Pioglitazone Prevents Hypertension and Reduces Oxidative Stress in Diet-Induced Obesity

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Abstract—The objective of this study was to determine the effect of pioglitazone on blood pressure (BP) and oxidative balance in obese, hypertensive, Sprague-Dawley rats and to identify some of the molecular mechanisms involved. After 12 weeks of a moderately high-fat diet, rats diverged into obesity-prone (OP) and obesity-resistant (OR) groups (n=6 per group). At the end of the diet, peroxisome proliferator activated receptor-γ (PPARγ) mRNA expression and activity in the renal cortex and medulla of OP rats were significantly lower compared with that in OR rats. Pioglitazone treatment increased PPARγ expression and activity in OP rats, suggesting a possible direct ligand-related effect of pioglitazone. As opposed to the untreated OP group, which showed moderate hypertension (systolic BP=159±5.3 mm Hg) after 12 weeks, pioglitazone-treated rats were normotensive (systolic BP=123.9±2.7 mm Hg). Insulin production was reduced by 2-fold in the OP group treated with pioglitazone. Urinary isoprostanes and renal lipid peroxides were also reduced in OP rats treated with pioglitazone compared with untreated counterparts. Also, expression of p47phox and gp91phox, both increased in OP versus OR rats, was reduced in the former by pioglitazone treatment. In addition, pioglitazone treatment increased nitrate/nitrite excretion and expression of renal endothelial and neuronal nitric oxide synthase. Collectively, the results show that pioglitazone treatment prevented hypertension and renal oxidative stress both by reducing free-radical production and by increasing nitric oxide production/availability. *(Hypertension. 2004;43:48-56.)*

Key Words: kidney ■ oxidative stress ■ free radicals ■ nitric oxide ■ vitamins

Obesity is a widespread and increasingly prevalent condition associated with a large number of comorbid diseases, one of the most important of which is obesity-induced hypertension. A pleiotropic class of molecules involved in regulation of gene expression in a variety of metabolic and cardiovascular conditions is the peroxisome proliferator activated receptor (PPAR). PPAR is ligand-activated transcription factors that form heterodimers with the 9-cis retinoic acid receptor RXRs. PPARγ is one of the three PPAR isoforms and is one of the major regulators of adipogenesis. In addition, PPARγ exerts pleiotropic effects on blood pressure, lipid metabolism, and insulin action.

Recent genetic analysis showed that 2 dominant-negative mutations in PPARγ were associated with severe hypertension in humans. Consistent with these findings, thiazolidinediones, the insulin-sensitizer drugs (pioglitazone, rosiglitazone) that are also high-affinity PPARγ ligands, have been shown to lower blood pressure (BP) in a variety of hypertensive animal models as well as in diabetic and nondiabetic hypertensive humans. However, the mechanism underlying the antihypertensive effects of PPARγ agonists is not known. PPARγ and RXR have been found constitutively expressed in the inner medullary collecting ducts, thick ascending limb, glomerulus, and renal medullary microvascular endothelial cells in rats, rabbits, and humans. Constitutive expression of PPARγ in these segments might have important regulatory effects on renal sodium and water reabsorption.

Free-radical production is associated with both obesity and hypertension in humans and animal models. One of the mechanisms by which free radicals can sustain hypertension is a reduction of nitric oxide (NO) availability at the level of the thick ascending limb or macula densa, therefore leading to increased sodium reabsorption. NO can readily interact with O2− anion, rendering the former unable to perform its physiologic vasodilatory effect. A recent report indicates that a major source of O2− in the rat kidney is a phagocytic-like NAD(P)H oxidase. All of the major NAD(P)H oxidase subunits have been identified in the rat kidney, and p47phox was shown to be increased in the prehypertensive spontaneously hypertensive rat, suggesting a possible causative role in this form of genetic hypertension.

Also, several recent reports showed the ability of PPARγ agonists to modulate expression of different NAD(P)H oxidase subunits. Inoue et al showed that PPARγ activators increase (Cu,Zn)superoxide dismutase expression while decreasing p22phox message in human endothelial cells. Also, activation of PPARγ by NO in macrophages was shown to downregulate p47phox and to attenuate the respiratory burst. In addition, troglitazone was shown to reduce reactive oxygen species generation by leukocytes and improve post-

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ischemic flow-mediated vasodilation in obese humans. However, there are no data on a potential PPARγ-related effect on free-radical or NO production in the kidney in obese, hypertensive animal models or humans. In this study, we aimed to assess the contribution of oxidative stress to BP regulation in a rat model of diet-induced obesity and hypertension and to identify some of the mechanisms underlying the hypotensive effects of pioglitazone treatment.

