Oral Intake of Rosiglitazone Promotes a Central Anti hypertensive Effect Via Upregulation of Peroxisome Proliferator-Activated Receptor-γ and Alleviation of Oxidative Stress in Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats


Abstract—Rosiglitazone, a synthetic ligand of transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ), possesses a blood pressure-lowering effect beyond insulin sensitizing and glucose lowering. Oxidative stress in rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of neurogenic vasomotor tone are located, contributes to neural mechanisms of hypertension. Activation of PPAR-γ protects against oxidative stress in RVLM by upregulation of mitochondrial uncoupling protein 2 (UCP2). We tested the hypothesis that oral intake of rosiglitazone exerts a central antihypertensive effect by ameliorating oxidative stress in RVLM via transcriptional upregulation of UCP2 after PPAR-γ activation. In adult spontaneously hypertensive rats but not normotensive Wistar-Kyoto rats, oral intake of rosiglitazone for 1 week resulted in vasodepression and a reduction in the vasomotor components of the systemic arterial pressure spectrum, our experimental index for sympathetic vasomotor tone. These antihypertensive effects of rosiglitazone in spontaneously hypertensive rats were abrogated by microinjection bilaterally into RVLM of PPAR-γ small interfering RNA. Oral intake of rosiglitazone also upregulated UCP2 and ameliorated the heightened superoxide anion level in RVLM of spontaneously hypertensive rats. Protection against oxidative stress in RVLM by rosiglitazone was abrogated by PPAR-γ small interfering RNA or by antisense oligonucleotide against ucp2 mRNA. Gene knockdown of ucp2 in RVLM also reversed the antihypertensive effect of rosiglitazone. These results suggest that oral intake of rosiglitazone promotes a central antihypertensive effect by decreasing sympathetic vasomotor activity through a PPAR-γ-dependent protection against oxidative stress in RVLM via transcriptional upregulation of the mitochondrial UCP2. (Hypertension. 2010;55:1444-1453.)

Key Words: peroxisome proliferator-activated receptor ■ thiazolidinedione ■ mitochondrial uncoupling protein ■ oxidative stress ■ blood pressure

Peroxisome proliferator-activated receptor (PPAR-γ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that plays an important role in the regulation of adipocyte differentiation and lipid and carbohydrate metabolism.1,2 Its well-characterized increase in insulin regulation of adipocyte differentiation and lipid and carbohydrate metabolism.1,2 Its well-characterized increase in insulin sensitizing and glucose lowering. Oxidative stress in rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of neurogenic vasomotor tone are located, contributes to neural mechanisms of hypertension. Activation of PPAR-γ protects against oxidative stress in RVLM by upregulation of mitochondrial uncoupling protein 2 (UCP2). We tested the hypothesis that oral intake of rosiglitazone exerts a central antihypertensive effect by ameliorating oxidative stress in RVLM via transcriptional upregulation of UCP2 after PPAR-γ activation. In adult spontaneously hypertensive rats but not normotensive Wistar-Kyoto rats, oral intake of rosiglitazone for 1 week resulted in vasodepression and a reduction in the vasomotor components of the systemic arterial pressure spectrum, our experimental index for sympathetic vasomotor tone. These antihypertensive effects of rosiglitazone in spontaneously hypertensive rats were abrogated by microinjection bilaterally into RVLM of PPAR-γ small interfering RNA. Oral intake of rosiglitazone also upregulated UCP2 and ameliorated the heightened superoxide anion level in RVLM of spontaneously hypertensive rats. Protection against oxidative stress in RVLM by rosiglitazone was abrogated by PPAR-γ small interfering RNA or by antisense oligonucleotide against ucp2 mRNA. Gene knockdown of ucp2 in RVLM also reversed the antihypertensive effect of rosiglitazone. These results suggest that oral intake of rosiglitazone promotes a central antihypertensive effect by decreasing sympathetic vasomotor activity through a PPAR-γ-dependent protection against oxidative stress in RVLM via transcriptional upregulation of the mitochondrial UCP2. (Hypertension. 2010;55:1444-1453.)

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The blood pressure–lowering effect of TZD has been attributed to a reduction in ROS production in the vascular smooth muscle cells\(^{11,17}\) and endothelial cells. Whether TZD, in particular, rosiglitazone, possesses a central antihypertensive action is currently unknown. A recent report\(^{19}\) on the neuroprotective effect of cerebral ischemia-induced brain infarction after oral intake of the PPAR-\(\gamma\) activator suggests a mechanism by which the PPAR-\(\gamma\) activator may exert its protective effect within the brain. Two pieces of observations further suggest that the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of vasomotor tone are located,\(^{20}\) presents itself as a reasonable candidate brain target for this PPAR-\(\gamma\) ligand. First, oxidative stress at the RVLM plays a pivotal role in the neural mechanism of hypertension.\(^{15,16,21}\) Second, transcriptional stress at the RVLM plays a pivotal role in the neural mechanism of hypertension.\(^{15,16,21}\) Second, transcriptional upregulation of UCP2 by PPAR-\(\gamma\) reduces arterial pressure by ameliorating oxidative stress in the RVLM.\(^{22}\) It follows that, on oral intake, rosiglitazone may exert a central antihypertensive effect by ameliorating oxidative stress in the RVLM via transcriptional upregulation of UCP2 after PPAR-\(\gamma\) activation. The present study was carried out to validate this hypothesis.

