Pioglitazone Reduces Vascular Lipid Accumulation in Angiotensin II-Induced Hypertensive Rat

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Aim: In an insulin-resistant state, excess lipids may accumulate in various non-adipose tissues, leading to histological and functional damage. It has been suggested that peroxisome proliferator-activated receptor-gamma (PPARγ) may ameliorate disorganized lipid balance. In the current study, we analyzed whether pioglitazone, an agonist of PPARγ, reduces angiotensin II-induced vascular lipid accumulation.

Methods: Angiotensin II was infused into rats at doses of 0.7 mg/kg/day via a subcutaneously implanted osmotic minipump for 7 consecutive days. Pioglitazone was orally given at a dose of 2.5 mg/kg/day for 7 days.

Results: Pioglitazone significantly reduced angiotensin II-induced enhanced lipid deposition and superoxide production in the adventitia of the aorta, as detected by oil red O and dihydroethidium (DHE) staining, respectively. Increased DHE signals, some observed at the site of lipid deposition, were mainly localized in ED-1-positive monocytes/macrophages. Angiotensin II-induced upregulation of the expression of LDL receptor and Nox1 was inhibited by pioglitazone treatment. In addition, angiotensin II significantly reduced the expression of PCSK9, and this reduction was ameliorated by pioglitazone. On the other hand, pioglitazone did not significantly alter the expression of the phosphorylated forms of AMPKα and ACC, which was downregulated by angiotensin II.

Conclusions: Pioglitazone treatment suppressed excess lipid accumulation and superoxide production in the aorta in an angiotensin II-induced rat model of hypertension.


Key words: Aorta, Lipid accumulation, Angiotensin II, Peroxisome proliferator-activated receptor

Introduction

Obesity and associated disorders, such as diabetes and atherosclerosis, are closely related to the elevated levels of lipids and chronic inflammation1). Recent reports have suggested that ectopic fat distribution might be a better predictor of future cardiovascular events than overall obesity itself2,3). Adipocytes have sufficient capacity to store overflowing lipids in the cytosol, whereas non-adipocyte cells have limited space to store excess fat4). Intracellular lipid accumulation in non-adipocyte cells and excess lipid utilization may disrupt cellular homeostasis and lead to cell death, a phenomenon defined as “lipotoxicity”5). Lipotoxicity occurs in multiple organs, including cardiac and vascular tissues. Perivascular fat may play an independent role in adverse vascular biology, such as the pathogenesis of arterial stiffness6). Lipotoxicity has been reported to impair endothelial cell function via diverse mechanisms, including vascular insulin resis-
Histological and Immunohistochemical Analysis

Oil red O staining was performed on sections of unfixed, freshly frozen vascular samples (3 μm in thickness). The area of lipid deposition was calculated by the image analysis software Photoshop (Adobe), and lipid deposition was semiquantified as described elsewhere. Staining with the oxidative fluorescent dye dihydroethidium (DHE) was performed as described previously. Images were obtained with a fluorescence microscope BX51 (Olympus), and the fluorescence intensity, obtained from at least five fields for each section, was calculated as a percentage of that of the untreated control. Immunohistochemistry was performed as described previously. Primary antibody against rat macrophage/monocyte (ED-1; BMA Biomedicals) was used at a dilution of 1/100.

Western Blot Analysis

Western blot analysis was performed as described previously. All layers of vascular samples were used for the analysis. Antibody against low-density lipoprotein (LDL) receptor (Abcam) was used at a dilution of 1/500. Antibodies against total and phosphorylated forms of AMP-activated protein kinase (AMPK) (Cell Signaling Technology), total and phosphorylated forms of acetyl-CoA carboxylase (ACC) (Cell Signaling Technology), sterol regulatory element-binding proteins (SREBP)-1c (Cell Signaling Technology), proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abcam), and β-actin (Sigma) were used at a dilution of 1/1000. Antibodies against fatty acid synthase (FAS) (Cell Signaling Technology) and Nox1 (Abcam) were used at a dilution of 1/2000. An ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized by a luminoanalyzer (Fuji Photo Film). Band intensity was calculated as a percentage of the control value.

Materials and Methods

Animal Models

All experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II-induced hypertension was induced in male Sprague–Dawley rats (250–300 g) by subcutaneous implantation of an osmotic minipump (Alza Pharmaceutical), as described previously. Briefly, Val5-Ang II (Sigma Chemical) was infused at doses of 0.7 mg/kg/day via a subcutaneously implanted osmotic minipump for 7 days. In some angiotensin II-infused rats, pioglitazone, an agonist of PPARγ, was orally given at a dose of 2.5 mg/kg/day for 7 consecutive days.