**Methods**

**Animals and Treatments**

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School. Male Sprague-Dawley rats (300 to 350 g) were fed a moderately high-fat diet (MHF; 32% kcal as fat; Research Diets) or a purified, low-fat diet (LF; 10.6% kcal as fat) (controls) for 12 weeks. After 1 week, randomly selected rats were placed on either the MHF or LF diet supplemented with either pioglitazone (0.1% wt/wt, or ~10 mg · kg−1 · d−1; Actos, Hanna Pharmaceuticals) or vitamin E (0.5% wt/wt as α-tocopherol acetate, or ~200 mg · kg−1 · d−1; Sigma). Rats that were fed the MHF diet with or without the indicated treatments diverged into distinct groups based on body weight gains. Assignment of rats into obesity-prone (OP, n = 6) and obesity-resistant (OR, n = 6) groups was performed as previously described. In brief, construction of body-weight–gain histograms was performed at the end of the study for MHF and LF diet groups. OR rats were defined as those with weight gains equal to or less than the heaviest controls, and the OP rats, as those with greater weight gains.

**BP Measurements**

The onset and development of hypertension were assessed by the tail-cuff method with a sphygmomanometer (Narco Biosystems Electro-Sphygmomanometer), as previously described.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections were processed for antigen retrieval and incubated with a primary polyclonal antibody for 1:1 Cys,His,Lys-4-hydroxy-2-nonenal (HNE) adducts (Calbiochem; 1:100 dilution). The slides were stained with reagents from a commercially available kit (ImmunoPure Metal Enhanced DAB substrate kit, Pierce).

**RNA Isolation and RT-PCR**

Kidneys were homogenized in ice-cold guanidinium isothiocyanate and processed for total RNA extraction according to Chomczynski. RNA (1 μg) was reverse-transcribed (RT) and amplified by polymerase chain reaction (PCR) as previously described. The following pairs of forward and reverse primers were used: PPARγ1L2, 5′-TCTTGTTATGGAAGACCACTC-3′ and 5′-CTCTTGGCATCTCCGACAAGC-3′; and β-actin, 5′-CGTAAAGACCTCTATGCCAA-3′ and 5′-CTCTTGGCCAGTGTA-3′. The neuronal (nNOS) and endothelial (eNOS) NO synthase primers were described previously. The bands corresponding to PCR products were measured by densitometry on an EagleEye system (Stratagene).

**Western Blotting**

The tissue was homogenized and fractionated by centrifugations at 800g (to pellet debris), at 10,000g (to pellet intracellular organelles), and at 150,000g, after which the membrane pellet was reconstituted in Tris-sucrose buffer, pH 7.4. Sample protein was assessed with the bicinchoninic acid method with a kit (Sigma). Equal amounts of protein (20 μg per lane) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and subsequently electroblotted. The following primary antibodies were used: anti-phox (Santa Cruz, 1:1000 dilution), anti-gp91phox (Transduction Laboratories, 1:2000 dilution), and anti-eNOS and anti-nNOS (Transduction Laboratories, 1:1500 dilution). Bands were visualized by enhanced chemiluminescence (ECL, Amersham Life Science).

**Other Assays**

Urinary free isoprostanes and thiobarbituric acid–reactive substances (TBARS) were measured as previously described. Plasma insulin and urine nitrite/nitrate (NOx) were measured with kits from Linco Research Inc and Cayman Chemical, respectively. Sodium excretion was assessed by flame photometry.

**Statistical Analysis**

Comparisons between group means were performed by 2-way ANOVA and Tukey post hoc analysis test with InStat software (GraphPad Software). The null hypothesis was rejected when P < 0.05.

**Results**

**Effect of Pioglitazone and Vitamin E Treatments on BP in OP, OR, and Control Rats**

In accordance with previous data, the OP rats showed a significant elevation in systolic BP after week 7 on the diet, which remained higher (159.3 ± 6.5 mm Hg) compared with that in OR (136.2 ± 4.5 mm Hg) and control (128.6 ± 3.2 mm Hg) groups until the end of the experiment (Figure 1A). When pioglitazone was added to the diets, OP rats maintained their BP.
within the normal range until the end of the experiment (131.3±4.5 mm Hg). Pioglitazone also had a moderate hypotensive effect in OR (125±2.2 mm Hg) and control (119.5±5.2 mm Hg) rats (Figure 1A).

To directly assess the effect of oxidative stress on the development of hypertension, 2 separate groups of rats were placed on either the MHF or LF diet supplemented with vitamin E treatment. Unlike pioglitazone, vitamin E failed to completely prevent the elevation in BP in OP rats; however, it significantly reduced it compared with the untreated OP group after 7 weeks (Figure 1B). At the end of the experiment, OP rats treated with vitamin E had an average systolic BP of 140.2±4.5 mm Hg. By the end of experiment, no significant differences were detected between the OR and corresponding untreated groups (Figures 1A through 1D). However, vitamin E treatment of OP, OR, and control rats did not significantly alter mRNA expression in either the cortex or medulla compared with corresponding untreated groups (Figures 2A through 2D).

