Transcriptional Upregulation of Mitochondrial Uncoupling Protein 2 Protects Against Oxidative Stress-Associated Neurogenic Hypertension


Rationale: Mitochondrial uncoupling proteins (UCPs) belong to a superfamily of mitochondrial anion transporters that uncouple ATP synthesis from oxidative phosphorylation and mitigates mitochondrial reactive oxygen species production.

Objective: We assessed the hypothesis that UCP2 participates in central cardiovascular regulation by maintaining reactive oxygen species homeostasis in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons that maintain vasomotor tone located. We also elucidated the molecular mechanisms that underlie transcriptional upregulation of UCP2 in response to oxidative stress in RVLM.

Methods and Results: In Sprague–Dawley rats, transcriptional upregulation of UCP2 in RVLM by rosiglitazone, an activator of its transcription factor peroxisome proliferator-activated receptor (PPAR)γ, reduced mitochondrial hydrogen peroxide level in RVLM and systemic arterial pressure. Oxidative stress induced by microinjection of angiotensin II into RVLM augmented UCP2 mRNA or protein expression in RVLM, which was antagonized by coinjection of NADPH oxidase inhibitor (diphenyleneiodonium chloride), superoxide dismutase mimetic (tempol), or p38 mitogen-activated protein kinase inhibitor (SB203580) but not by extracellular signal-regulated kinase 1/2 inhibitor (U0126). Angiotensin II also induced phosphorylation of the PPARγ coactivator, PPARγ coactivator (PGC)-1α, and an increase in formation of PGC-1α/PPARγ complexes in a p38 mitogen-activated protein kinase–dependent manner. Intracerebroventricular infusion of angiotensin II promoted an increase in mitochondrial hydrogen peroxide production in RVLM and chronic pressor response, which was potentiated by gene knockdown of UCP2 but blunted by rosiglitazone.

Conclusions: These results suggest that transcriptional upregulation of mitochondrial UCP2 in response to an elevation in superoxide plays an active role in feedback regulation of reactive oxygen species production in RVLM and neurogenic hypertension associated with chronic oxidative stress. (Circ Res. 2009;105:886-896.)

Key Words: uncoupling proteins • mitochondrion • peroxisome proliferator-activated receptor • oxidative stress • blood pressure

Living organisms possess a variety of physiological protective mechanisms to counteract oxidative stress and to restore redox balance. Oxidative damage to cells that results from an imbalance of production over degradation of the reactive oxygen species (ROS), particularly superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), is associated with a variety of cardiovascular diseases, including heart failure, atherosclerosis, and hypertension. Of note is that overproduction of O$_2^-$ and H$_2$O$_2$ in the central nervous system contributes to neural mechanisms of hypertension by increasing sympathetic outflow to the peripheral blood vessels.

In addition to the degradative enzymes (eg, superoxide dismutase [SOD] and catalase) and low-molecular-weight antioxidants (eg, ascorbic acid and glutathione), the uncoupling proteins (UCPs) have emerged as important natural antioxidants in the maintenance of ROS homeostasis. UCPs belong to a superfamily 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 elicited by the UCPs mitigates mitochondrial ROS production. In mammals, 5 homologues, UCP1 to UCP5, have so far been cloned. Among them, dysfunction of UCP2 is suggested to be of considerable importance in cardiovas-
cular pathophysiology associated with oxidative stress. Knockdown of ucp2 gene increases mitochondrial membrane potential and ROS production in murine endothelial cells. Oxidative stress is also greater in the thoracic aorta of mice that are subject to bone marrow transplant derived from UCP2−/− mice. Adenovirus-mediated overexpression of UCP2, on the other hand, decreases ROS generation in human aortic endothelial cells.

In the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of vasmotor tone are located, emerging evidence supports a pivotal role for oxidative stress in neural mechanism of hypertension. On the other hand, whereas UCP2 is expressed in a wide array of tissues, including the brain, the physiological significance of its antioxidant role in central regulation of cardiovascular phenotypes is yet to be identified. The present study was undertaken to assess the hypothesis that mitochondrial UCP2 participates in central cardiovascular regulation by maintaining ROS homeostasis in RVLM. We also elucidated the molecular mechanisms that underlie transcriptional regulation of UCP2 expression in response to oxidative stress in RVLM.

