Cardioprotective effects of rosiglitazone are associated with selective overexpression of type 2 angiotensin receptors and inhibition of p42/44 MAPK

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Molavi, Behzad, Jiawei Chen, and J. L. Mehta. Cardioprotective effects of rosiglitazone are associated with selective overexpression of type 2 angiotensin receptors and inhibition of p42/44 MAPK. Am J Physiol Heart Circ Physiol 291: H687–H693, 2006. First published March 31, 2006; doi:10.1152/ajpheart.00926.2005.—Current evidence points to renin-angiotensin system as a key mediator in ischemia-reperfusion injury. Rosiglitazone, a peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand, has recently been shown to confer cardioprotection against ischemia-reperfusion in animal models. We sought to examine the expression of ANG II receptors during PPAR-γ-mediated cardioprotection. Male Sprague-Dawley rats (nondiabetic) were fed either regular rat chow (control diet group, n = 9) or rosiglitazone-rich diet (rosiglitazone-rich diet group, n = 9) and were subjected to 1 h of myocardial ischemia followed by 1 h of reperfusion. A third group of rats had only thoracotomy and pericardiotomy and served as a sham control group (n = 9). Hemodynamics, infarct size, and expression of ANG II type 1 and type 2 receptors (AT1 and AT2) were measured in all groups. There was a 58% reduction of infarct size in the rosiglitazone-rich diet group (P < 0.01 vs. control diet group). Increased myocardial expression of AT1 receptors in the ischemic-reperfused myocardium was attenuated in the rosiglitazone-rich diet group (P < 0.05 vs. control diet group). Importantly, myocardial AT2 mRNA and protein expression were significantly increased (by >100-fold) in the rosiglitazone-rich diet group (P < 0.05). These changes were accompanied by inhibition of p42/44 MAPK in the rosiglitazone-rich diet group, while the Akt1 expression, believed to mediate insulin sensitization, remained similar in all three groups. The cardioprotective effects of rosiglitazone against myocardial ischemia-reperfusion injury are independent of its insulin-sensitizing properties and are associated with significant overexpression of AT2 receptors along with inhibition of p42/44 MAPK.

Peroxosme proliferator-activated-γ receptors; angiotensin receptors; ischemia-reperfusion injury; rosiglitazone

THE IMPLEMENTATION of immediate reperfusion strategies to salvage the ischemic myocardium has reduced mortality and morbidity in the setting of acute coronary syndromes (8) but has also brought the phenomenon of ischemia-reperfusion injury into focus. This phenomenon can present clinically as acute congestive heart failure or malignant ventricular arrhythmias after successful revascularization. Previous studies have shown that ANG II type 1 (AT1) receptors are overexpressed during ischemia-reperfusion and exert deleterious effects on myocardial contractility (37, 38). Although a few studies have failed to show any cardioprotective effects by angiotensin receptor blockers, the bulk of current evidence suggests that AT1 receptor blockade is associated with cardioprotection (28, 33). In addition, AT1 receptor blockade during ischemia-reperfusion injury in rat hearts is associated with cardioprotection, increased AT2 expression and signaling through inositol 1,4,5-trisphosphate (IP3) pathway activation (22). The IP3 pathway has also been described to be a component of insulin signaling and glucose utilization in the cardiomyocytes (11). In fact, some studies indicate that ANG II receptor activation leads to insulin resistance in vasculature through IP3 pathway inhibition (9, 18, 31). In addition, recent clinical trials of renin-angiotensin-aldosterone system (RAAS) modulation in coronary artery disease and heart failure suggest a significant reduction (14–30%) in incidence of diabetes with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (26, 27, 41). Therefore, a cross-talk between RAAS and glucose utilization pathways appears to exist.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor family (2, 29). There are three categories of PPARs: α, γ, and δ (β) (17). The cardioprotective effects of PPAR-γ agonists, which are currently used to treat Type 2 diabetes, against ischemia-reperfusion injury have been recently demonstrated in diabetic and nondiabetic animals (40, 42). We sought to explore the molecular aspects of the cardioprotective effects of PPAR-γ ligand activation against ischemia-reperfusion injury with particular regard to changes in ANG II receptor expression.

