Oxidative stress, induced by intracellular accumulation of reactive oxygen species, is implicated as a causal role in atherosclerosis, microvascular complications of diabetes as well as in beta cell failure in type 2 diabetes [1, 2]. In rodents, PPARγ agonists, which are clinically used for treatment of diabetes, can eliminate oxidative stress both in vitro [3] and in vivo [4, 5], and also increase the expression of catalase, a major antioxidant which converts hydrogen peroxide to oxygen and water [6, 7]. Moreover, low expression levels of catalase and PPARγ mRNAs are associated with elevated oxidative stress in adipose tissues of obese mice [8]. These data suggest that PPARγ is pharmacologically and pathologically associated with the expression of catalase and oxidative stress. Previous studies from our laboratories and those of others showed that the expression of catalase in mouse [6] and rat [7] is directly regulated by PPARγ through PPARγ binding elements in the promoter region of catalase gene.

Several studies have reported the effects of PPARγ agonists on oxidative stress in human. In obese subjects, treatment with PPARγ agonist reduces the generation of reactive oxygen species (ROS) from leukocytes and lipid peroxidation [9]. PPARγ agonist also lowers $O_2^-$ production from diabetic plaques or monocytes in diabetics [10]. However, there is little information on whether PPARγ mediates the expression of catalase in human and if so, whether it is mediated via a mechanism similar to that in rodents.

In the present study, we investigated the regulation of catalase through PPARγ in human. The re-
Point mutations were introduced into -12472/+6-Luc using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primer used in mutagenesis was 5'-CATAACACAAAAATGTTT-GACaaTTGCAATGATTTCCTTT-3'. Tandem repeats of human PPRE1, PPRE2, PPRE3 or the sequence corresponding to mouse catalase PPREs were connected to tk-Luc vector (a kind gift from Dr. David Mangelsdorf, University of Texas Southwestern Medical Center, Dallas, TX). Expression plasmids encoding mouse PPARγ1 (pCMX-mPPARγ), mouse retinoid X receptor α (RXRα) (pCMX-mRXRα), VP16-PPARγ (pCMX-VP16-PPARγ), VP16-RXRα (pCMX-VP16-RXRα), β-galactosidase (pCMX-β-gal), pCMX control plasmid and pCMX-VP16 control plasmid were prepared as described previously [11]. These constructs were transfected into COS-1 cells, together with the expression plasmids encoding VP16-PPARγ (pCMX-VP16-PPARγ) and VP16-RXRα (pCMX-VP16-RXRα), a constitutive active form of PPARγ and RXRα, or control pCMX-VP16 plasmid.

Luciferase reporter assay

Reporter plasmids, pCMX-mPPARγ, pCMX-mRXRα, pCMX-β-gal or pCMX were transfected into 3T3-L1 adipocytes at 4 days after induction of differentiation using LipofectAMINE 2000 reagent (Invitrogen, San Diego, CA). The medium was replaced 4 hours later by 10% fetal bovine serum (FBS)-supplemented Dulbecco’s Modified Eagle’s Medium (DMEM) with or without pioglitazone. The cells were harvested 24 hours later. Luciferase activities were normalized by β-galactosidase activities.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously [11] with anti-PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or non-immune goat IgG (Sigma, St. Louis, MO). Nuclear extracts were prepared from differentiated 3T3-L1 adipocytes using Nuclear Extract Kit (Active Motif Japan, Tokyo).

Statistical Analysis

Student’s t-test was used to determine statistical significance. Values are expressed as means±SEM. A P value less than 0.05 denoted the presence of a statistically significant difference.
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and RXRα significantly transactivated -18926/+6-Luc in differentiated 3T3-L1 adipocytes (Fig. 3a). In our search of putative PPREs, whose consensus sequence is AGGNCAAGGTCA [13], within -18926/+6 of human catalase promoter, we found three putative PPREs located at -11710/-11698 (PPRE1), -6304/-6292 (PPRE2), -5161/-5149 (PPRE3) (Fig. 3B). Next, we subcloned a battery of clones with various deletions in the human catalase promoter and analyzed their activities. The -12472/+6-Luc showed a significant response to PPARγ/RXRα+pioglitazone, similar to -18926/+6-Luc, but additional 1 kb deletion or longer deletion from the 5’ end of -12472/+6-Luc (-11335/+6-Luc, -7991/+6-Luc, -6806/+6-Luc) diminished this response (Fig. 3a). These findings indicate that PPRE1 is necessary for pioglitazone-induced transactivation of human catalase promoter, while PPRE2, PPRE3, and PPREs corresponding to mouse PPREs are not. Human PPRE1 was completely conserved in chimpanzee, but poorly conserved in mouse or rat (Fig. 3c).