Table 1. Oligonucleotide primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Nox1</td>
<td>MN_053683</td>
<td>TGGACGAATTAGGCAAACCG</td>
<td>TTGGGGTGAGGCAGTCTAT</td>
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<tr>
<td>SREBP-1c</td>
<td>XM_213329</td>
<td>CTGATGGAGACCGGAGTTC</td>
<td>ATCACCAAGGCCTGACTGT</td>
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<tr>
<td>FAS</td>
<td>M76767</td>
<td>CTGGAAGCGAACATGTCTC</td>
<td>TTTCAAGAGGATCTCTG</td>
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<tr>
<td>LDL receptor</td>
<td>X13722</td>
<td>CGGAGGAGCAGATCCACATT</td>
<td>AAAACGTCTCTTCTGCTAA</td>
</tr>
<tr>
<td>PPARγ</td>
<td>NM_013124</td>
<td>ATCACCTGCTGGACGCTCTC</td>
<td>AGGCTCTACTCTTGAAGC</td>
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<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>TGAACGGAAGGCTACTGG</td>
<td>TCCCACCACCCTGCTCTGA</td>
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</tbody>
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SREBP, sterol regulatory element-binding proteins; FAS, fatty acid synthase; LDL, low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Statistical Analysis

Data are expressed as the mean ± SEM. We used ANOVA followed by a multiple comparison test to compare raw data, before expressing results as a percentage of the control value using the statistical analysis software Dr. SPSS II for Windows (SPSS Inc.). A value of \( p < 0.05 \) was considered to be statistically significant.

Results

Accumulation of Lipids in the Aorta

The body weight, blood pressure, and serum lipid
was suppressed by pioglitazone treatment (87 ± 5%, p = NS versus control) (Fig. 2). In the aorta of angiotensin II-infused rats, increased DHE signals were observed in the adventitia of the aorta among cells that were mainly positive for ED-1; thus, they were judged to be monocytes/macrophages. Some adventitial cells with increased DHE signals were detected at the site of lipid deposition (Fig. 3). In addition, angiotensin II significantly increased the protein expression of Nox1 (227 ± 18%, p < 0.001 versus control), a component of NADPH oxidase, and this upregulation was suppressed by pioglitazone treatment (154 ± 19%, p = NS versus control). Angiotensin II-induced upregulation of rats in each group have been described previously14). Oil red O staining of vascular sections showed no apparent lipid deposition in untreated control rats and control rats treated with pioglitazone alone (Fig. 1A, B). An increase in oil red O-positive lipid droplets was observed in the adventitia of the aorta in angiotensin II-infused rats (Fig. 1C), and this increase was suppressed by pioglitazone administration (Fig. 1D).

**Superoxide Production in the Aorta**

Compared with untreated control rats, DHE staining-positive signals were increased by angiotensin II (285 ± 34%, p < 0.001 versus control), and this increase was suppressed by pioglitazone treatment (87 ± 5%, p = NS versus control) (Fig. 2). In the aorta of angiotensin II-infused rats, increased DHE signals were observed in the adventitia of the aorta among cells that were mainly positive for ED-1; thus, they were judged to be monocytes/macrophages. Some adventitial cells with increased DHE signals were detected at the site of lipid deposition (Fig. 3). In addition, angiotensin II significantly increased the protein expression of Nox1 (227 ± 18%, p < 0.001 versus control), a component of NADPH oxidase, and this upregulation was suppressed by pioglitazone treatment (154 ± 19%, p = NS versus control). Angiotensin II-induced upregulation
of mRNA expression of Nox1 (565 ± 95%, \( p < 0.001 \) versus control) was also inhibited by pioglitazone treatment (298 ± 57%, \( p = \text{NS} \) versus control) (Fig. 4).