MATERIALS AND METHODS

Ischemia-Reperfusion Experiments

Male Sprague-Dawley rats (nondiabetic) were fed either regular rat diet (control diet group, n = 9) or the PPAR-γ ligand rosiglitazone-rich diet (rosiglitazone, 3 mg·kg⁻¹·day⁻¹ for 7 days; n = 9) and were subjected to ischemia-reperfusion. Rosiglitazone is a highly selective PPAR-γ agonist and has been shown to increase PPAR-γ transcriptional activity in a dose-dependent manner by up to 35-fold (1, 15). A third group of rats was subjected to only thoracotomy and pericardiotomy and served as a sham control group (n = 9). The study protocol was reviewed and approved by the animal care and use committee at the Central Arkansas Veterans Healthcare System. The methodology for the induction of ischemia-reperfusion injury has been described earlier (5). Briefly, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), incubated, and mechanically ventilated on a positive-pressure respirator with room air. Body temperature was maintained at 36–37°C with a heating blanket. A small plastic catheter was placed into the left ventricle via the right carotid artery with the other end of the catheter attached to a transducer. The transducer was connected to a physio-
logical probe (Grass 7400, AstroMed) to monitor contractility as measured by positive and negative first derivative of left ventricular pressure (±dP/dt). Arterial pressure was monitored by placing a catheter in the right common iliac artery. Heart rate was recorded by surface ECG. A left thoracotomy was performed via the fifth intercostal space to expose the heart. The main left coronary artery (LCA) was ligated 2–3 mm proximal to the origin of first diagonal branch with a 6-0 silk suture. Induction of ischemia was indicated by elevation of ST segment on ECG and cyanosis of anterior wall of the left ventricle. Hearts of rats on both the control diet and the rosiglitazone-rich diet were subjected to 60 min of ischemia followed by 60 min of reperfusion (release of ligature). In the sham control group of rats, thoracotomy and pericardiectomy were performed, and silk thread was passed around LCA without ligation. Blood samples for determination of plasma glucose levels were withdrawn at the time of the procedure in all three groups of rats.

**Determination of Infarct Size**

At the end of ischemia-reperfusion, the hearts were quickly removed and mounted on a Langendorff apparatus and then flushed with saline for 60 s. The LCA was reoccluded with a snare, and Evans blue dye was infused into the perfusate to mark the area at risk. The heart was then frozen and cut into 2 mm transverse slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in 0.2 M PBS buffer (pH 7.4) for 5 min (32). The area of infarcted tissue (TTC-negative tissue) and the area at risk were determined by planimetry.

**Measurement of Plasma Glucose Levels**

Plasma glucose levels were quantitatively measured by the glucose oxidase method by using a Beckman Glucose Analyzer-2 machine.

**Immunohistochemical Localization of ANG II Receptors**

Immunohistochemical characterization of AT1 and AT2 receptors was made in formalin-fixed samples (n = 4) in each group. (Seven to ten discontinuous slides were used in every heart.) A biotin-streptavidin detection system with dianamino benzidine as the chromogen (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunohistochemical localization of AT1 and AT2 receptors. The primary antibody for AT1 was rabbit polyclonal IgG (200 µg/ml), which recognizes the NH2-terminal extracellular domain and is reactive against rat AT1 receptors (Santa Cruz Biotechnology). The primary antibody for AT2 receptors was a rabbit polyclonal IgG (200 µg/ml), which recognizes the COOH-terminal domain of AT2 receptor (amino acids 221–363) and is reactive against rat AT2 receptors (Santa Cruz Biotechnology). The primary rabbit polyclonal antibodies to AT1 and AT2 were subsequently diluted 1:50. Rabbit serum was used instead of primary antibody for the negative control specimens. A second anti-rabbit antibody was used with the biotin-streptavidin detection system.

**Real-Time PCR for ANG II Receptor Gene Expression**

RNA extraction and cDNA preparation. The methodology for RNA extraction has been described earlier (6). Briefly, 500 mg of myocardial tissue was homogenized in 300 µl of warmed Ultraspec RNA extraction reagent (Biotec Laboratories, Houston, TX). The samples were then centrifuged and mixed with chloroform and subsequently isoproopyl alcohol to fully precipitate RNA. The samples were subsequently centrifuged at 15,000 rpm, and the resulting pellets were washed twice with 70% ethanol. After brief centrifugation, the remaining fluid was drained, and the pellets were allowed to dry for 5 min. The pellets were subsequently rehydrated with diethyl pyrocarbonate water, and the resultant RNA was quantitated by spectrophotometry and light absorption at 260 nm (A260) and 280 nm (A280) wavelength. The A260/A280 ratio of 1.8–2.0 was considered optimal. A high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) was used to produce cDNA copies of myocardial RNA. In brief, 50 µl of RNA solution was mixed with 50 µl of 2× RT master mix (Applied Biosystems-Foster City, CA) containing 2× RT buffer, 2× dNTP mixture, 2× random primers, multiScribe RT at 50 U/µl and RNase-free water. The solution was mixed and incubated at 25°C for 10 min and 37°C for 2 h.