**Results**

We examined the effects of pioglitazone on catalase gene expression in human primary adipocytes, obtained from two manufacturers, Zen-Bio and ScienCell Research Laboratories. In both cells, pioglitazone increased the mRNA expression of catalase as well as that of adiponectin, one of the PPARγ target genes (Fig. 1).

Next, we assessed the conservation across species on the PPREs of mouse catalase promoter, using the UCSC Genome Browser (http://genome.ucsc.edu). The results showed complete conservation of the mouse catalase PPREs in rat, but poor conservation in primates (Fig. 2). These results suggest that the expression of human catalase is regulated by PPARγ, but is mediated through a distinct mechanism from mouse.

To determine this mechanism, we analyzed the activity of the 5’ flanking region of the human catalase gene using luciferase reporter assay. Treatment with pioglitazone together with overexpression of PPARγ and RXRα significantly transactivated -18926/+6-Luc in differentiated 3T3-L1 adipocytes (Fig. 3a). In our search of putative PPREs, whose consensus sequence is AGGNCAAGGTCA [13], within -18926/+6 of human catalase promoter, we found three putative PPREs located at -11710/-11698 (PPRE1), -6304/-6292 (PPRE2), -5161/-5149 (PPRE3) (Fig. 3B). Next, we subcloned a battery of clones with various deletions in the human catalase promoter and analyzed their activities. The -12472/+6-Luc showed a significant response to PPARγ/RXRα+pioglitazone, similar to -18926/+6-Luc, but additional 1 kb deletion or longer deletion from the 5’ end of -12472/+6-Luc (-11335/+6-Luc, -7991/+6-Luc, -6806/+6-Luc) diminished this response (Fig. 3a). These findings indicate that PPRE1 is necessary for pioglitazone-induced transactivation of human catalase promoter, while PPRE2, PPRE3, and PPREs corresponding to mouse PPREs are not. Human PPRE1 was completely conserved in chimpanzee, but poorly conserved in mouse or rat (Fig. 3c).

**Fig. 1.** Effects of pioglitazone on mRNA expression of catalase in human primary adipocytes.

The mRNA expression levels of catalase and adiponectin were measured in human primary adipocytes from Zen-Bio (n=9) (A) and ScienCell Research Laboratories (n=6) (B), treated with vehicle or 10 μM of pioglitazone for 24 hours. Values are normalized to the level of 36B4 and expressed as mean ± SEM. *P<0.05, **P<0.001 compared with the control treatment.
Okuno et al. example, the lipoprotein lipase (LPL) gene is regulated by PPARα through a PPRE functionally conserved between human and mouse [14]. The human cytochrome P450 1A1 (CYP1A1) gene is regulated by PPARα through two PPREs, whose sequences are highly conserved among human, mouse, and rat [15]. On the other hand, PPREs in the promoter of several genes are not conserved between human and rodents. In human, expression of apolipoprotein a-i (apoA-I) is regulated by PPARα through a PPRE, while in rat, it is not regulated by PPARα and no PPRE exists in its promoter [16]. PPARα induces the expression of mouse glyoxylate reductase/hydroxypyruvate reductase (GRHPR) through a PPRE, but it does not regulate human GRHPR since its promoter lacks PPRE [17]. In these cases, the lack of PPRE in their promoters leads to the lack of responsiveness to PPARα. In human, expression of apolipoprotein A-I (apoA-I) is regulated by PPARα through a PPRE, while in rat, it is not regulated by PPARα and no PPRE exists in its promoter [16]. PPARα induces the expression of mouse glyoxylate reductase/hydroxypyruvate reductase (GRHPR) through a PPRE, but it does not regulate human GRHPR since its promoter lacks PPRE [17]. In these cases, the lack of PPRE in their promoters leads to the lack of responsiveness to PPARα. In contrast, PPREs in the mouse catalase promoter are not conserved in human catalase promoter, but human catalase still maintains similar responsiveness to PPARα through another functional PPRE in a position different from that of mouse promoter. A similar case was reported by Ludwig et al. [18] about the promoter of even-skipped stripe 2 gene, which expresses in the second transverse stripe in Drosophila embryos. The even-skipped stripe 2 element, which controls the expression of even-skipped stripe 2 gene, had undergone a considerable evolutionary change between D. melanogaster and D. pseudoobscura in its binding-site sequences, although the expression of even-skipped stripe 2 is strongly conserved. In catalase promoter, sequence differences of PPREs between species would be masked by other coevolved PPRE as is the case in even-skipped stripe 2 gene. However, the physiological and evolutionary significances of the distinct positions of PPREs among species in catalase remain to be elucidated.