**Effect of Pioglitazone on Oxidative Stress and NAD(P)H Oxidase Subunit Expression in OP, OR, and Control Rats**

We tested whether PPARγ activation had an effect on 2 different parameters of oxidative stress as well as on NO production. Results indicated that both urine free isoprostanes, a recognized index for systemic free-radical production, and kidney TBARS, an index of lipid peroxidation, were moderately though significantly inhibited by pioglitazone treatment after 12 weeks of diet feeding in OP rats (Figure 3). To compare the effect of pioglitazone with that of a known antioxidant, we measured the effect of vitamin E treatment on oxidative stress in OP, OR, and control groups. Vitamin E had a comparable effect in reducing urine free isoprostane formation (Figure 3A) and a more potent effect in reducing lipid peroxidation in renal tissue (Figure 3B). In addition, HNE immunostaining of renal tissue showed strong staining of the proximal and distal tubules in the cortex of OP compared with OR rats (Figure 4). In OP rats treated with pioglitazone, there was a noticeable reduction in HNE staining compared with nontreated counterparts (Figure 4A). Vitamin E had a comparable effect in the ability to reduce HNE staining in OP rats (not shown).

NAD(P)H oxidase has been shown to be one of the major sources of free radicals in the kidney. To test whether pioglitazone exerted a genomic effect on different NAD(P)H oxidase components, we measured protein expression for p47phox,
p67phox, gp91phox, and p22phox by Western blotting in OP, OR, and control groups of rats with or without pioglitazone treatment. Both p47phox and gp91phox showed increased expression in OP compared with OR rats, and pioglitazone treatment decreased p47phox and gp91phox expression in the former (Figure 5), suggesting a possible relation between the level of expression for PPARγ and the 2 NAD(P)H oxidase subunits. Neither p22phox nor p67phox significantly differed in OP versus OR rats or changed with pioglitazone treatment (not shown). To verify the specificity of the pioglitazone effect, we also measured p47phox and gp91phox protein expression in the vitamin E–treated groups. Vitamin E treatment did not significantly change p47phox or gp91phox protein expression in OP, OR, or control groups (Figure 5).

NOx Production and NOS Expression in OP Rats With or Without Pioglitazone or Vitamin E Treatment

In accordance with previous reports, NOx excretion was lower in OP versus OR and control rats, suggesting reduced NO production and/or availability in the former (Table). Pioglitazone treatment increased the excreted NO metabolites
in OP rats, with no significant effect in OR and control rats, whereas vitamin E did not significantly alter NOx excretion in any of the 3 groups (Table). To further test whether changes in NOx production were related to a change in expression of the constitutively expressed NOS isoforms in the kidney, eNOS and nNOS expression was measured in OP, OR, and control rats with or without pioglitazone or vitamin E treatment. We previously reported that OP rats have increased eNOS expression in the renal cortex and medulla. When treated with pioglitazone, OP rats displayed a further increase in both eNOS and nNOS mRNA in both kidney cortex and medulla (Figure 6). Also, pioglitazone treatment increased eNOS and especially nNOS expression in both the cortex and medulla of OR and control rats. In contrast, vitamin E treatment did not significantly change either eNOS or nNOS mRNA expression in the cortex and medulla of OP, OR, and control groups (Figure 6). The change in mRNA expression was mirrored by a similar increase in protein expression for both NOS isoforms (Figure 7). The most prominent changes were detected for the eNOS isoform in both the cortex and medulla (Figure 7).

**Discussion**

In this study, we showed that pioglitazone treatment prevented the development of hypertension and renal oxidative stress in a rat model of diet-induced obesity. Also, our results indicate that compared with vitamin E, a well-known antioxidant, pioglitazone is more efficient in reducing BP and increasing sodium excretion in obese versus lean rats, while being equally efficient in reducing urinary isoprostanes, kidney TBARS, and HNE adducts. One possible explanation for these different effects is the ability of pioglitazone, as opposed to vitamin E, to increase renal NOS expression and NOx excretion in treated OP rats. In addition, pioglitazone, but not vitamin E treatment, significantly reduced expression of p47phox and gp91phox in OP rats, suggesting that both a reduction in oxidative stress and an improvement in NO production/bioavailability are important in efficiently reducing BP in this model.

