a possibility to better assess the individual benefit of the treatment with fibrates or thiazolidinediones.

Both PPAR ligands and HO-1 have been described to block the inflammatory response in vascular cells and to potentially inhibit VSMC proliferation.^{6,8,17,21,26,27,32,48–50} Furthermore, inhibition of COX-2 expression and prostaglandin synthesis by HO-1 was reported.^{51–56} We determined the contribution of HO-1 to the described anti-inflammatory and antiproliferative effects of PPAR α ligands in VSMCs²⁷ and show that inhibition of HO-1 enzymatic activity by zinc protoporphyrin abrogated the inhibitory effect of PPAR α ligands on cytokine-induced expression of COX-2 as well as on proliferation in VSMCs. Interestingly, Wy-14,643-induced HO-1 did not affect COX-2 expression in endothelial cells, and while the expression of vascular cell adhesion molecule-1 in endothelial cells was strongly inhibited by the PPAR α ligand, inhibition of HO-1 did not reverse this effect (data not shown). Moreover, we show that treatment of SMCs with the PPAR α -ligand Wy-14,643 inhibited platelet-derived growth factor and serum-induced proliferation, while concomitant inhibition of HO-1 reversed this effect. Interestingly, it has been shown that upregulation of HO-1 in endothelial cells increases proliferation.⁵⁷ These data strongly indicate cell type specific mechanistic differences in the anti-inflammatory action of PPAR ligand-induced HO-1.

Since PPARs have been recognized as key transcription factors regulating the expression of genes involved in lipid and glucose metabolism, HO-1 represents a novel type of PPAR target gene. The finding that HO-1 expression is transcriptionally regulated by PPAR α or PPAR γ provides an important additional link between these ligand-activated transcription factors and their described anti-inflammatory and antiproliferative properties and thus contributes to our understanding of the pleiotropic beneficial effects exerted by PPAR ligands in inflammatory vascular disorders.

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Disclosures

None.

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