**Methods**

An expanded Methods section is available in the Online Data Supplement http://circres.ahajournals.org.

**Animals**

Experiments were carried out in adult male Sprague–Dawley rats (280 to 305 g, n = 345) purchased from the Experimental Animal Center of the National Applied Research Laboratories, Taiwan. All experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee.

**PPARγ-Dependent Transcriptional Upregulation of Mitochondrial UCP2 in RVLM**

Upregulation of mitochondrial UCP2 expression in RVLM was elicited by microinjection bilaterally of an activator of its transcription factor PPARγ, rosiglitazone into this medullary site. Ligand specificity was ascertained by comicroinjection of a selective PPARγ inhibitor, GW9662. The temporal changes in UCP2 mRNA or protein expression in RVLM after rosiglitazone treatment were determined by real-time RT-PCR and Western blot analysis.

**Reduction in Oxidative Stress and Arterial Pressure After Transcriptional Upregulation of Mitochondrial UCP2 in RVLM**

The effect of transcriptional upregulation of UCP2 on mitochondrial level of H2O2 in RVLM and basal systemic arterial pressure (SAP) was examined at various time intervals after microinjection bilaterally into RVLM of rosiglitazone. H2O2 in the mitochondrial fraction was measured by an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probe, Eugene, Ore). SAP was determined under conscious conditions by radio telemetry. The specificity of PPARγ-induced transcriptional upregulation of UCP2 on H2O2 production in RVLM or SAP was confirmed by coadministration of GW9662.

**Transcriptional Upregulation of Mitochondrial UCP2 Expression in RVLM by O2−**

We reported previously that angiotensin II (Ang II) induces O2− production in RVLM via activation of NADPH oxidase. To evaluate the role of NADPH oxidase–derived O2− in the expression of mitochondrial UCP2, Ang II was microinjected bilaterally into RVLM, alone or together with a NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (1 nmol); a SOD mimetic, tempol (50 nmol); or by gene knockdown of p22phox or p47phox subunit of the NADPH oxidase with their respective antisense oligonucleotide (ASON) (100 pmol). The involvement of cytosolic or mitochondrial O2− in Ang II–induced mitochondrial UCP2 expression was further confirmed in animals that received gene transfer into RVLM of adenovirus encoding the cytosolic copper/zinc SOD (AdSOD1), mitochondrial manganese SOD (AdSOD2), or catalase (AdCAT).

**Involvement of Mitogen-Activated Protein Kinases in Transcriptional Upregulation of UCP2 in RVLM by O2−**

We reported previously that activation of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated protein kinase (ERK)1/2 by NADPH oxidase–derived O2− mediates the cellular responses to Ang II in RVLM. To examine the engagement of these 2 signaling molecules in transcriptional regulation of UCP2 by O2−, the temporal expression of UCP2 mRNA or protein in RVLM was determined on coadministration of p38 MAPK or ERK1/2 inhibitor SB203580 or U0126 bilaterally into RVLM with Ang II.

**Phosphorylation of PGC-1α by MAPKs and UCP2 Upregulation in RVLM**

We determined whether PGC-1α, a PPAR-interacting protein for the induction of UCP2, is a target of the activated MAPKs that leads to UCP2 upregulation in RVLM. Expression of phosphorylated PGC-1α or the formation of PGC-1α/PPARγ complex in nuclear protein extracted from RVLM was determined by Western blot.
analysis or immunoprecipitation followed by immunoblot, after microinjection bilaterally in RVLM of Ang II, alone or together with SB230580 or U0126.

Protective Role for UCP2 Against Ang II–Induced Pressor Response and Oxidative Stress in RVLM

We established a protective role for \( \text{O}_2^{\cdot-}/\text{H}_2\text{O}_2 \)-dependent transcriptional activation of mitochondrial UCP2 in central cardiovascular regulation and ROS homeostasis in RVLM by determining the effect of microinjection into the bilateral RVLM or intracisternal infusion (1 or 5 nmol) by osmotic minipump of rosiglitazone or an ASON against UCP2 on pressor responses to chronic intracerebroventricular (ICV) infusion of Ang II for 7 days and the elicited elevation in \( \text{H}_2\text{O}_2 \) in RVLM.