**Materials and Methods**

All of the experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee. Adult (12-week–old) male spontaneously hypertensive rats (SHRs) or age-matched normotensive Wistar-Kyoto (WKY) rats, purchased from the Experimental Animal Center of the National Applied Research Laboratories (Taiwan) were used. Animals received oral administration of rosiglitazone, amlodipine, or saline (once per day between 12:00 and 1:00 PM via gastric gavage) for 1 week. The key experimental procedures included measurement of systemic arterial pressure (SAP) and heart rate (HR) under conscious condition by radiotelemetry.\(^{22,23}\) computation of power density of vasomotor components (0 to 0.8 Hz) of the SAP spectrum as an index for sympathetic vasomotor tone,\(^{24,25}\) detection of \(\text{O}_2^-\)\(^{22,23}\) microinjection into RVLM of small interfering RNA (siRNA) or antisense oligonucleotide (ASON), determination of mRNA or protein expression by real time RT-PCR or Western blot,\(^{22,23}\) and characterization of metabolic indices.\(^{26}\) Detailed procedures are provided in the expanded Materials and Methods section in the online Data Supplement at http://hyper.ahajournals.org.

**Statistic Analysis**

Data are expressed as mean±SEM. The statistical software SigmaStat (SPSS Inc) was used for data analysis. One-way or 2-way ANOVA with repeated measures was used, as appropriate, to assess group means, to be followed by the Scheffé multiple-range test for post hoc assessment of individual means. \(P\) value <0.05 was considered statistically significant.

**Results**

**Oral Intake of Rosiglitazone Decreases SAP and Sympathetic Vasomotor Activity in SHRs**

Our first series of experiments established the critical premise that rosiglitazone exerts an antihypertensive effect. Compared with saline control, oral intake of rosiglitazone (20 mg · kg\(^{-1}\) · day\(^{-1}\)) for 1 week resulted in a significant decrease in SAP in conscious SHRs that reached a maximum on day 7 after the commencement of treatment that was not found in WKY rats (Figure 1A). There was a concomitant reduction in power density of the vasomotor components of the SAP spectrum detected in animals maintained under propofol anesthesia on days 4 (Figure S1A, available in the online Data Supplement at http://hyper.ahajournals.org), 7 (Figure 1B), or 10 (Figure S1A) after rosiglitazone. Oral intake of rosiglitazone for 1 week, on the other hand, induced a minimal effect on HR in SHRs or WKY rats (Figure 1C). In control experiments, oral intake for 1 week of a calcium channel blocker amlodipine (16 mg · kg\(^{-1}\) · day\(^{-1}\), which exhibits minimal central antihypertensive effects,\(^{27}\) resulted in a hypotensive response that was comparable in the temporal profile to that of rosiglitazone in SHRs (Figure S1B), without a concomitant reduction in sympathetic vasomotor tone.
Upregulation of PPAR-γ in the RVLM Underlies Central Antihypertensive Effects of Oral Intake of Rosiglitazone in SHRs

Our third series of experiments further ascertained a causal role for the upregulation of PPAR-α or -γ in the RVLM in its central antihypertensive effect of oral rosiglitazone. Microinjection bilaterally into the RVLM of PPAR-γ siRNA (0.1, 0.5, or 1.0 nmol) on day 4 after the commencement of rosiglitazone treatment abrogated the antihypertensive effect promoted by the PPAR-γ activator in a concentration-dependent manner (Figure 3C). The effectiveness of PPAR-γ siRNA (0.5 nmol) was confirmed by realtime RT-PCR and Western blot analyses, which showed a significant antagonism of rosiglitazone-induced upregulation of PPAR-γ mRNA and protein in the RVLM of treated SHRs (Figure S2A). PPAR-γ mRNA or protein in the RVLM of rosiglitazone-treated WKY rats was not changed, nor was it affected by PPAR-γ siRNA treatment (Figure S2B). Microinjection bilaterally into the RVLM of GW9662 (0.5 nmol), GW9471 (0.5 nmol), or PPAR-γ siRNA (1 nmol), on the other hand, had no effect on basal mean SAP (MSAP) in SHRs or WKY rats (data not shown). Treatment with PPAR-γ siRNA also did not affect the pressor response induced by microinjection bilaterally into the RVLM of L-glutamate (2 nmol), evaluated under anesthetic conditions 0, 12, 24, or 48 hours after silencing PPAR-γ transcription in SHRs (Figure S3).

Amelioration of Oxidative Stress in the RVLM of SHRs After Oral Intake of Rosiglitazone

As observed previously,23 tissue level of O$_2^-$ in the RVLM of SHRs, which contributes to heightened sympathetic vasomotor tone and hypertension,21,23 was significantly higher than that in WKY rats (Figure 4A). This augmented O$_2^-$ production in the RVLM of SHRs was reversed to a level comparable to that in WKY rats after oral intake of rosiglitazone, detected by the lucigenin-chemiluminescence method (Figure 4A) on day 4, 7, or 10, or by dihydroethedium fluorescence method (Figure 4B) on day 7 after the initiation of oral rosiglitazone. This induced alleviation of the elevated O$_2^-$ level in RVLM, measured on day 7, was antagonized by microinjection bilaterally of PPAR-γ siRNA (1 nmol) into the RVLM 3 days before measurement. Oral intake of rosiglitazone alone or together with PPAR-γ gene knockdown in RVLM by siRNA, on the other hand, exerted minimal effect on the O$_2^-$ level in the RVLM of WKY rats (Figure 4A and 4B). Likewise, amiodipine ingestion for 1 week did not alter the O$_2^-$ level in the RVLM of SHRs (Figure 4B).
products of the mitochondrial translation of an array of downstream target genes, of which protein
expression is a transcription factor to regulate expression of mitochondrial UCPs. We
detected basal expression of UCP2, 3, and 4, but not UCP1 or 5, in the RVLM of SHRs and WKY rats (Figure 5). The expression of UCP2 in the RVLM of SHRs, as well as UCP3 in the RVLM of SHRs or WKY rats, measured on day 7 after the beginning of oral treatment with rosiglitazone, was significantly upregulated (Figure 5). Oral intake of amlodipine, on the other hand, did not affect UCP2, 3, or 4 expression in the RVLM of SHRs or WKY rats.