**Regulation of Genes Related to Lipid Metabolism**

Compared with untreated control rats, angiotensin II significantly decreased PCSK9 expression (41 ± 3%, \( p < 0.005 \) versus control), and this decrease was ameliorated by pioglitazone (72 ± 10%, \( p = \text{NS} \) versus control). Furthermore, the protein expression of LDL receptor was significantly increased by angiotensin II (195 ± 25%, \( p < 0.005 \) versus control), and this upregulation was ameliorated by pioglitazone treatment (145 ± 30%, \( p = \text{NS} \) versus control) (Fig. 5). Although the expression of total and phosphorylated forms of AMPK\( \alpha \) and ACC was significantly reduced by angiotensin II, this downregulation was not affected by pioglitazone treatment (Fig. 6). The protein expression of FAS was significantly decreased by angiotensin II (43 ± 6%, \( p < 0.05 \) versus control), and this reduction was ameliorated by pioglitazone treatment (57 ± 9%, \( p = \text{NS} \) versus control). Concomitant treatment of pioglitazone and angiotensin II significantly increased the protein expression of SREBP-1c (132 ± 11%, \( p < 0.05 \) versus control).

Angiotensin II-induced upregulation of LDL receptor mRNA expression (757 ± 227%, \( p < 0.01 \) versus control) was suppressed by pioglitazone (314 ± 75%, \( p = \text{NS} \) versus control), as with the protein expression. In addition, angiotensin II significantly reduced the expression of FAS mRNA (32 ± 5%, \( p < 0.05 \) versus control), and this reduction was ameliorated by concomitant pioglitazone treatment (61 ± 9%, \( p = \text{NS} \) versus control). Although not statistically significant, angiotensin II suppressed SREBP-1c mRNA expression (61 ± 7%, \( p < 0.005 \) versus control), and concomitant treatment with pioglitazone significantly upregulated the expression of SREBP-1c mRNA (204 ± 28%, \( p < 0.005 \) versus control). The expression of PPAR\( \gamma \) mRNA was not altered either by angiotensin II alone (54 ± 7%, \( p = \text{NS} \) versus control) or angiotensin II plus pioglitazone (134 ± 30%, \( p = \text{NS} \) versus control) (Fig. 7).

**Discussion**

In the current study, we demonstrated that pioglitazone suppressed excess lipid accumulation and superoxide production induced by angiotensin II in the rat aorta. Angiotensin II significantly decreased the expression of FAS in the rat aorta, but this decrease was restored by pioglitazone treatment. In addition, concomitant administration of angiotensin II and pioglitazone significantly increased the mRNA expression of SREBP-1c, even though pioglitazone treatment reduced vascular lipid accumulation. Pioglitazone did not alter the suppressed expression of phosphorylated AMPK\( \alpha \) and ACC induced by angiotensin II, which might rather act to enhance vascular lipid deposition. On the other hand, angiotensin II-induced upregulation of the LDL receptor and downregulation of PCSK9 were both improved by pioglitazone treatment. The expression of NADPH oxidase, which was increased by...
lipotoxicity may decrease endothelial NO synthase (eNOS) gene expression and eNOS catalytic activity, which leads to vascular endothelial cell dysfunction, by several key mechanisms, including inflammation and oxidative stress as well as insulin resistance. Under an activated renin-angiotensin system, NADPH oxidase-mediated superoxide production may reduce NO bioavailability and induce vascular dysfunction. Our current study demonstrated that in the angiotensin II-infused rat, DHE signals indicating superoxide production were increased at the site of lipid deposition and were mainly localized in ED-1-positive monocytes/macrophages. Lipids are ligands for the transcriptional regulators of PPARs, which belong to the nuclear hormone receptor superfamily. Under normal conditions, PPARγ is mainly expressed in adipose tissue and regulates various functions, including the development of fat cells and their capacity to store lipids. Because PPARγ plays a pivotal role in adipogenesis, its mutation causes a failure of adipogenesis and leads to insulin resistance. Lipotoxicity may decrease endothelial NO synthase (eNOS) gene expression and eNOS catalytic activity, which leads to vascular endothelial cell dysfunction, by several key mechanisms, including inflammation and oxidative stress as well as insulin resistance. Under an activated renin-angiotensin system, NADPH oxidase-mediated superoxide production may reduce NO bioavailability and induce vascular dysfunction. Our current study demonstrated that in the angiotensin II-infused rat, DHE signals indicating superoxide production were increased at the site of lipid deposition and were mainly localized in ED-1-positive monocytes/macrophages. Lipids are ligands for the transcriptional regulators of PPARs, which belong to the nuclear hormone receptor superfamily. Under normal conditions, PPARγ is mainly expressed in adipose tissue and regulates various functions, including the development of fat cells and their capacity to store lipids. Because PPARγ plays a pivotal role in adipogenesis, its mutation causes a failure of adipogenesis and leads to insulin resistance.
acid metabolism-related genes, such as SREBP-1c and FAS and the phosphorylated forms of AMPK and ACC. In some cases, it rather changed the expression to exacerbate lipid deposition. This finding may be, at least in part, compatible with the previous report that PPARα and PPARγ/δ, but not PPARδ, favorably modulate the expression of genes associated with cardiac fatty acid metabolism.