ROS Production and SAP After Transcriptional Downregulation of Mitochondrial UCP2 in RVLM

We investigated whether mitochondrial UCP2 in RVLM participates in tonic regulation of tissue level of ROS or SAP by determining the effect of microinjection bilaterally into RVLM of mitochondrial UCP2 protein detected in RVLM 12 hours after coadministration into the bilateral RVLM of rosiglitazone (5 nmol) and the PPAR\( \gamma \) antagonist GW9662 (C). Values are means ±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. Changes in UCP2 mRNA in A are presented in folds with reference to the sham group (designated 1). *\( P<0.05 \) vs vehicle control (C) group; #\( P<0.05 \) vs rosiglitazone group in the Scheffe multiple-range analysis. In this and Figures 4 and 5, protein expression of cytochrome c oxidase (Mt. COX) was determined as loading control for the mitochondrial extract.

Statistical Analysis

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

Results

Transcriptional Upregulation of Mitochondrial UCP2 in RVLM by PPAR\( \gamma \) Activator

The fundamental premise for UCP2 to play a role in ROS homeostasis in RVLM and cardiovascular regulation is its presence in this neural substrate. Results from our first series of experiments established this premise by showing a moderate basal expression of UCP2 mRNA or protein in the mitochondrial fraction of tissues from RVLM (Figure 1). We further showed that determined 6 or 12 hours postinjection, microinjection bilaterally into RVLM of rosiglitazone (1 or 5 nmol), an activator of the transcription factor PPAR\( \gamma \), significantly upregulated UCP2 mRNA (Figure 1A) or protein.
expression. Comicroinjection bilaterally into RVLM of a PPARγ antagonist, GW9662 (100 or 500 pmol), discernibly attenuated the induced upregulation of UCP2 protein expression by rosiglitazone (5 nmol) (Figure 1C). GW9662 alone, however, did not affect UCP2 protein level in RVLM.

**Transcriptional Activation of Mitochondrial UCP2 Decreases H2O2 Level in RVLM and Reduces SAP**

Our second series of experiments investigated whether transcriptional upregulation of UCP2 regulates tissue level of ROS in RVLM. Given the abundance of manganese SOD in the mitochondria that rapidly dismutates O2− to H2O2,20 the latter was used as an index of ROS production. Activation of UCP2 by rosiglitazone (1 or 5 nmol) significantly decreased H2O2 level in the mitochondrial fraction of RVLM (Figure 2A). The reduction in mitochondrial H2O2 detected 12 hours after administration of the PPARγ activator (5 nmol) was significantly reversed by comicroinjection into RVLM of GW9662 (100 or 500 pmol) (Figure 2B). Moreover, our third series of experiments showed that microinjection of rosiglitazone (1 or 5 nmol) bilaterally into RVLM resulted in a dose-related decrease in mean (M)SAP (Figure 3A), as measured by radiotelemetry under conscious conditions. This rosiglitazone-induced vasodepressor response, which was significant for at least 27 hours and returned to baseline 48 hours postinjection, was reversed by coadministration of GW9662 (500 pmol) into RVLM (Figure 3B). Transcriptional activation of UCP2, on the other hand, exerted minimal effect on HR (data not shown). Microinjection of rosiglitazone into areas adjacent to the confines of RVLM (eg, lateral reticular nucleus or spinal trigeminal nucleus) did not affect basal MSAP (+3.6±2.4 versus −4.5±3.1 mm Hg, n=5) or HR (−5±3 versus −7±4 bpm, n=5). Furthermore, we found in a separate experiment that pressor response induced by L-glutamate (2 nmol) in RVLM of anesthetized animals was comparable before and after microinjection of rosiglitazone (+16.3±2.5 versus +18.7±3.0 mm Hg, n=4) into the RVLM.