Oral Intake of Rosiglitazone Protects Against Oxidative Stress in the RVLM in SHRs Via Transcriptional Upregulation of Mitochondrial UCP2

To establish a causal role for the upregulated mitochondrial UCP2 and UCP3 in rosiglitazone-induced protection against oxidative stress in the RVLM of SHRs, an ASON against UCP2 or UCP3 was microinjected bilaterally into the RVLM on day 4, and O$_2^{-}$ production was measured on day 7 after the commencement of rosiglitazone ingestion. Compared with sense oligonucleotide controls, pretreatment with UCP2 but not UCP3 ASON abrogated the reduction in O$_2^{-}$ production detected by the lucigenin-chemiluminescence method (Figure 6) after oral intake of rosiglitazone. Gene knockdown of uc$p2$ or uc$p3$ in the RVLM of WKY rats, on the other hand, did not affect the tissue level of O$_2^{-}$, which was not altered by rosiglitazone treatment.

Transcriptional Upregulation of Mitochondrial UCP2 in the RVLM Is Involved in a Central Antihypertensive Effect Induced by Oral Intake of Rosiglitazone in SHRs

Microinjection bilaterally on day 4 into the RVLM of an ASON against UCP2 (Figure 7A) but not UCP3 (Figure 7B) significantly antagonized the reduction in SAP or the power density of vasomotor components of SAP signals (Figure 7C) in SHRs, measured on day 7 after the initiation of oral rosiglitazone administration. UCP2 or UCP3 ASON alone had no discernible effect on baseline MSAP or the sympathetic vasomotor activity of SHRs. Real-time RT-PCR and Western blot analyses confirmed that UCP2 or UCP3 ASON effectively blunted the mRNA and protein expression of individual UCP (Figure S4).

Rosiglitazone Elicits a Central Antihypertensive Effect in SHRs via Activation of the PPAR-γ/UCP2 Pathway in RVLM

Our eighth series of experiments further ascertained that rosiglitazone elicits a central antihypertensive effect in SHRs via activation of the PPAR-γ/UCP2 pathway in RVLM. Microinjection bilaterally into the RVLM of a dose of rosiglitazone (1 nmol) that was ineffective by the intravenous route induced a significant reduction in MSAP (Figure S5A) measured under conscious condition by radiotelemetry and power density of the vasomotor components of SAP signals (Figure S5B) recorded in anesthetized SHRs or WKY rats. These cardiovascular depressive responses of rosiglitazone in SHRs, which were greater in amplitude and longer in duration
than those in WKY rats, were significantly blunted after gene knockdown of PPAR-γ in the RVLM by siRNA (1 nmol) or UCP2 ASON (100 pmol; Figure S5C and S5D). Microinjection of the same dose of rosiglitazone into areas outside the confine of the RVLM (eg, dorsolateral or ventromedial medulla, lateral reticular nucleus, or lateral paragigantocellular nucleus) resulted in minimal changes in the MSAP in SHRs or WKY rats (data not shown).

Effect of Oral Intake of Rosiglitazone on Body Weight, Food Intake, Food Efficiency, and Plasma Level of Insulin

Our final series of experiments measured various metabolic indices to confirm that, as a therapeutic agent for type 2 diabetes mellitus,4 the dose of rosiglitazone used in the present study was within its therapeutic window. Oral intake of rosiglitazone (20 mg · kg⁻¹ · day⁻¹) for 1 week significantly increased food intake and body weight in SHRs and WKY rats (Table S1). Although the food intake and body weight gain were greater in SHRs than in WKY rats, the increase in food efficiency, defined as grams of body weight gain per 100 grams of food ingested, was comparable in both strains of animals. SHRs were hyperinsulinemic when compared with age-matched WKY rats. Oral intake of rosiglitazone significantly reduced the fasting plasma insulin level in SHRs.

Discussion

The present study provided novel results to demonstrate that, on oral intake, rosiglitazone induces a central antihypertensive effect in SHRs via depression of sympathetic vasomotor activity through cellular mechanisms that involve amelioration of oxidative stress by upregulation of transcription factor PPAR-γ and increase in expression of mitochondrial UCP2 in the RVLM. To our knowledge, ours is the first report on the cellular and molecular mechanisms of a central antihypertensive effect of the oral administration of rosiglitazone.