**RT-PCR for AT1 and AT2 receptors.** Applied Biosystems (Foster City, CA) 7700 Sequence Detection System and TaqMan probes were used for real-time PCR (RT-PCR). The AT1 (reference sequence: AK087228), AT2 (reference sequence: AK086334) and ribosomal 18S (control) primers and TaqMan probe, as well as the TaqMan Universal Master Mix, were purchased form Applied Biosystems. The cDNA templates (9 µl in each tube) were mixed with 10 µl of TaqMan Universal Master Mix and 1 µl of 20× working stock of AT1, AT2, or ribosomal 18S genes, bringing the final volume to 20 µl. The PCR conditions were 50°C for 2 min and 95°C for 10 min followed for 40 cycles. Thereafter, the data were processed by using the accompanying software, and amplification plots for fluorescence responses were obtained for AT1 and AT2 genes. The ribosomal 18S gene expression was used as the housekeeping gene to normalize the data.

**Western Blot Analyses for AT1, AT2, p42/44 MAPK, and Akt1**

Monoclonal mouse antibody to p42/44 MAPK was purchased from Cell Signaling Technology (Beverly, MA). This antibody detects endogenous levels of p44 and p42 MAP kinase (ERK1 and ERK2) phosphorylated at threonine 204 and tyrosine 204. This antibody does not cross-react with the corresponding phosphorylated residues of either stress-activated protein kinase/JNK or p38 MAP kinase and therefore selectively binds the activated form of 42/44 MAPK. Monoclonal mouse antibody to Akt1, which recognizes amino acids 345–480 of this kinase, was purchased form Santa Cruz Biotechnology. Monoclonal rabbit antibodies to AT1 and AT2 receptors, which recognize the NH2 terminus and COOH terminus of type 1 and type 2 angiotensin receptors in rats and humans were purchased from Santa Cruz Biotechnology. Equal amounts of protein (50 µg) from the hearts in each group were separated by 10% SDS-PAGE and transferred to nitrocellulose filters (Sigma). After incubating in blocking solution (5% nonfat milk, Sigma), membranes were incubated in a buffer containing 2.0 µg/ml of the antibodies to phosphorylated p42/44 MAPK AT1, AT2, and Akt1. Anti-mouse and anti-rabbit alkaline phosphatase-conjugated antibodies were used as a secondary antibody at 1:5,000 dilutions. Protein bands of interest were detected by chemiluminescence system, and relative intensities of protein bands were analyzed by MSF-300G Scanner, as described earlier (16).

**Statistical Analysis**

All data are expressed as means ± SD. Means of different groups were compared with ANOVA followed by Student’s t-test for paired samples.
and unpaired observations. A *P* value of <0.05 was considered significant. Morphological and immunohistochemical differences were assessed by three different individuals observing the same slides blindly.

**RESULTS**

There was no significant difference in plasma glucose levels at the time of the experiment (nonfasting levels) among different groups of rats [285 ± 125 vs. 315 ± 148 vs. 305 ± 179 mg/dl for sham, control diet, and rosiglitazone-rich diet groups, respectively; *P* = nonsignificant (NS)]

**Hemodynamic Findings**

Baseline hemodynamic profiles (mean arterial pressure and ±dP/dt) were similar in the sham control, the control diet group exposed to ischemia-reperfusion, and the rosiglitazone-rich diet group of rats exposed to ischemia-reperfusion (Table 1).

Ischemia caused a decline in mean arterial pressure and ±dP/dt in the control-diet and rosiglitazone-rich diet groups of rats. The ischemia-induced reduction in cardiac function was similar in the rats fed the rosiglitazone-rich diet or the control-diet groups; however, the decline in cardiac hemodynamics during reperfusion was 75% less in the rats fed the rosiglitazone-rich diet (*P* < 0.01 vs. control-diet fed rats) (Table 1).

**Infarct Size**

The area of the myocardium at risk was determined by planimetry in five hearts in both the control-diet and rosiglitazone-rich diet groups. The means at risk in both groups were similar (64 ± 4 vs. 61 ± 6% of left ventricle, *P* = 0.42).

After ischemia-reperfusion, the infarct size was 39 ± 6% of the area at risk in the control-diet group and 16 ± 9% in the rosiglitazone-rich diet group of rats, which reflects a 58% reduction in infarct size in the rosiglitazone-rich diet group compared with the control diet group (*P* = 0.002) (Fig. 1).