**NADPH Oxidase–Derived O2− Induces Transcriptional Upregulation of Mitochondrial UCP2 in RVLM**

In our search for cellular signals that upregulate UCP2, we noted that an elevated production of O2− induces mitochondrial UCP expression.21 Our previous work17 further indicated that activation of NADPH oxidase by Ang II is an important source of O2− in RVLM. The fourth series of experiments therefore investigated whether NADPH oxi-
dase‐derived O$_2^{−−}$ upregulates mitochondrial UCP2 transcription in RVLM. Microinjection bilaterally into RVLM of Ang II (100 pmol) resulted in upregulation of UCP2 mRNA (Figure 4A) or protein (Figure 4B) expression in RVLM that endured at least 24 hours. The Ang II‐induced increase in UCP2 mRNA or protein level, detected 12 or 24 hours postinjection, was significantly antagonized by coadministration into RVLM of DPI (1 nmol) or tempol (50 nmol) (B) or mitochondrial UCP2 protein in RVLM 12 hours after microinjection bilaterally into RVLM of Ang II (100 pmol) in rats that received gene knockdown by p22$^{phox}$ or p47$^{phox}$ ASON (100 pmol), administered bilaterally into the RVLM 24 hours before Ang II administration (C) or gene transfer of AdSOD1, AdSOD2, or AdCAT, administered bilaterally into the bilateral RVLM 7 (second bar in each group) or 14 (third bar in each group) days before Ang II administration (D). Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. Changes in UCP2 mRNA in A are presented in folds with reference to the sham group (designated 1). *P<0.05 vs vehicle control (C) group; #P<0.05 vs Ang II group in the Scheffé multiple‐range analysis.

Figure 4. Temporal changes in UCP2 mRNA expression (A) and representative gels (inset) or densitometric analysis of UCP2 protein level detected in RVLM after microinjection bilaterally into RVLM (at time 0) of Ang II (100 pmol), given alone or in combination with DPI (1 nmol) or tempol (50 nmol) (B) or mitochondrial UCP2 protein in RVLM 12 hours after microinjection bilaterally into RVLM of Ang II (100 pmol) in rats that received gene knockdown by p22$^{phox}$ or p47$^{phox}$ ASON (100 pmol), administered bilaterally into the RVLM 24 hours before Ang II administration (C) or gene transfer of AdSOD1, AdSOD2, or AdCAT, administered bilaterally into the bilateral RVLM 7 (second bar in each group) or 14 (third bar in each group) days before Ang II administration (D). Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. Changes in UCP2 mRNA in A are presented in folds with reference to the sham group (designated 1). *P<0.05 vs vehicle control (C) group; #P<0.05 vs Ang II group in the Scheffé multiple‐range analysis.

Involvement of p38 MAPK in UCP2 Upregulation in RVLM by NADPH Oxidase‐Derived O$_2^{−−}$

We reported previously$^{16,17}$ that phosphorylation of p38 MAPK and ERK1/2 via NADPH oxidase‐derived O$_2^{−−}$ mediates Ang II‐induced cellular responses in RVLM. Thus, our fifth series of experiments examined the roles of p38 MAPK or ERK1/2 in transcriptional upregulation of UCP2 by NADPH oxidase‐derived O$_2^{−−}$ in RVLM. Coadministration bilaterally into RVLM of p38 MAPK inhibitor, SB203580 (500 nmol), but not ERK1/2 inhibitor, U0126 (500 nmol), significantly antagonized the Ang II‐induced upregulation of UCP2 mRNA (Figure 5A) or protein (Figure 5B) expression. Neither inhibitor, however, affected the basal expression of UCP2 mRNA or protein in RVLM.

Phosphorylation of PGC‐1α by p38 MAPK is Essential for UCP2 Upregulation in RVLM

By being phosphorylated on activation of p38 MAPK,$^{22}$ the nuclear coactivator PGC‐1α, a PPAR‐interacting protein for the induction of UCP2,$^{18}$ presents itself as a reasonable interposing signal in the cascade of events that lead to
transcriptional upregulation of UCP2 by NADPH oxidase–
derived $O_2^-$ . Microinjection bilaterally into RVLM of Ang II (100 pmol) induced significant phosphorylation of PGC-1α at its threonine residue (Figure 6A) and an increase in the expression of PGC-1α/PPARγ complexes (Figure 6B), examined 15, 30, or 60 minutes posttreatment. Changes in UCP2 mRNA in A are presented in folds with reference to the sham group (designated 1). $^*$$P<0.05$ vs vehicle control group; $^#$$P<0.05$ vs Ang II group in the Scheffé multiple-range analysis.