Rosiglitazone is a synthetic, high-affinity PPAR-γ ligand of the TZD class3 clinically used for management of type 2 diabetes mellitus because of its properties to enhance insulin-mediated glucose uptake and to improve insulin sensitivity.1,4 Accumulating evidence indicates that rosiglitazone also possesses potent blood pressure–lowering effects in patients or
animal models of both diabetes mellitus/metabolic and non-diabetes/metabolic syndrome,7–11 although the underlying mechanisms are not fully elucidated. All hitherto implicated mechanisms, including the ability to inhibit the proliferation of arterial smooth muscle cells,29 prevent augmented vasoconstriction to vasoactive compounds,30 protect endothelial-dependent vasodilation,31 or increase in NO production/availability,32 depict peripheral effects of rosiglitazone. However, controversy still exists on the role of PPAR-γ in vascular smooth muscle cells and endothelial cells in hypertension. It is noted that endothelium-specific PPAR-γ knockout mice do not manifest an apparent hypertensive phenotype unless otherwise induced by challenges such as high-salt water or a high-fat diet.33 Moreover, vascular smooth muscle

Figure 5. Representative gels and densitometric analysis of results from Western blots showing amount of UCP 1, 2, 3, 4, and 5 protein relative to mitochondrial cytochrome c oxidase (Mt. COX) detected from RVLM on day 7 after commencement of oral intake of rosiglitazone (RSG; 20 mg·kg⁻¹·day⁻¹), amlodipine (AMLO; 16 mg·kg⁻¹·day⁻¹), or saline for 1 week in SHRs or WKY rats. Values are mean±SEM of quadruplicate analyses on samples pooled from 4 to 5 animals in each group. *P<0.05 vs baseline control group, and #P<0.05 vs WKY group in the Scheffe multiple-range analysis.
cell-selective PPAR-γ gene deletion results in a paradoxical hypotensive phenotype.34

Results from the present study thus provided novel insights into the neural mechanism of rosiglitazone-induced hypotension. We found that oral intake of rosiglitazone for 1 week significantly decreased SAP in SHRs by reducing sympathetic vasomotor tone. This vasodepressor response may represent a central antihypertensive action of rosiglitazone at the RVLM for 4 reasons. First, the spectral components of SAP signals measured in the present study take origin from RVLM, and their power densities reflect the prevailing sympathetic vasomotor activity to the blood vessels.24 We are aware that changes in baroreflex or anesthesia may influence the power of those low-frequency spectral signals. In this regard and in line with a recent report,37 our preliminary results showed that SHRs exhibited an insignificant change in baroreflex after oral intake of rosiglitazone for 1 week. Furthermore, propofol infusion in our acute experiments provides satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation.38

Second, oral intake of amlodipine, a calcium channel blocker that induces hypotension via peripheral vasodila-

tion,37 resulted in a reduction in SAP in SHRs that was temporally similar to that elicited by rosiglitazone, without affecting sympathetic vasomotor tone. Third, blockade of PPARs in the RVLM by siRNA or its receptor antagonist

Figure 6. Temporal changes in tissue level of $O_2^-$ in RVLM of SHRs or WKY rats on day 7 after commencement of oral intake of rosiglitazone (RSG; 20 mg·kg$^{-1}$·day$^{-1}$) for 1 week, alone or with additional treatment of microinjection bilaterally into RVLM of ASON or sense (SON) oligonucleotide against UCP2 or UCP3 mRNA on day 4 after rosiglitazone. Values are mean±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs baseline group, and #P<0.05 vs rosiglitazone group in the Scheffé multiple-range analysis.

Figure 7. Temporal changes in MSAP (A and B) or power density of vasomotor components of SAP spectrum (C) detected on day 7 after commencement of oral intake of rosiglitazone (RSG; 20 mg·kg$^{-1}$·day$^{-1}$) or saline for 1 week, alone or with additional treatment of microinjection bilaterally into RVLM of UCP2 (A) or UCP3 (B) ASON on day 4 after rosiglitazone. Values are mean±SEM, n=5 to 7 animals per group, or quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs saline group, and #P<0.05 vs rosiglitazone group in the Scheffé multiple-range analysis. Horizontal bar indicates the duration of drug treatment and arrow denotes time during which microinjection was executed.
appreciably abrogates the antihypertensive effect on oral intake of rosiglitazone. Fourth, microinjection of rosiglitazone directly into the RVLM, at an intravenous dose that did not promote cardiovascular responses, duplicated the antihypertensive effects of oral intake of rosiglitazone in SHRs. The exhibition of hypotension that was greater in amplitude and longer in duration in SHRs compared with WKY rats further implicates a deficiency in the endogenous ligands to PPAR-γ in the RVLM under hypertensive condition. The site specificity of the central antihypertensive effect of rosiglitazone was confirmed when microinjection of this PPAR activator to areas adjacent to the confines of RVLM elicited minimal cardiovascular responses. Comparable magnitudes of vasodepression induced by rosiglitazone in WKY rats and Sprague-Dawley rats further confirm the specificity of rosiglitazone effects in SHRs. It should be noted, however, that our results could not rule out the effect of PPAR activation on blood vessels or insulin sensitivity in the RVLM or exclude the participation of other brain sites in the central antihypertensive action of rosiglitazone.

Mechanistically, our results revealed that the central antihypertensive effect of rosiglitazone depends on activation of PPAR-γ in the RVLM. Oral intake of rosiglitazone upregulated PPAR-α and PPAR-γ protein expression in the RVLM of SHRs. This upregulation was not secondary to vasodepression, because SHRs that received oral intake ofamlodipine exhibited no comparable alteration in PPAR-γ or PPAR-α protein expression in the RVLM. Antagonism by pharmacological blockade with specific inhibitors or gene knockdown by siRNA further ascertained that selective activation of PPAR-γ in the RVLM underpins the central antihypertensive action of rosiglitazone. The lack of effect of siRNA treatment on 1-glutamate–induced pressor response further ascertained its specificity in silencing the PPAR-γ mRNA. Our results also indicated that, despite being upregulated, PPAR-α in the RVLM is not involved in the central antihypertensive action of rosiglitazone. The role of PPAR-α and its agonist on blood pressure is still controversial. The PPAR-α agonist docosahexaenoic acid prevents the development of hypertension in angiotensin II–treated young rats, but another agonist, clofibrate, is ineffective under normal salt condition. In randomized, controlled trials, the PPAR-α agonist fenofibrate shows minor hypotensive effects in patients with type 2 diabetes mellitus. The reason that expression of PPARs in SHRs, the likelihood of a strain-dependent effect of this PPAR activator is greatly reduced. The observation that siRNA affected PPAR-γ mRNA or the protein level in RVLM of SHRs only when activated by rosiglitazone is intriguing and suggests that the upregulated PPAR-γ was primarily derived from newly transcribed mRNA. This suggestion is supported by the lack of effects of PPAR-γ siRNA treatment in rosiglitazone-treated WKY rats, which did not exhibit upregulation of PPAR-γ mRNA or protein in the RVLM.