On the other hand, we demonstrated that the upregulation of the LDL receptor and downregulation of PCSK9 induced by angiotensin II were both attenuated by pioglitazone treatment. PCSK9, which is primarily synthesized by the liver and intestine, is a crucial protein in LDL cholesterol metabolism. Chan et al. demonstrated that anti-PCSK9 antibody increased LDL receptor protein levels both in HepG2 cells and in the liver of wild-type mice. Duan et al. reported that pioglitazone increased PCSK9 protein expression in the liver of wild-type mice, although it also increased hepatic LDL receptor protein expression. In contrast, it has been shown that hepatic protein expression, but not mRNA expression, of the LDL receptor is significantly inhibited by pioglitazone in obese diabetic mice. Although not statistically significant, the expression of PCSK9 was borderline increased by the administration of pioglitazone alone in the current study; in addition, angiotensin II-induced downregulation of PPARγ led to a significant increase in LDL receptor protein expression, which was not observed with pioglitazone treatment.

Fig. 7. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA levels of the low-density lipoprotein receptor (LDLR) (A), fatty acid synthase (FAS) (B), sterol regulatory element-binding proteins (SREBP)-1c (C), and peroxisome proliferator-activated receptor (PPAR)-γ (D). Data represent the mean ± SEM of data from nine to ten rats in each group. Abbreviations are the same as in Fig. 1.

*p < 0.05 versus untreated control.
**p < 0.01 versus untreated control.
***p < 0.005 versus untreated control.
related PCSK9 expression was restored by concomitant treatment with pioglitazone. Recently, the accumulation of both cholesterol and fatty acid has been suggested to be associated with lipotoxicity in pancreatic \( \beta \)-cells\(^{38}\). Together with these previous observations, present results may suggest that the modulation of genes related to cholesterol metabolism by PPAR\( \gamma \) activation may underlie to some extent the amelioration of vascular lipotoxicity.

In the current study, angiotensin II infusion significantly reduced the expression of the phosphorylated forms of AMPK\( \alpha \) and ACC. We previously demonstrated that the phosphorylated form of AMPK\( \alpha \) was also decreased by angiotensin II in the rat liver\(^{39}\). However, angiotensin II upregulated the cardiac and renal expression of phosphorylated AMPK\( \alpha \) and ACC\(^{14,15}\). Kim \textit{et al.} reported that, in vascular smooth muscle cells of spontaneously hypertensive rats, angiotensin II inhibited the activation of AMPK\( \alpha \)\(^{40}\). Taking these observations together, we propose the hypothesis that vascular tissue may be, at least in part, an organ that provides the capacity to store excess lipids to protect other organs, such as the heart and kidney, against damage from lipotoxicity in a state of aggravated lipid homeostasis. Additionally, although not statistically significant, pioglitazone decreased the expression ratio of phosphorylated AMPK\( \alpha \) in the current study. We previously reported that the phosphorylated form of AMPK\( \alpha \) was increased by pioglitazone in the rat heart and kidney, although there was no statistical significance in the rat heart\(^{14,15}\). Boyle \textit{et al.} found that rosiglitazone significantly upregulated the expression of the phosphorylated form of AMPK\( \alpha \) in human aortic endothelial cells\(^{41}\). As it has been widely recognized that thiazolidinediones activate AMPK in various organs, our current finding seems to contradict previous reports. However, in our current study, the expression ratio of phosphorylated AMPK\( \alpha \) to total AMPK\( \alpha \) was increased by pioglitazone treatment irrespective of the presence or absence of angiotensin II as compared with untreated control, indicating that pioglitazone may promote the activation of AMPK\( \alpha \). Although pioglitazone suppressed the expression of total AMPK\( \alpha \) in the current study, this point is a subject for future investigation to elucidate the role of pioglitazone in the amelioration of vascular lipotoxicity.

In summary, pioglitazone treatment reduced vascular lipid accumulation induced by angiotensin II, an effect that was not accompanied by favorable modulation of the expression of fatty acid metabolism-related genes. The current findings suggest that the amelioration of vascular lipotoxicity and aggravated oxidative stress by pioglitazone may be related to a vasculoprotective effect during the activation of the renin-angiotensin system.

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**Disclosures**

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