Superoxide-Dependent Upregulation of Mitochondrial UCP2 in RVLM During Chronic Oxidative Stress

We reported previously that chronic infusion of Ang II induces p38 MAPK phosphorylation and oxidative stress in RVLM. Our seventh series of experiments therefore examined whether expression of mitochondrial UCP2 is regulated by $O_2^-$ under this condition of chronic oxidative stress. Compared with animals that received artificial cerebrospinal fluid (aCSF) infusion, protein expression of UCP2 in RVLM was significantly upregulated 7 days after animals were subject to ICV infusion of Ang II (100 μg·μL$^{-1}$·h$^{-1}$) (Online Figure II). This Ang II–induced UCP2 upregulation was blunted by microinjection bilaterally into RVLM of an Ang II type 1 receptor antagonist, losartan (2 nmol), or by gene transfer of AdSOD1 or AdSOD2 into RVLM (Online Figure II).

UCP2 Protects Against Chronic Oxidative Stress in RVLM and Hypertension

Our eighth series of experiments established a causal role for $O_2^-$–dependent transcriptional activation of mitochondrial UCP2 in central cardiovascular regulation and ROS homeostasis in RVLM. Consistent to our previous findings, chronic ICV infusion of Ang II (100 μg·μL$^{-1}$·h$^{-1}$) for 7 days elicited a gradual increase in MSAP that became significant between days 5 and 7 (Figure 7A). The Ang II–induced long-term pressor response was significantly potentiated in animals subject to microinjection bilaterally into RVLM of an Ang II type 1 receptor antagonist, losartan (2 nmol), or by gene transfer of AdSOD1 or AdSOD2 into RVLM (Online Figure II).

Figure 5. Temporal changes in UCP2 mRNA expression (A) and representative gels (inset) or densitometric analysis of UCP2 protein level (B) detected in RVLM after microinjection bilaterally into RVLM (at time 0) of Ang II (100 pmol), given alone or in combination with SB230580 (1 nmol) or U0126 (50 nmol). Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. Changes in UCP2 mRNA in A are presented in folds with reference to the sham group (designated 1). $^*$$P<0.05$ vs vehicle control group; $^#$$P<0.05$ vs Ang II group in the Scheffé multiple-range analysis.

Figure 6. Representative gels (inset) or densitometric analysis of results from immunoblot (IB) assays following immunoprecipitation (IP) showing temporal changes in expression of phosphorylated threonine residue or total PGC-1α (A) or association between PPARγ and PGC-1α (B) in nuclear fraction of samples from RVLM after microinjection bilaterally into RVLM (at time 0) of Ang II (100 pmol), given alone or in combination with aCSF, tempol (50 nmol), SB230580 (1 nmol), or U0126 (50 nmol). Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. $^*$$P<0.05$ vs aCSF-control (C) group; $^#$$P<0.05$ vs Ang II group in the Scheffé multiple-range analysis. ND indicates below detection limit.
expression in the mitochondrial but not cytosolic fraction from RVLM (Online Figure III), significantly attenuated the long-term pressor response (Figure 7B) that was reversed by UCP2 ASON treatment (Figure 7C). In addition, whereas gene knockdown of UCP2 (100 pmol) in RVLM enhanced, chronic infusion of rosiglitazone blunted the elevated tissue level of hydrogen peroxide detected in RVLM on day 7 after Ang II infusion (Figure 7D).

Endogenous UCP2 Does Not Tонically Affect Mitochondrial H2O2 Level in RVLM or SAP

We further used gene knockdown to decipher whether endogenous UCP2 in RVLM exerts a tonic regulatory effect on ROS level and cardiovascular functions. Microinjection bilaterally into RVLM of UCP2 ASON or SON (100 pmol) on day 4 after Ang II infusion (A), intracisternal infusion of rosiglitazone (1 or 5 μg·μL⁻¹·h⁻¹) immediately following ICV infusion of Ang II (B), or both (C) or mitochondrial level of hydrogen peroxide detected in RVLM on day 7 after ICV infusion of Ang II in rats treated with UCP2 ASON or SON or rosiglitazone (D). Values are means±SEM; n=4 to 7 animals in each group (A through C) or quadruplicate analyses on samples pooled from 4 to 6 animals in each group (D). *P<0.05 vs control or aCSF group; #P<0.05 vs Ang II group at corresponding time points in the Scheffe multiple-range analysis. Open arrows in A and C denote time during which microinjection was executed, and arrows in B and C denote the commencement of intracisternal infusion.