Oxidative stress in the RVLM plays a pivotal role in the neural mechanism of hypertension. An increase in \(O_2^{--}\) production in RVLM induces sympathoexcitation and hypertension in normotensive WKY rats. Overexpression of adenovirus encoding superoxide dismutase in the RVLM, on the other hand, ameliorates oxidative stress and promotes vasodepression in SHRs. Our present results revealed that protection via activation of PPAR-γ against oxidative stress in the RVLM of SHRs contributes to the blood pressure–lowering effect by rosiglitazone. We observed that suppression of the augmented tissue level of \(O_2^{--}\) in RVLM by oral intake of rosiglitazone but not amlodipine exhibited a temporal profile that coincided with its elicited cardiovascular depressive responses in SHRs. More importantly, silencing PPAR-γ transcription with siRNA rendered rosiglitazone ineffective. The design of the present study, however, did not allow us to decipher whether the \(O_2^{--}\)–producing cells affected by PPAR-γ siRNA were bulbospinal RVLM cells. The lack of effect of PPAR-γ siRNA on basal the \(O_2^{--}\) level in RVLM of WKY rats implicates a minor role for endogenous PPAR-γ in the maintenance of redox balance, hence baseline SAP and sympathetic vasomotor tone, under normotensive conditions.

Glutamatergic and angiotensinergic transmission in the RVLM plays a significant role in the hypertensive state of SHRs. Of interest is that transcriptional upregulation of mitochondrial UCP2 by PPAR-γ in response to elevated \(O_2^{--}\) induced by angiotensin II at glutamate-sensitive loci in the RVLM plays an active role in feedback regulation of ROS production. Intriguingly, the present study revealed that this novel mechanism also underpins the antioxidant effect of rosiglitazone on oral administration. Oral intake of this PPAR-γ activator resulted in upregulation of UCP2 in the RVLM of SHRs, as well as UC3 in the RVLM of SHRs and WKY rats. Because gene knockdown of UCP3 did not affect the antioxidant effect of rosiglitazone, we reason that the protective effect of this PPAR-γ activator against oxidative stress in the RVLM of SHRs is primarily attributable to the upregulation of mitochondrial UCP2. This notion is corroborated by observations that knockdown of the \(ucp2\) but not \(ucp3\) gene rendered rosiglitazone ineffective in reducing sympathetic vasomotor activity and SAP.

UCP2 is a homologue of the UCP protein family of mitochondrial anion transporters that uncouple ATP synthesis from oxidative phosphorylation by causing proton leakage across the mitochondrial inner membrane, leading to energy dissipation and heat production. More importantly, the resultant decrease in proton electrochemical gradient across the inner mitochondrial membrane mitigates the production of mitochondrial-derived ROS. It has been demonstrated that gene knockdown of \(ucp2\) increases mitochondrial membrane potential and \(O_2^{--}\) production in murine endothelial cells. Adenovirus-mediated overexpression of UCP2, on the other hand, decreases \(O_2^{--}\) generation in human aortic endothelial cells. We reported recently that mitochondrial UCP2 is involved in ROS homeostasis in the RVLM. Our present results further suggest that oral intake of rosiglitazone promotes a long-term antihypertensive effect via transcriptional upregulation of mitochondrial UCP2 to protect brain RVLM tissues against oxidative stress. Because PPAR-γ and UCP2 are also expressed in brain endothelial and vascular smooth mus-
cle cells, the contribution of these sources of PPAR-γ/UCP2 signaling to the observed protection against oxidative stress by oral intake of rosiglitazone remains to be determined.

We confirmed that the dosage of rosiglitazone used in the present study was within its therapeutic range by showing a significant increase in insulin sensitivity in SHRs. Nonetheless, as a therapeutic agent against type 2 diabetes mellitus, these observations may argue for the antihypertensive effect of rosiglitazone to be secondary to its antihyperinsulinemic action. This argument is deemed unlikely because P465L mutation in PPAR-γ in mice elicits a significant increase in blood pressure while maintaining normal blood glucose and insulin levels. In addition, we observed recently that oral intake of a low dose (10 mg·kg⁻¹·day⁻¹) of rosiglitazone for 14 days induced a central antihypertensive effect without increasing insulin sensitivity (unpublished data). We also noted that food intake in SHRs that received rosiglitazone treatment was greater than that in WKY rats. Because feed efficiency was comparable between these 2 strains of animals, the central antihypertensive action of orally administered rosiglitazone may not be related to its dietary effect.

**Perspectives**

Recent studies suggest that peripheral administration or oral ingestion of PPAR activators may exert central antihypertensive effects. Because of the pivotal role of oxidative stress in the neural mechanism of hypertension, our demonstration that PPAR-γ-dependent transcriptional upregulation of the mitochondrial antioxidant UCP2 in RVL primates the central antihypertensive action of oral intake of rosiglitazone therefore opens a new vista for novel therapeutic strategies against hypertension. More importantly and in the spirit of translational medicine, our results are of particular clinical relevance because a significant proportion of patients with diabetes mellitus/metabolic syndrome is hypertensive, and rosiglitazone is widely prescribed as an insulin sensitizer.

