Pioglitazone Stimulates Apolipoprotein A-I Production Without Affecting HDL Removal in HepG2 Cells
Involvement of PPAR-α

Shucun Qin, Tianjiao Liu, Vaijinath S. Kamanna, Moti L. Kashyap

Objective—Pioglitazone, an antihyperglycemic drug, increases plasma high-density lipoprotein (HDL)-cholesterol in patients with type 2 diabetes. The mechanisms by which pioglitazone regulate HDL levels are not clear. This study examined the effect of pioglitazone on hepatocyte apolipoprotein A-I (apoA-I) and apoA-II production and HDL-protein/cholesterol ester uptake.

Methods and Results—in human hepatoblastoma (HepG2) cells, pioglitazone, dose-dependently (0.5 to 10 μmol/L), increased the de novo synthesis (up to 45%), secretion (up to 44%), and mRNA expression (up to 59%) of apoA-I. Pioglitazone also increased apoA-II de novo synthesis (up to 73%) and mRNA expression (up to 129%). Pioglitazone did not affect the uptake of HDL₃-protein or HDL₃-cholesterol ester in HepG2 cells. The pioglitazone-induced apoA-I lipoprotein particles increased cholesterol efflux from THP-1 macrophages. The pioglitazone-induced apoA-I secretion or mRNA expression by the HepG2 cells was abrogated with the suppression of PPAR-α by small interfering RNA or a specific inhibitor of PPAR-α, MK886.

Conclusions—The data indicate that pioglitazone increases HDL by stimulating the de novo hepatic synthesis of apoA-I without affecting hepatic HDL-protein or HDL-cholesterol removal. We suggest that pioglitazone-mediated hepatic activation of PPAR-α may be one of the mechanisms of action of pioglitazone to raise hepatic apoA-I and HDL.

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Key Words: apolipoproteins ■ lipids ■ lipoproteins ■ high density lipoprotein ■ glitazones

A growing body of evidence indicates that high-density lipoprotein (HDL) bears an inverse relationship to the development of atherosclerotic coronary heart disease.¹ The antithrombogenic effects of HDL and apolipoprotein A-I (apoA-I, major protein of HDL) are mainly attributed to its ability to facilitate reverse cholesterol transport pathway,²,³ enhance fibrinolysis, and inhibit platelet aggregation,⁴ LDL oxidation,⁵ endothelial transmigration of monocytes,⁶ and proinflammatory cytokine-mediated expression of endothelial cell adhesion molecules.⁷

Glitazones (a class of thiazolidinediones agents such as pioglitazone and rosiglitazone) are antihyperglycemic drugs used in the treatment of type 2 diabetes. These oral antidiabetic agents reduce blood glucose levels by sensitizing peripheral tissues to insulin.⁸ In addition to improving glycemic control, glitazones also influence plasma lipid and lipoprotein levels.⁹ Treatment of type 2 diabetic patients with pioglitazone consistently increased plasma HDL-cholesterol and decreased triglycerides with generally minimal to no effect on total and LDL-cholesterol.¹⁰–¹³ Clinically, the effect of pioglitazone on HDL-cholesterol (about 10% to 15%) is greater than that observed with statins and several fibrates, and just below that of niacin. Although the mechanisms are not clearly understood, the binding and activation of nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) by glitazones has been suggested to explain their effects on glucose and lipid metabolism.¹⁴–¹⁶ PPARs (PPAR-α, -γ, -δ) are members of the nuclear receptor family, and by ligand activation events, modulate transcription of various genes involved in lipid metabolism and atherosclerosis (reviewed in references 17,18).

PPAR-α agonists (such as fibrates), through heterodimerizing with retinoid receptor and binding to PPAR response element in the promoter region of apoA-I gene, were shown to increase the transcription of human apoA-I.¹⁹ It was also shown that the activation of PPAR-α regulates nuclear factor kB (NFκB)-induced reduction in apoA-I and HDL-cholesterol.²⁰ Although pioglitazone has been originally identified as
high affinity ligand for PPAR-γ, an earlier report indicated that pioglitazone serves as a weak human PPAR-α activator. Despite consistent beneficial effect of pioglitazone on HDL levels, the hepatocellular mechanisms by which pioglitazone raise apoA-I and HDL concentrations are not clearly understood. In this study, we examined the effect of pioglitazone on apoA-I de novo synthesis, secretion, and mRNA expression, and the participation of PPAR-α in apoA-I production in human hepatoblastoma cells (HepG2). Additional studies were performed to examine the effect of pioglitazone HDL-apoA-I/cholesterol ester uptake by HepG2 cells to determine whether the rise in apoA-I/HDL observed clinically with this agent could be a result of decreased hepatic HDL removal.