Transcriptional Up- or Downregulation of Mitochondrial UCP2 in RVLM Does Not Affect Mitochondrial Respiratory Enzyme Activity or ATP Production

Enzyme activity of mitochondrial respiratory complex I to V, NADH cytochrome c reductase, or succinate cytochrome c
reductase, as well as tissue ATP content in RVLM, assessed 12 or 24 hours after microinjection bilaterally into RVLM of rosiglitazone (5 nmol) or UCP2 ASON (100 pmol), was comparable to those from their corresponding control groups (Online Figure V).

Discussion

The present study provided novel evidence for an active role for mitochondrial UCP2 in RVLM in ROS homeostasis and central cardiovascular regulation under conditions of oxidative stress. Our results support the notion that mitochondrial UCP2 participates actively in a cellular adaptive program for feedback control of ROS production in RVLM and the associated neurogenic hypertension during oxidative stress. To our knowledge, this is the first report that unveils the functional significance of mitochondrial UCP2 in protection against brain oxidative stress-associated hypertension.

Several antioxidant systems are present in the cell to counteract oxidative effects and to restore redox balance. In addition to the documented ROS scavenging enzymes and low molecular weight antioxidants, whether mitochondrial UCP functions as a natural antioxidant defense against oxidative stress is still debatable. The present study provided novel in vivo evidence to support an antioxidant role for UCP2 and revealed its functional significance in neural control of cardiovascular phenotype. We found in RVLM, where mitochondrial oxidative stress plays a pivotal role in neural mechanism of hypertension, that the rosiglitazone-promoted decrease in H2O2 level was completely reversed by the PPARγ inhibitor GW9662 at a time point when MSAP was statistically insignificant from vehicle controls. Furthermore, observations of temporally correlated sequential up-regulation of UCP2 mRNA and protein by rosiglitazone at low dose, together with the antagonism of the prolong duration of rosiglitazone-induced hypotension by gene knockdown of UCP2, strongly support the notion that transcriptional upregulation of mitochondrial UCP2 underpins the reduction in mitochondrial H2O2 and the induced hypotension. These observations also deemed unlikely the possibility that the effects demonstrated are the consequences to a potential pleiotropic action of rosiglitazone. A less than 20% decrease in mitochondrial membrane potential by UCP is able to inhibit H2O2 production by more than 50%. Mitochondrial ROS production in the brain is also significantly lower in transgenic mice that overexpress UCP2 or after adenovirus-mediated gene transfer of UCP2 in aortic endothelial cells.

It is generally accepted that O2•− induces expression of UCPs, including UCP2, although the underlying cellular events remain largely unknown. In this regard, we reported previously that Ang II induces O2•− in RVLM via activation of NADPH oxidase. The present study took advantage of this cellular event to unveil the molecular mechanism that underlies the O2•−-dependent transcriptional upregulation of mitochondrial UCP2. We found that Ang II at a dose that induces O2•− production also increased UCP2 mRNA and protein expression in RVLM. The delayed antagonism of Ang II–induced UCP2 protein upregulation 24 hours after treatment with the NADPH oxidase inhibitor DPI or the SOD mimetic tempol, together with the blockade of Ang II–induced UCP2 mRNA expression 12 hours after the same treatment, again suggests that NADPH oxidase–derived O2•− regulates UCP2 protein expression at the transcriptional level. It is intriguing to note that the Ang II–induced UCP2 upregulation was significantly blunted by p22phox or p47phox ASON and gene transfer of AdSOD1, indicating that O2•− derived from extramitochondrial compartments may play an active role in transcriptional upregulation of mitochondrial UCP2 in RVLM. The extramitochondrial origin of the NADPH oxidase–derived O2•− was further confirmed by observations that p22phox and p47phox subunits are only found in the membranous and cytosolic fractions from RVLM. In myocardium of the failing heart, upregulation of UCP2 is closely associated with an increase in NADPH oxidase–derived O2•−. Furthermore, we found that dismutation of O2•− to H2O2 by the SOD1 transgene reversed, whereas conversion of H2O2 to H2O after overexpression of catalase by gene transfer did not affect the Ang II–induced UCP2 upregulation. These observations are interpreted to suggest that it is Ang II–induced O2•− but not H2O2 that induces the expression of mitochondrial UCP2 in RVLM. It is noteworthy that Ang II–induced UCP2 upregulation was also appreciably blunted by overexpression of SOD2 transgene in the RVLM. These results indicate that in addition to extramitochondrial sources, UCP2 expression may be regulated by O2•− generated in the mitochondrial compartment. We recognize that O2•− derived from xanthine oxidase increases the expression of UCP3, a homolog of UCP2 in skeletal muscle cells. Whereas its role in O2•−-dependent transcriptional upregulation of mitochondrial UCP2 in RVLM remains to be identified, we noted that that xanthine oxidase plays a minor role in Ang II–induced O2•− production.