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**Disclosures**

None.

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Oral Intake of Rosiglitazone Promotes Central Antihypertensive Effect via Upregulation of PPAR-γ and Alleviation of Oxidative Stress in RVLM of SHR

Expanded Methods

Animals
Adult, male spontaneously hypertensive rats (SHR, 10-11 week old, n = 238) or age-matched normotensive Wistar-Kyoto (WKY, n = 234) rats were purchased from the Experimental Animal Center of the National Applied Research Laboratories, Taiwan. Animals were maintained under temperature control (24±0.5°C) and 12-hour light-dark cycle (lights on during 08:00-20:00), and provided with standard chow and tap water ad libitum. Animals were allowed to acclimatize for at least 7 days prior to experimental manipulations. All experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee.

Drug treatment scheme
SHR or WKY rats were housed singly and dosed orally (once per day between 12:00 and 13:00 via gavage) with a PPARγ agonist, rosiglitazone (RSG, 20 mg • kg⁻¹ • day⁻¹), a calcium channel blocker, amlodipine (AMLO, 16 mg • kg⁻¹ • day⁻¹) or saline for 1 week. Rosiglitazone was dissolved in saline and administered orally as a suspension. The body weight and daily food intake were measured before, during and after the 1-week treatment period.

Measurement of Systemic Arterial Pressure and Heart Rate by Radiotelemetry
Systemic arterial pressure (SAP) and heart rate (HR) were measured in rats under conscious condition using a radiotelemetry system (Data Sciences International, Minneapolis, MN) according to the procedures described previously.¹⁻³ For long-term recording that continued for a maximum of 27 days, the averaged mean SAP (MSAP) recorded for 60 minutes every day between 13:00 and 15:00 was used as the daily value. For experiments of short-term recording that continued for 24 hours, the MSAP recorded every 2 hours was averaged.

Evaluation of Sympathetic Vasomotor Tone
For the measurement of sympathetic vasomotor tone, the femoral artery was cannulated and pulsatile SAP was recorded according to procedures described previously.¹⁻³ In brief, the arterial catheter was connected to a pressure transducer (Becton, Dickinson and Company, Franklin Lakes, NJ) and in turn to a pressure processor amplifier (Biopac Systems, Goleta, CA) via which SAP signals were amplified and filtered (frequency range: DC to 100 Hz). The SAP signals were simultaneously subject to on-line power spectral analysis as described previously.⁴ We were particularly interested in the very low-frequency (0-0.25 Hz) and low-frequency
(0.25-0.8 Hz) components in the SAP spectrum. The sum of the power density of these two spectral components recorded continuously for 30 minutes was used to reflect the prevailing sympathetic vasomotor tone. During the experiment, animals received continuous intravenous infusion of propofol (20 mg \( \cdot \) kg\(^{-1} \cdot \) h\(^{-1} \); Zeneca, Macclesfield, UK), which provided satisfactory anesthetic maintenance while exerting minimal effect on MSAP or HR and preserving the capacity of central cardiovascular regulation as indicated by the power of the vasomotor components.

**Microinjections of Test Agents into RVLM**

Microinjection bilaterally of test agents into RVLM was carried out according to procedures described previously. In brief, animals were maintained under propofol anesthesia during the entire microinjection procedure. The skull overlying the cerebellum and caudal medulla oblongata was removed to expose the dorsal surface of the brain. Microinjection was carried out with a glass micropipette (external tip diameter: 50-80 µm) connected to a 0.5-µl Hamilton (Reno, NV) microsyringe. The stereotaxic coordinates for RVLM were: 4.5 to 5.0 mm posterior to lambda, 1.8 to 2.1 mm lateral to midline and 8.0 to 8.5 mm below dorsal surface of cerebral cortex. These coordinates were selected to cover the extent of ventrolateral medulla in which functionally identified sympathetic premotor neurons reside. Functional location of RVLM neurons was carried out at the beginning of each experiment by the elicitation of a transient increase in SAP (15-20 mmHg) on microinjection of L-glutamate (2 nmole) to bilateral RVLM at 10-minute intervals. Subsequent microinjection bilaterally of test agents was executed sequentially and stereotaxically by another micropipette to the identified pressor loci in RVLM. As a routine, a total volume of 50 nl was delivered over 2-3 minutes to allow for complete diffusion of the test agents. Accuracy of the microinjection was confirmed by histological examination of the injection sites. The chemicals used included L-glutamate, rosiglitazone, PPAR\(\gamma\) inhibitor, GW9662, PPAR\(\alpha\) inhibitor, GW6471, specific PPAR\(\gamma\) small interfering RNA (siRNA, AUUUGUCUGUUGUCUUUCC) or scrambled RNA (scRNA, GGAAGACAACAGACAAAAU) (Ambion, Austin, TX), antisense (ASON) or sense (SON) oligonucleotide against mitochondrial uncoupling protein 2 or 3 (UCP2 or UCP3; Genemed Biotechnologies, San Francisco, CA). The extent of PPAR\(\gamma\) or UCP knockdown by siRNA or ASON was quantified by reverse-transcription (RT) real-time polymerase chain reaction (PCR). All the test agents were dissolved in artificial cerebrospinal fluid (aCSF), except rosiglitazone, GW9662 and GW6471, which were dissolved by 0.5% DMSO.