**Myocardial ANG II Receptor Expression**

The expression of ANG II AT1 and AT2 receptors in the sham-control group was low with minimal staining for both receptors. Ischemia-reperfusion was associated with slight up-regulation of AT1 receptors in the control-diet group of rats exposed to ischemia-reperfusion, and this upregulation was ameliorated in the rosiglitazone-rich diet group of rats (Fig. 2). More importantly, hearts from the rosiglitazone-rich diet group and exposed to ischemia-reperfusion showed an intense overexpression of myocardial AT2 receptors (vs. hearts from the control diet group) (Fig. 3). The immunohistochemical findings were subsequently verified with Western blotting, indicating suppression of AT1 protein expression with rosiglitazone supplementation in the diet. The AT1 protein expression in the
control diet group exposed to ischemia-reperfusion did increase but did not reach a statistically significant different level compared with the sham group. The rosiglitazone-rich diet group also demonstrated a significant increase in AT2 protein expression (Fig. 4).

AT1 and AT2 receptor gene expression was determined by RT-PCR. Note that myocardial ischemia-reperfusion caused an eightfold upregulation of the AT1 mRNA in the control diet group of rats, and this effect was attenuated in the rats fed rosiglitazone-rich diet (Fig. 5). In contrast, AT2 receptor mRNA expression was ~100-fold higher in the hearts from rosiglitazone-rich diet group than in those from the control diet group ($P < 0.02$) (Fig. 6). These data support the observations on immunostaining and Western blotting for AT1 and AT2 receptors.

**Myocardial p42/44 MAPK and Akt1 Expression**

Ischemia-reperfusion induced a significant rise in p42/44 MAPK levels in the hearts from the control diet group. The ischemia-reperfusion-induced increase in p42/44 MAPK activity was significantly attenuated in the hearts from rats on the rosiglitazone-rich diet group ($P < 0.05$) (Fig. 7).

The Akt1 expression was similar in the hearts from the sham control, control diet, and rosiglitazone-rich diet groups, indicating no significant alterations with ischemia-reperfusion or type of diet ($P > 0.5$) (Fig. 8).

**DISCUSSION**

This study confirms the results of previous studies showing the protective effect of PPAR-γ ligand rosiglitazone against myocardial ischemia-reperfusion injury (40). The protective effect was evident by attenuation of decrease in $\Delta\Delta p/dt$ and reduction in infarct size in the rats given rosiglitazone-rich diet.

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**Fig. 3.** Representative examples of AT2 receptor expression in the myocardium (supplied by the left coronary artery) by immunostaining. A: negative control with normal rabbit serum in the sham-control group. B: negative control in the regular-rat chow group. C: negative control in the rosiglitazone-rich diet group. D–F: staining with AT2 antibody in a heart from a sham-control group rat (D), from the ischemic-reperfused region of a rat from the control diet group (E), and from the ischemic-reperfused region from a rosiglitazone-rich group rat (F). Note that the AT2 receptor immunostaining is markedly increased in rosiglitazone-rich group.

**Fig. 4.** Myocardial AT2 (A) and AT1 (B) protein expression in the 3 experiment groups (sham control, control diet, and rosiglitazone-rich diet). *$P < 0.05$. 

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**H690 ROSIGLITAZONE, ISCHEMIA-REPERFUSION, AND ANG II RECEPTORS**

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Activation of AT1 receptors has been implicated in the pathogenesis of ischemia-reperfusion injury (12, 38). In fact, previous studies have indicated that AT1 receptor antagonists are cardioprotective in models of ischemia-reperfusion injury (20, 24). Likewise, antisense directed at the AT1 receptor mRNA protects the hearts from the adverse effects of ischemia-reperfusion (38). It has been suggested that a part of the beneficial effect of AT1 receptor blockers may be mediated by upregulation of AT2 receptors (14, 35). Matsubara (18a) have reviewed the mechanisms of AT2 receptor-mediated cardioprotection, which involve stimulation of protein tyrosine or serine/threonine phosphatases in a Gi protein-dependent manner. Some studies have indicated that AT2 receptor overexpression may have deleterious effects on left ventricular systolic function and lead to heart failure (36). However, these studies were performed in a chronic setting on transgenic mice that were evaluated by echocardiography at 18 wk and did not involve acute ischemia-reperfusion injury. Yet multiple other studies have confirmed a cardioprotective role for AT2 receptor activation during ischemia-reperfusion and after acute myocardial infarction (3, 39). Diep et al. (7) showed that PPAR-γ ligands are capable of modulating ANG II receptor expression in rats, and the resultant PPAR-γ-mediated vasodilation is in part due to modulation of AT2 receptor expression. We wondered if rosiglitazone-mediated cardioprotection would involve alterations in ANG II receptor expression. Indeed, we observed that rosiglitazone-fed rats had >100-fold increase in AT2 receptor mRNA and a marked reduction in AT1 receptor mRNA. The results of alterations in mRNA expression were also evident on immunostaining and Western blot assays of the reperfused myocardium.