Discussion

In general, important regulatory elements, including PPRE, tend to be conserved among species. For example, the lipoprotein lipase (LPL) gene is regulated by PPARs through a PPRE functionally conserved between human and mouse [14]. The human cytochrome P450 1A1 (CYP1A1) gene is regulated by PPARα through two PPREs, whose sequences are highly conserved among human, mouse, and rat [15]. On the other hand, PPREs in the promoter of several genes are not conserved between human and rodents. In human, expression of apolipoprotein a-i (apoA-I) is regulated by PPARα through a PPRE, while in rat, it is not regulated by PPARα and no PPRE exists in its promoter [16]. PPARα induces the expression of mouse glyoxylate reductase/hydroxypyruvate reductase (GRHPR) through a PPRE, but it does not regulate human GRHPR since its promoter lacks PPRE [17]. In these cases, the lack of PPRE in their promoters leads to the lack of responsiveness to PPARα. In contrast, PPREs in the mouse catalase promoter are not conserved in human catalase promoter, but human catalase still maintains similar responsiveness to PPARα through another functional PPRE in a position different from that of mouse promoter. A similar case was reported by Ludwig et al. [18] about the promoter of even-skipped stripe 2 gene, which expresses in the second transverse stripe in Drosophila embryos. The even-skipped stripe 2 element, which controls the expression of even-skipped stripe 2 gene, had undergone a considerable evolutionary change between D. melanogaster and D. pseudoobscura in its binding-site sequences, although the expression of even-skipped stripe 2 is strongly conserved. In catalase promoter, sequence differences of PPREs between species would be masked by other coevolved PPRE as is the case in even-skipped stripe 2 gene. However, the physiological and evolutionary significances of the distinct positions of PPREs among species in catalase remain to be elucidated.

To evaluate whether PPARγ can bind to these putative PPREs in vivo, we assessed whether VP16-PPARγ and VP16-RXRα, constitutive active forms of PPARγ and RXRα, respectively, transactivate human PPRE1, PPRE2, PPRE3. VP16-PPARγ/VP16-RXRα significantly transactivated a tandem repeat of human PPRE1, but not that of PPRE2, PPRE3, or the sequence corresponding to mouse catalase PPREs (Fig. 3D), indicating that PPARγ can bind to human PPRE1, but not the other sequences. Next, to assess the significance of human PPRE1 in the regulation of catalase by pioglitazone, we introduced point mutations into -12472/+6-Luc. Mutations in human PPRE1 significantly attenuated transactivation of -12472/+6-Luc by PPARγ/RXRα+pioglitazone (Fig. 3E).

To further confirm the binding of PPARγ to human PPRE1 in vitro, we performed electrophoretic mobility shift assay (EMSA) experiments. A double-strand oligonucleotide of human PPRE1 was 32P-radiolabeled and incubated with nuclear extracts prepared from differentiated 3T3-L1 adipocytes. A shifted band was formed (lane 1, white arrow), but was abrogated by 10-fold molar excess of the unlabeled probe of PPRE1 (lane 2) or consensus PPRE from human adiponectin promoter (lane 4), but not by that of mutant PPRE1 (lane 3), reflecting the specificity of the DNA binding complex. In addition, the specific bands were immunodepleted and supershifted using anti-PPARγ antibody (lane 6, black arrow), but not using non-immune goat IgG (lane 5), indicating that the endogenous PPARγ protein in adipocytes binds to PPRE1 in human catalase promoter.