For experiments in which GW9662 or GW6471 was microinjected bilaterally into RVLM, animals were maintained under propofol anesthesia on day 7 after the
commencement of oral ingestion of rosiglitazone. Changes in the hemodynamic parameters were recorded for 60 minutes postinjection. For treatment of PPARγ siRNA, UCP2 ASON or their controls, test agent was microinjected bilaterally into RVLM on day 4 after the commencement of rosiglitazone in animals that were anesthetized with a bolus injection of pentobarbital sodium (50 mg/kg). Following the completion of microinjection, the wound was closed in layers and animals were allowed to recover in individual cages. SAP and HR were measured for another 3 days under conscious condition using radiotelemetry.

Collection of Tissue Samples from Ventrolateral Medulla and Hypothalamus
At various time intervals after experimental treatment, rats were killed with pentobarbital sodium and perfused intracardially. The brain was rapidly removed and immediately frozen on dry ice. Medulla oblongata covering the RVLM was blocked between 0.5 and 1.5 rostral to the obex, which served as the anatomical landmark, and forebrain tissue between optic chiasm and entry of the optic tract was collected for the hypothalamus. The medullary tissue was then sectioned into 3 slices at 300- to 350-µm in thickness by a cryostat, and both sides of the ventrolateral medulla covering RVLM (approximately at 1.5- to 2.5-mm lateral to the midline and medial to the spinal trigeminal tract) from each slice were collected by micropunches with an 1-mm inner diameter burr. A total of 6 punches for RVLM were taken from each animal. The hypothalamic tissue was collected similarly by micropunches using the third ventricle as an anatomical guide. Medullary or hypothalamic tissues collected form the same experimental groups were pooled and stored at -80°C prior to mRNA or protein analysis.  

RNA Isolation and Reverse-Transcriptase Real-Time Polymerase Chain Reaction
Total RNA from RVLM was isolated with TRIzol reagent according to the manufacturer’s protocol. All RNA isolated was quantified by spectrophotometry and the optical density 260/280 nm ratio was determined. Expression of PPARγ or UCP mRNA in the isolated RVLM was evaluated by RT real-time quantitative PCR. RT reaction was performed using a SuperScript Preamplification System (Invitrogen) for the first-strand cDNA synthesis. Real-time PCR for amplification of cDNA was performed by a LightCycler® (Roche Diagnostics, Mannheim, Germany). PCR reaction for each sample was carried out in duplicate for all cDNA and for the GAPDH control. The PCR mixture (total volume 20 µL), which was prepared with nuclease free water, contained 2 µL of LightCycler® FastStart DNA Master SYBR Green 1 (Roche Diagnostics), 3 mM MgCl₂ and 5 µM of each primer, together with 5
µL of purified DNA or negative control. The primer pairs for amplification of UCP2 cDNA (GenBank accession no. U69135) were 5’-TCCCCTGTTGATGTGGTCAA-3’ for the forward primer, and 5’-CAGTGACCTGCAGCTGGTA-3’ for the reverse. Primer pairs for UCP3 cDNA (GenBank accession no. AB010742) were 5’-CTTACACATCATCTAGGAGAAAGTT-3’ for the forward primer, and 5’-TCCAAAGGGCAGGACAAAAATGTAA-3’ for the reverse. Primer pairs for PPARγ (GenBank accession no. NM_181024) were 5’-AGGAGAACGATTCGGCTGAAGC-3’ for the forward primer, and 5’-AAAGGCGGTTGTTGTTGTC-3’ for the reverse. GAPDH cDNA (GenBank accession no. NM017008) were 5’-GCCAAAAGGGTCATCATCTC-3’ for the forward primer, and 5’-GGCCATCCACAGTCTTCT-3’ for the reverse. The amplification protocol for cDNA was a 10-minute denaturation step at 95°C for polymerase activation, a "touch down" PCR step of 10 cycles consisting of 10 seconds at 95°C, 10 seconds at 65°C and 30 seconds at 72°C, followed by 40 cycles consisting of 15 seconds at 95°C, 10 seconds at 55°C, and 30 seconds at 72°C. After slow heating (0.1°C per second) the amplified product from 65°C to 95°C to generate a melting temperature curve, which serves as a specificity control, the PCR samples were cooled to 40°C. The PCR products were subsequently subjected to agarose gel electrophoresis for further confirmation of amplification specificity. Fluorescence signals from the amplified products were quantitatively assessed using the LightCycler® software program (version 3.5). Second derivative maximum mode was chosen with baseline adjustment set in the arithmetic mode. The relative change in UCP2 mRNA expression was determined by the fold-change analysis, in which Fold change = 2^{-[\Delta \Delta \text{Ct}]}, where \Delta \Delta \text{Ct} = (Ct_{UCP/PPARγ} - Ct_{GAPDH})_{oligonucleotide treatment} - (Ct_{UCP/PPARγ} - Ct_{GAPDH})_{control}. Note that Ct value is the cycle number at which fluorescence signal crosses the threshold.

**Western Blot Analysis**

RVLM tissue was homogenized in isolation buffer. Protein extract from RVLM homogenates was used to analyze PPARα, PPARβ/δ, PPARγ, UCP1-5 expression by Western blot, as described previously. In brief, proteins (50 µg) from RVLM were separated by using 10-12% SDS-PAGE and transferred to PVDF membrane. The primary antiserum used for Western blot analysis included a rabbit monoclonal antiserum against PPARγ, PPARβ/δ or PPARγ (1:1000; Cell Signaling), a rabbit polyclonal antiserum against UCP1-5 (1:1000; Calbiochem) or β-tubulin (1:1000; Cell Signaling, Danvers, MA). This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoReserach, West Grove, PA). Specific antibody-antigen complex was detected
using an enhanced chemiluminescence Western Blot detection system (NEN Life Science Products, Boston, MA). The amount of detected protein was quantified by Photo-Print Plus software (ETS Vilber-Lourmat, France), and was expressed as the ratio to β-tubulin protein or mitochondrial cytochrome c oxidase.