Materials and Methods

Materials
Tissue culture materials, media, and FBS were obtained from Sigma Chemical Company. L-[4,5-3H]Leucine and [14C]cholesterol were purchased from Amersham Corporation. HepG2 cells and human macrophage THP-1 cell line were obtained from American Type Culture Collection. The polyclonal antibodies for human apoA-I and apoA-II were purchased from Boehringer Mannheim Biochemicals. All other chemicals used were of analytical grade.

Studies on Secretion of ApoA-I in HepG2
HepG2 cells were grown in high-glucose DMEM (containing 10% FBS, 1% glutamine-penicillin-streptomycin, and 1% fungizone) for 3 to 4 days to attain 75% to 80% confluence. Cells were incubated with various amounts of pioglitazone (0 to 10 μmol/L) at 37°C for 48 hours. Culture medium was removed, the cell monolayer washed with PBS, and collected for cellular protein measurement. A 50-μL sample of culture medium was used to measure apoA-I and apoA-II were purchased from Boehringer Mannheim Biochemicals.

Studies on De Novo Synthesis of ApoA-I
Studies examining the effect of pioglitazone on the de novo synthesis of apoA-I by HepG2 cells were performed by measuring the incorporation of radiolabeled leucine into apoprotein secreted into the media.22 HepG2 cells were incubated with varying concentrations of pioglitazone (0 to 10 μmol/L) in DMEM media containing 10% FBS for 48 hours. The medium was replaced with leucine-poor DMEM (5% leucine of normal media) without FBS containing the corresponding amounts of pioglitazone and 3H-leucine (5 μCi/mL) and incubated for 18 hours at 37°C. The medium was collected and used for immunoprecipitation using monospecific antibodies for apoA-I. The incorporation of 3H-leucine into apoA-I was expressed as cpm/mg of cellular protein.

RT-PCR Analysis for ApoA-I mRNA Expression
HepG2 cells were incubated with various amounts of pioglitazone (0 to 10 μmol/L) at 37°C for 24 hours. Culture medium was removed, the cell monolayer washed with PBS and collected for total RNA isolation. Cells were collected by trypsinization and total RNAs were extracted using RNeasy mini kit (Qiagen). cDNA was synthesized from 850 ng of total RNA in 20 μL using random hexamers and Murine Moloney leukemia virus reverse transcriptase (Life Technologies Inc). The apoA-I primers used were as follows: sense, 5′-ATG AAA GCT GGC GTG CTG ACC-3′ and antisense, 5′-GGA GCT CTA CCG CCA GAA GGT G-3′. Semiquantitative RT-PCR analysis was performed starting with first strand cDNA from reverse transcription with 150 nmol/L of sense and antisense primer in a final volume of 50 μL. Sense and antisense primer for β-actin (150 nmol/L) were also included in the reaction mixture. PCR amplification products at different cycles were separated on 1.2% agarose gel and were visualized with ethidium bromide (cycle 19 was found be in linear phase). The band intensity was measured using Eagle Eye gel documentation system.

Studies on the Participation of PPAR-α in ApoA-I Expression and Secretion
The involvement of PPAR-α in pioglitazone-induced apoA-I mRNA expression was examined using pioglitazone suppression in Hep G2 cells with either a specific inhibitor of PPAR-α or small interference RNA (siRNA). The same protocols described above in “RT-PCR analysis for apoA-I mRNA expression” was used for studies with MK886, a specific inhibitor of PPAR-α.20 In brief, Hep-G2 cells were preincubated with MK886 (10 μmol/L) for 60 minutes. After the pretreatment, pioglitazone was added to cells and incubated for 24 hours. Total RNA was isolated and used for apoA-I mRNA expression using RT-PCR analysis as described above.

Suppression of PPAR-α expression in HepG2 with siRNA was accomplished as described by Shoda et al21 and according to the transfection kit instructions. Cells (70% to 80% confluent) were transfected with siRNA using Lipofectamine 2000 (Invitrogen) and distributed into 6-well plate. After 24 hours, cells were treated with vehicle or pioglitazone (10 μmol/L) for 24 hours. Total RNA and cell lysates were then prepared, and mRNA levels of PPAR-α and medium apoA-I protein level were determined as described above.