Thiazolidinediones are known to exert pleiotropic actions both in vivo and in vitro, some of which occur through their ability to activate PPARγ. To the best of our knowledge, this...
is the first study to address the in vivo regulation of PPARγ expression in the kidney in an obese, hypertensive model in response to a dietary challenge. Our results indicate that a high-fat diet upregulated PPARγ mRNA expression in lean (OR) but not in obese (OP) rats. A similar finding was reported in a genetic mouse model of obesity, in which lean but not obese mice exposed to a high-fat diet displayed a 4-fold increase in adipose tissue PPARγ expression.30 Nevertheless, because PPARγ expression was measured only as an end point in the experiment when the rats were already obese, further studies are necessary to delineate whether the reduced PPARγ expression in response to a high-fat diet in OP rats is a late effect induced by the obese state per se or whether it occurs earlier, before the onset of obesity. Interestingly, although the high-fat diet failed to upregulate PPARγ expression in OP rats, pioglitazone treatment elevated PPARγ expression in OP rats. A possible explanation is that pioglitazone indirectly affects PPARγ expression by its ability to act as an insulin sensitizer. Circulating insulin levels were ≈3.5-fold higher in the nontreated versus pioglitazone-treated OP group. One major intracellular signaling pathway for insulin involves activation of the phosphatidylinositol 3-kinase (PI3K) cascade.31 A recent report demonstrates that PI3K overexpression decreases PPARγ activity in 3T3-L1 adipocytes.32 It is possible that increased circulating insulin, through activation of intracellular PI3K, is responsible for the reduced PPARγ expression measured in OP rats, and pioglitazone treatment indirectly increases PPARγ expression by reducing circulating insulin and improving insulin sensitivity. However, at least in part, pioglitazone seems to act independently of insulin, because the control normoinsulinemic group treated with pioglitazone had increased PPARγ acti-
vation compared with the nontreated control. Also, oxidative stress does not seem to be responsible for the reduced PPARγ expression and activity in OP rats, because vitamin E significantly lowered oxidative stress in the treated OP group but failed to increase the expression or activity of renal PPARγ in the latter.

Interestingly, recent studies suggest a direct role for free radicals and NO availability in the renal cortex and medulla in sodium reabsorption and development of hypertension. The inability of vitamin E, as opposed to pioglitazone treatment, to increase sodium excretion or totally prevent the development of hypertension in OP rats could be explained.
by its inability to upregulate NOS expression or increase NO excretion. Our results suggest that vitamin E treatment, despite reducing oxidative stress, could not significantly improve NO bioavailability. In contrast, pioglitazone treatment both reduced oxidative stress and significantly increased NO excretion in OP rats. The result could be explained by the ability of pioglitazone to also enhance NO availability by a mechanism independent of free-radical production. Our results indicate that pioglitazone treatment upregulates the expression of eNOS and nNOS at both the RNA and protein levels in the renal cortex and medulla of OP rats. The effect might or might not be directly mediated by PPARγ activation.

Finally, the mechanisms involved in the reduction of oxidative stress by pioglitazone are largely unknown. A recent article has demonstrated the ability of both pioglitazone and rosiglitazone to reduce nitrotyrosine formation in a mouse model of rheumatoid arthritis. Yet another mechanism suggested by our data seems to be via downregulation of gp91phox and p47phox protein expression. The results showed upregulation of these 2 NAD(P)H oxidase subunits in OP versus OR and control groups without treatment. The pioglitazone-treated OP group had reduced expression of both proteins in the kidney compared with their nontreated counterparts. It is difficult to conclude whether the effect of pioglitazone on NAD(P)H oxidase subunits was mediated by PPARγ activation. One recent article indicated that activation of PPARγ in macrophages results in downregulation of p47phox and attenuation of the respiratory burst. Also, p47phox was identified in a variety of renal epithelial, mesangial, and vascular cells and was shown to be increased in prehypertensive spontaneously hypertensive rats. Therefore, the latter could be a key target involved in regulation of superoxide production by PPARγ agonists in both renal and vascular cells.
Perspectives

This study shows for the first time the in vivo regulation of PPARγ expression and activity in the kidney by a high-fat diet and pioglitazone treatment. Also, to the best of our knowledge, this is the first report on the ability of pioglitazone, possibly via a PPARγ ligand–dependent mechanism, to prevent renal oxidative stress and to improve NO production in diet-induced obese rats. This effect is possibly achieved by upregulation of renal eNOS and nNOS, as by-as-yet-unknown mechanisms, as well as by reduced superoxide production because of down-regulation of NAD(P)H oxidase subunits. Owing to the ability of pioglitazone to prevent hypertension and increase sodium excretion, further studies will be important to determine the major cell types involved and the exact mechanisms by which PPARγ could regulate sodium and water handling by the kidney.

Acknowledgments

This study was supported by National Institutes of Health grants HL68726 and HD38240 and a postdoctoral fellowship from the Mid-Atlantic Affiliate of the American Heart Association to Dr Anca Dobrian.

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*Hypertension.* 2004;43:48-56; originally published online November 24, 2003;
doi: 10.1161/01.HYP.0000103629.01745.59

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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