**Measurement of Superoxide Anion**

Production of O$_2^•^−$ in the RVLM was measured by the lucigenin-enhanced chemiluminescence (ECL) assay$^{1-3}$ or by microinjection of dihydroethidium (DHE; Molecular Probes, 1 µmol/L) into RVLM,$^{13}$ as described previously. ECL signal was detected by a luminometer (Sirius Luminometer, Berthold, Germany) and DHE fluorescence was visualized by a laser confocal microscope equipped with a krypton/argon laser (Olympus Fluorview 300, Japan).

**Serum Determinations**

Plasma glucose concentration was measured by the glucose oxidase method with the ACCU-CHEK® Advantage II glucose analyzer (Roche Diagnostics, Indianapolis, IN).$^{11}$ Plasma insulin was measured by ELISA (Mercodia AB, Uppsala, Sweden).$^{14}$
References


### ADDITIONAL TABLE

**Table S1.** Final body weight, cumulative food intake, feed efficiency, and plasma levels of glucose and insulin in SHR or WKY rats that received oral intake of rosiglitazone (RSG, 20 mg • kg\(^{-1}\) • day\(^{-1}\)), amlodipine (AMLO, 16 mg • kg\(^{-1}\) • day\(^{-1}\)) or saline for 7 days.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHR</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>RSG</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>295±8</td>
<td>329±14</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>23±6</td>
<td>37±4(^\star),(^\dagger)</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>106±5</td>
<td>124±4(^\star),(^\dagger)</td>
</tr>
<tr>
<td>Feed efficiency (%)(^a)</td>
<td>22±3</td>
<td>30±2(^\star)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>8.1±0.3</td>
<td>8.0±0.4</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>194±18(^\dagger)</td>
<td>136±14(^\star)</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n = 6-8 rats per group. \(^*\)P<0.05 vs. saline group, \(^\dagger\)P < 0.05 vs. WKY rats.

\(^a\)Calculated as grams body weight gain per 100 g food ingested and expressed in %.
Figure S1. Effects of oral intake of rosiglitazone (RSG; 20 mg • kg⁻¹ • day⁻¹), amloidipine (AMLO; 16 mg • kg⁻¹ • day⁻¹) or saline for 1 week on changes in power density of vasomotor components of systemic arterial pressure (SAP) spectrum recorded on day 4 or 10 in anesthetized spontaneously hypertensive rats (SHR) or Wistar-Kyoto (WKY) rats (A) or time-course changes in mean SAP (MSAP) recorded from conscious (B) SHR or WKY rats by radiotelemetry. Values are means ± SEM, n = 4 to 6 animals per group. *P < 0.05 versus corresponding saline group in the Scheffé multiple range analysis. Data on MSAP from RSG- or saline-treated animals are duplicated from Figure 1A for comparison.
Figure S2. Real-time polymerase chain reaction results in fold changes of PPARγ mRNA expression or representative gels (insets) or densitometric analysis of protein expression level of PPARγ detected from RVLM of SHR (A) or WKY rats (B) on day 7 after commencement of oral administration of rosiglitazone (20 mg · kg⁻¹ · day⁻¹) or saline for 1 week with additional treatment of microinjection bilaterally into RVLM of PPARγ small interfering RNA (1.0 nmole) on day 4 after rosiglitazone. Values are means ± SEM of quadruplicate analyses on samples pooled from 4 to 5 animals in each group. *P < 0.05 versus baseline group, and †P < 0.05 versus RDG group in the Scheffé multiple-range analysis.
Figure S3: Maximal changes in MSAP evoked by microinjection bilaterally into RVLM of L-glutamate (2 n mole) at 0, 12, 24, or 48 hours after microinjection of PPARγ siRNA (1 n mole) into bilateral RVLM. Values are means ± SEM, n = 4 to 5 animals in each group. No significant difference among groups in one-way ANOVA.
Figure S4: Temporal change in mRNA expression and representative gels (inset) or
densitometric analysis of protein expression level of UCP2 and UCP3 detected in
RVLM after microinjection bilaterally into RVLM (at time 0) of antisense (ASON) or
sense (SON) oligonucleotide against UCP2 or UCP3 mRNA or artificial cerebrospinal
fluid (aCSF). Values are means ± SEM of quadruplicate analyses on samples pooled
from 3 to 4 animals in each group. *P < 0.05 versus corresponding aCSF or baseline
(C) group in the Scheffé multiple range analysis.
Figure S5. Temporal changes in MSAP measured by radiotelemetry under conscious condition (A) or power density of vasomotor components of SAP spectrum recorded under anesthetized condition (B) at various time intervals over 24 hours after microinjection bilaterally of rosiglitazone (RSG; 1 nmole) into RVLM; or 24 hours after co-microinjection of RSG and PPARγ siRNA (1 nmole) or UCP2 ASON (100 pmole) into bilateral RVLM (C,D). Values are means ± SEM, n = 4 to 6 animals in each group *P < 0.05 versus DMSO group, and †P < 0.05 versus rosiglitazone group in the Scheffé multiple-range analysis.