Studies on ApoA-II Synthesis and mRNA Expression
Studies examining the effect of pioglitazone on the de novo synthesis of apoA-II by Hep-G2 cells was performed by measuring the incorporation of radiolabeled leucine into apoprotein secreted into the media, as described in apoA-I de novo synthesis. ApoA-II mRNA expression measurement was done by real-time PCR. Hep-G2 cells were incubated with various amounts of pioglitazone (0 to 10 μmol/L) at 37°C for 24 hours. The cell monolayer was washed with PBS and collected for total RNA isolation. Total RNAs were extracted using RNeasy mini kit (Qiagen). CDNA was synthesized from 1.4 μg of total RNA in 20 μL using random hexamers and Ommi-script R. Transciptase (Qiagen). The apoA-II primers were purchased from SuperArray. The real-time PCR was performed with the use of the PCR Master Mix RT2 SYBR Green /Fluorescein (SuperArray). Sequence-specific amplification was detected with an increased fluorescence signal of SYBR during the amplification cycle. Amplification of the human GAPDH gene was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. ApoA-II mRNA levels were normalized to GAPDH mRNA levels, and were presented as fold difference of treated cells against untreated cells.

Studies on the Uptake of HDL-Protein
Studies examining the uptake by HepG2 cells were performed by using radiolabeled HDL total protein or apoA-I-HDL.22 Radio iodination of HDL total protein was carried out by incubating freshly isolated HDL, with carrier-free 125I.22 After the iodination, unreacted 125I-HDL were also included in the reaction mixture. PCR amplification products at different cycles were separated on 1.2% agarose gel and were visualized with ethidium bromide (cycle 19 was found be in linear phase). The band intensity was measured using Eagle Eye gel documentation system. The uptake of radiolabeled HDL-protein by HepG2 cells was expressed in terms of cellular protein.

Uptake of 3H-Cholesterol Esters Labeled HDL
For these studies, radiolabeled HDL-cholesterol ester was prepared by incubating 4 μCi of [1α,2α(n)-3H] cholesterol with the serum...
HDL fraction for 18 hours at 37°C (through LCAT enzyme reaction mediated cholesterol-ester formation; 22). The [3H] cholesterol ester-HDL was isolated by ultracentrifugation at d/H11005 1.210g/mL and dialyzed extensively against 0.15 mol/L NaCl. Uptake studies were performed by preincubating HepG2 cells pioglitazone (0 to 10 /H9262 mol/L) for 48 hours. Medium was removed, and fresh DMEM containing 5 mg/mL FBA (fatty acid free) and [3H] cholesterol ester–labeled HDL (50 /H9262 g HDL protein/mL) was added. Cells were harvested 6 hours later, washed thoroughly, and digested with 1 mL of 1N NaOH. Radioactivity was measured and expressed as cpm per mg cellular protein.

Measurement of Cholesterol Efflux

Experimental protocols for these studies were exactly same as described for apoA-I secretion studies. After the incubation of HepG2 cells with pioglitazone, the medium was collected and used for cholesterol efflux measurements.24 An aliquot of culture medium (5 mL) was concentrated to 1 mL by lyophilization and dialyzed against DMEM to remove excess salt present in the concentrated sample. The ability of these media (containing secreted apoA-I lipoprotein particles) to efflux free cholesterol was measured using [3H]cholesterol-labeled human macrophage THP-1 cells. Cholesterol efflux assay were initiated by incubating concentrated culture medium with [3H]cholesterol-labeled THP-1 cells for 20 hours as described previously.24 Quantitative analysis of the ability of HepG2 cell culture medium (in the presence or absence of pioglitazone) to efflux cholesterol was performed by measuring the [H]cholesterol radioactivity appearing in the medium per milliliter of incubation medium per mg of THP-1 cellular protein.

Statistical Analysis

Data presented are the mean±SE of 3 separate experiments done in duplicate. Statistical significance was calculated by using the Student t test, and a value of P<0.05 was considered significant.

Results

As shown in Figure 1, a significant increase in apoA-I secretion by HepG2 cells was noted at 1.0 μmol/L pioglitazone (40% over control), and pioglitazone at 10 μmol/L increased apoA-I secretion by 44% as compared with control. The percent increase in apoA-I secretion by pioglitazone (10 μmol/L) in various experiments were 44%, 60%, and 52%. Data from the de novo synthesis of apoA-I studies show that the incorporation of radiolabeled leucine into apoA-I increased in a dose-dependent manner by HepG2 cells incubated with pioglitazone (Figure 2). Pioglitazone as low as 0.5 μmol/L increased apoA-I de novo synthesis (19% over control), and the maximum effect was observed at 10 μmol/L pioglitazone (45% increase compared with control level, Figure 2).