Discussion

In general, important regulatory elements, including PPRE, tend to be conserved among species. For example, the lipoprotein lipase (LPL) gene is regulated by PPARs through a PPRE functionally conserved between human and mouse [14]. The human cytochrome P450 1A1 (CYP1A1) gene is regulated by PPARα through two PPREs, whose sequences are highly conserved among human, mouse, and rat [15]. On the other hand, PPREs in the promoter of several genes are not conserved between human and rodents. In human, expression of apolipoprotein A-I (apoA-I) is regulated by PPARα through a PPRE, while in rat, it is not regulated by PPARα and no PPRE exists in its promoter [16]. PPARα induces the expression of mouse glyoxylate reductase/hydroxypyruvate reductase (GRHPR) through a PPRE, but it does not regulate human GRHPR since its promoter lacks PPRE [17]. In these cases, the lack of PPRE in their promoters leads to the lack of responsiveness to PPARα. In contrast, PPREs in the mouse catalase promoter are not conserved in human catalase promoter, but human catalase still maintains similar responsiveness to PPARα through another functional PPRE in a position different from that of mouse promoter. A similar case was reported by Ludwig et al. [18] about the promoter of even-skipped stripe 2 gene, which expresses in the second transverse stripe in Drosophila embryos. The even-skipped stripe 2 element, which controls the expression of even-skipped stripe 2 gene, had undergone a considerable evolutionary change between D. melanogaster and D. pseudoobscura in its binding-site sequences, although the expression of even-skipped stripe 2 is strongly conserved. In catalase promoter, sequence differences of PPREs between species would be masked by other coevolved PPRE as is the case in even-skipped stripe 2 gene. However, the physiological and evolutionary significances of the distinct positions of PPREs among species in catalase remain to be elucidated.
Fig. 3. Analysis of human catalase promoter.

(A, E) Activities of the indicated reporter constructs of human catalase promoter in 3T3-L1 adipocytes transfected with PPARγ/RXRα expression plasmids or pCMX control plasmid followed by treatment with vehicle or 10 μM pioglitazone for 24 hours. Normalized luciferase activities are expressed as mean ± sEM (n=3). (B) Sequence of the human PPRE1, PPRE2 and PPRE3. Arrows: direction of PPRE elements. (C) Sequence alignment of the human PPRE1 across species. Arrows: direction of PPRE elements. Asterisks: nucleotides different from human PPRE1. (D) Activities of tk-Luc vector containing two copies of the indicated sequences in COS-1 cells transfected with VP16-PPARγ/VP16-RXRα expression plasmids or control VP16 plasmid. Normalized luciferase activities are shown as mean ± SEM (n=4). h1, h2, h3; human PPRE1, PPRE2, PPRE3. m1, m2; sequences corresponding to mouse PPRE1, PPRE2.
The PPREs in mouse catalase promoter are localized at -9 kb [6], which is one of the farthest from the transcription initiation sites among PPREs identified previously, and we found a more remote PPRE on human catalase promoter in the present study. Many studies have identified distal regulatory elements [21]. Human plasminogen activator inhibitor 1 (PAI-1) is regulated by TNFα through a distal NF-κB-binding site at -15 kb upstream of the transcription start site [22]. A recent study using chromatin immunoprecipitation combined with tiled microarrays showed that the majority of the estrogen receptor binding sites are mapped very distant (from -10 kb to -500 kb) from the transcription start sites [23]. Thus, although most PPREs have been mapped on the proximal promoter, many PPREs may remain unidentified.

In conclusion, we have demonstrated in the present study that PPARγ directly regulates the expression of catalase in human, similar to mice, although in vivo binding of endogenous human PPARγ to this PPRE has not verified by chromatin immunoprecipitation (ChiP) assay. These data provide support to the favorable effects of PPARγ agonists on oxidative stress in human. PPARγ agonists can be potentially useful clinically to increase the expression of catalase and treatment of diseases associated with oxidative stress.

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