At the molecular level, the deleterious effects of ANG II have been shown to be mediated through AT1 receptors and subsequent downstream activation of stress-responsive MAPKs (23). MAPK activation has been shown to be a pivotal pathway in the setting of myocardial ischemia-reperfusion injury and can exert deleterious effects on myocardial contractile performance (25, 30). The upregulation of AT2 receptors in our study was associated with inhibition of p42/44 MAPK. p42/44 MAPKs are extracellular signal-regulated kinases that mediate a wide spectrum of intracellular signaling pathways, including those initiated by AT1 receptor activation (34).
inhibition of p42/44 MAPK through AT2 receptor can potentially antagonize the deleterious effects of ANG II and AT1 receptor activation on the myocardium during ischemia-reperfusion. This hypothesis is supported by the work of Nakajima and colleagues (23), who found that AT2 receptor overexpression by gene transfer prevented neointimal proliferation in balloon-injured rat carotid arteries. They were also able to demonstrate that the antiproliferative effects of AT2 overexpression were accompanied by a significant reduction of MAPK (23). Inhibition of p42/44 MAPK may relate to AT2 receptor-mediated activation of protein tyrosine phosphatase, which dephosphorylates the threonine 202 and tyrosine 204 residues of p42/44 MAPK (10).

It is noteworthy that we also observed that the AT1 receptor expression was attenuated in rosiglitazone-fed rats. It is unclear from these studies whether the cardioprotective effect of rosiglitazone is primarily due to inhibition of AT1 or upregulation of AT2 receptor expression. The 100-fold increase in AT2 receptor mRNA levels in the rosiglitazone-rich diet group of rats draws attention to the strong association between cardioprotective effects of PPAR-γ ligands and upregulation of AT2 receptors. Yet this study cannot establish a direct causal relationship between upregulation of AT2 receptors and cardioprotection, and further experiments with genetically modified species expressing various levels of angiotensin receptors are needed in this regard. The precise basis of rosiglitazone-mediated upregulation of AT2 downregulation of AT1 receptors is not known but may be related to a reduction in free radical generation during ischemia-reperfusion. In other studies, we have shown that rosiglitazone decreases P67 NADPH oxidase in ischemic-reperfused hearts (21). In recent studies, we have demonstrated that another PPAR-γ ligand, pioglitazone, decreases superoxide anion generation in endothelial cells exposed to oxidative stress (19). Furthermore, pioglitazone also decreases adhesion of inflammatory cells to the activated endothelial cells, a common denominator in ischemia-reperfusion-mediated tissue injury (19). AT1 receptor activation has been shown to activate NADPH oxidase in a variety of tissues (4). We propose that PPAR-γ ligands break the positive feedback between AT1 receptor activation and reactive oxygen species generation. However, it should be noted that the cardioprotective effects of PPAR-γ ligands may also stem from several other simultaneous modulations in signaling mechanisms, including NF-kB expression, which has been shown to be downregulated by PPAR-γ ligands (21). Nonetheless, the most important information in this study is the dramatic increase in AT2 receptor expression at the transcriptional level in the rats on rosiglitazone-rich diet.

In addition to the modifications in AT1 and AT2 receptor expression, the cardioprotective effects of rosiglitazone in our study could be explained by its glucoregulatory effects. Although insulin sensitization and glucose-insulin infusion during acute myocardial infarction have been a time-honored strategy in myocardial preservation, it is unlikely that the cardioprotective effects of rosiglitazone in this study were related to improved glucose homeostasis. First, the animals in our study were nondiabetic, and there was no significant difference in plasma glucose levels among the sham-control, control diet, and rosiglitazone-rich diet groups. Second, the Akt expression was unaltered in all three groups of animals. Akt1 is an intracellular kinase that has been known to mediate insulin-sensitizing effects of rosiglitazone (13).

In summary, we show that the PPAR-γ ligand rosiglitazone protects the myocardium against reperfusion injury and reduces infarct size in a nondiabetic setting. The cardioprotective effects of rosiglitazone are accompanied by downregulation of AT1 and overexpression of AT2 receptors. The upregulation of AT2 receptors was accompanied by reduced activity of p42/44 MAPK in the rosiglitazone-rich diet group. However, there was no significant change in insulin signaling as evidenced by similar levels of Akt1 in all three groups.

REFERENCES