As shown in representative RT-PCR, the incubation of varying amounts of pioglitazone with HepG2 cells induced dose-dependently the mRNA expression of apoA-I (Figure 3, top panel). Quantitative analysis of apoA-I mRNA message indicated that the treatment of HepG2 cells with pioglitazone as low as 0.1 μmol/L concentration stimulated apoA-I mRNA levels by 36% compared with control, the maximal effect noted at 10 μmol/L by 59% compared with control (Figure 3, lower panel). Because PPARα also regulates apoA-II expression, we examined whether pioglitazone affects apoA-II synthesis and mRNA expression in HepG2 cells. The data...
indicated that pioglitazone (0.5 to 10 μmol) also dosage-dependently stimulated apoA-II de novo synthesis by 28% to 73% (Figure 2B) and apoA-II mRNA levels by 31% to 129% (Figure 3B).

To determine whether pioglitazone increases HDL-cholesterol by decreasing HDL clearance, additional studies were performed to assess the effect of pioglitazone on uptake of HDL and its components by HepG2 cells. As shown in Figure 4, the preincubation of HepG2 cells with pioglitazone at varying concentrations (5 to 10 μmol/L) did not alter the uptake of either HDL-protein or HDL-CE.

The ability of pioglitazone-induced apoA-I–containing lipoprotein particles to efflux cholesterol was examined by using [3H]cholesterol-labeled human macrophage cell line (THP-1 cells). Cholesterol efflux studies using conditioned medium obtained from HepG2 cells treated with varying amounts of pioglitazone (0.5 to 10 mmol/L) showed a dose-dependent increase in cholesterol efflux by 10% to 39% compared with control, as measured by the release of [3H]cholesterol from THP-1 cells into the culture medium (Figure 5).

Activation of PPAR-γ by agonists (such as fibrates) has been previously shown to participate in increasing apoA-I mRNA expression. In the present study, we have also shown that fenofibrate increased apoA-I secretion by 68% (apoA-I secretion as μg/mg cell protein data: control=0.25±0.02, 10 μmol/L fenofibrate=0.42±0.05) and mRNA expression by 73%. This served as a positive control for PPARα-mediated effects on apoA-I expression relevant to the current study. In addition to strong activation of PPAR-γ, pioglitazone was also shown to activate human PPAR-α. Using MK-886 (a specific inhibitor of PPAR-α activation), we have examined whether pioglitazone-induced apoA-I mRNA expression was mediated by activation of PPAR-α.

As shown in Figure 6A and 6C, preincubation of HepG2 cells with MK-886 almost completely blocked apoA-I mRNA expression (92%) and secretion (80%) induced by pioglitazone. MK-886 in control cells did not alter apoA-I mRNA levels (Figure 6A) or apoA-I secretion in the medium (Figure 6C). In additional experiments, we used gene silencing with siRNA transfection to further confirm the participation of PPAR-α in pioglitazone-induced apoA-I production. Using cotransfection with GFP, we estimated that the transfection efficiency was about 54%. As shown in Figure 6B, siRNA transfection markedly reduced PPAR-α mRNA levels by...
66% as compared with control vector transfection. PPAR-α mRNA levels were reduced by 69% when cells were transfected with PPAR-α siRNA and subsequently treated with pioglitazone, an effect similar to that of control cells (without pioglitazone treatment) transfected with sRNA. Furthermore, pioglitazone-induced increase in the apoA-I secretion was significantly abrogated in cells transfected with the PPAR-α siRNA complex (P=0.014; Figure 6D).

**Discussion**

Several studies clearly established that pioglitazone significantly increased serum HDL-cholesterol and decreased triglycerides, and pioglitazone produced more favorable lipid profiles than rosiglitazone in patients with type 2 diabetes.\(^{25-27}\) However, the effect of pioglitazone on apo-AI level is not clear. In a small number of nondiabetic patients with low HDL-cholesterol and metabolic syndrome, Szapary et al have recently shown that pioglitazone treatment for 6 weeks significantly increased mean apo-AI by 6.8% and apo-AII by 7.7%; however, by 12 weeks of treatment, only the change in apo-AII remained significant.\(^{26}\) Further studies are required in large number of patients to clearly understand the effect of pioglitazone on apo-AI levels.

In this study, using HepG2 cell system, we have delineated the effect of pioglitazone on various cellular processes involved in HDL metabolism that in turn determine the overall concentration of apoA-I/HDL mass. The data indicate that pioglitazone by increasing apoA-I mRNA levels increased the de novo synthesis and secretion of apoA-I particles in the culture medium. Additionally, we have shown that the apoA-I-containing lipoprotein particles secreted in the culture medium of HepG2 cells treated with pioglitazone
were able to significantly increase cholesterol efflux from THP-1 macrophages, suggesting that these particles are functionally active in initiating reverse cholesterol transport. We have also shown that pioglitazone increases apoA-II synthesis and mRNA expression in HepG2 cells. Pioglitazone had no effect on HDL uptake, suggesting that pioglitazone increases apoA-I/HDL by increasing apoA-I synthesis but not HDL catabolism in HepG2 cells. These findings, at least in part, define hepatic mechanisms of action of pioglitazone to increase apoAI, apoA-II, and HDL seen in humans.26 However, as the findings of this study were derived from in vitro studies using HepG2 cells (a hepatocarcinoma cell line but not primary hepatocytes), real limitations exist in extrapolating in vitro findings of this study to in vivo conditions in humans.

An increasing number of transcription factors have been reported to be involved in the regulation of apoA-I gene expression. These transcription factors include members of a steroid/thyroid nuclear receptor superfamily, such as hepatocyte nuclear factor-4 of a steroid/thyroid nuclear receptor superfamily, such as hepatocyte nuclear factor-4 (HNF-4α), apoA-I regulatory protein-1 (ARP-1), and retinoid X receptor (RXRα); the HNF-3/forkhead family of transcription factors, such as HNF-3α/β, early growth response factor (Egr-1), and transcription factor Sp1. PPAR-α activators (such as fibrates), through heterodimerizing with retinoid receptor and binding to PPAR response element in the promoter region of apoA-I gene, were shown to increase the transcription of human apoA-I.19 Recent data also indicated that inactivation of nuclear factor-kB enhances the expression and secretion of apoA-I from HepG2 cells through activation of PPAR-α.20 However, the role of PPAR-γ in apoA-I transcription is not clearly understood. Troglitazone, a PPAR-γ agonist, had no effect on apoA-I mRNA transcription in Hep-G2 cells.20 Our data also indicate that 15-dPGJ2, a natural ligand and activator of PPAR-γ, had no effect on apoA-I mRNA expression in HepG2 cells (data not shown). Our findings and the current literature point that PPAR-α, but not PPAR-γ, regulates apoA-I mRNA levels and subsequent apoA-I production.

Although pioglitazone is known to exhibit high-affinity binding and activation of PPAR-γ, it is interesting to note that pioglitazone also acts as a weaker activator for PPAR-α.21 Sakamoto et al have shown that both pioglitazone and rosiglitazone (but not troglitazone) transactivated PPAR-α by 5.4-fold and 4.2-fold over control, respectively, in COS-1 cells.21 Using competition-binding assays with radioligand, it was shown that the activation of PPAR-α by pioglitazone was attributable to direct binding of pioglitazone to PPAR-α.21 Because PPAR-α regulates apoA-I transcription, we investigated whether the effect of pioglitazone on apoA-I mRNA expression is mediated through PPAR-α. Using specific inhibitor and siRNA approaches, we have shown that inhibition of PPAR-α in Hep G2 cells significantly inhibited pioglitazone-induced apoA-I production. These findings suggest that the activation of hepatic PPAR-α by pioglitazone regulates apoA-I mRNA expression and subsequent apoA-I protein synthesis and secretion. It is also important to note that the effective concentrations of pioglitazone used in our studies (0.1 to 10 µmol/L) are comparable to the plasma levels observed in patients treated with pioglitazone,31 suggesting clinical relevance to the in vivo situation.

Although used for glycemic control, thiazolidinediones should be considered also as part of dyslipidemia treatment in diabetes, in combination with lipid regulating drugs including statins, niacin, and fibrates. Whereas fibrates and statins act via PPAR-α activation, niacin decreases apoA-I catabolism.22,23 This information is important in using combination therapy using drugs with complementary mechanisms of action to achieve aggressive HDL goals. Additional research is needed to assess the additive efficacy of such combinations not only on lipids, but also on reducing cardiovascular events in the high risk population of diabetics beyond monotherapy.

In summary, we suggest that pioglitazone, at least in part through PPAR-α-mediated events, increases hepatic apoA-I mRNA expression resulting in increased plasma levels of physiologically active apoA-I/HDL particles. Pioglitazone, by activating both PPAR-γ and PPAR-α, beneficially regulates both glucose and HDL levels. Additionally, our findings may be useful in forming the rationale for combination therapy using pioglitazone and other HDL raising agents with different mechanisms to additively increase HDL levels in diabetic patients.

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Disclosures

None.

References


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