The O2•−-induced UCP2 expression is not antagonized by inhibitors of mitochondrial ATP-sensitive potassium channel, adenine translocase, or mitochondrial permeability transition pores, suggesting that it is specific to the UCP. We reported previously that activation of p38 MAPK and ERK1/2 underlies the manifestation of Ang II–induced cellular responses in RVLM via NADPH oxidase–derived O2•−. Of those 2 MAPKs, the present study demonstrated that p38 MAPK, but not ERK1/2, acts as a key regulator of O2•−-dependent UCP2 transcription in RVLM under oxidative stress. We found that p38 MAPK inhibitor (SB203580), but not ERK1/2 inhibitor (U0126), significantly prevented the Ang II–induced upregulation of mitochondrial UCP2 mRNA or protein. Our results further indicated that the induction of UCP2 transcription by p38 MAPK is accomplished at 2 levels: phosphorylation of threonine residue of the PPARγ coactivator, PGC-1α, and enhancement of the ability of the latter to bind with PPARγ. Activated p38 MAPK directly phosphorylates PGC-1α at its threonine 262, serine 265, and threonine 298 residues. The resultant potentiation of PGC-1α docking to PPARγ leads to a conformational change that permits the induction of UCPs by its transcription factors. Our observations of an increase in formation of PGC-1α/PPARγ complex in nuclear extract from RVLM
after Ang II in a p38 MAPK–dependent manner are in line with those observations.

Functional evaluations in the present study further demonstrated, for the first time, that O$_2^-$–dependent transcriptional upregulation of mitochondrial UCP2 in RVLM plays an active role in feedback antagonism of oxidative stress and the associated hypertension. Production of O$_2^-$ and H$_2$O$_2$ via activation of NADPH oxidase in RVLM plays an important role in mediating chronic pressor response after ICV infusion of Ang II.$^{16}$ Using a loss-of-function approach, we found that Ang II–induced production of mitochondrial H$_2$O$_2$ and chronic pressor response were further augmented after gene knock down of UCP2 in RVLM by its ASON. Conversely, gain-of-function experiments revealed that transcriptional upregulation of mitochondrial UCP2 in RVLM by acute and chronic administration of the PPARγ activator rosiglitazone attenuated the tissue level of H$_2$O$_2$ and ameliorated the pressor response induced by Ang II. In addition, UCP2 gene knockdown attenuated the rosiglitazone-promoted inhibition of Ang II–induced hypertension. These results, together with our demonstration of a minor role for endogenous UCP2 at RVLM in regulation of basal ROS production and SAP, strongly support the notion that O$_2^-$–dependent transcriptional upregulation of mitochondrial UCP2 in RVLM participates actively in a feedback adaptive program to reduce ROS production in RVLM and hypertension associated with chronic oxidative stress. Rosiglitazone treatment was reported to reduce blood pressure in hypertensive patients$^{31}$ via reduction in ROS production in the vascular smooth muscle cells$^{32}$ or endothelial cells.$^{33}$ Our observations that rosiglitazone-induced vasodpressor response returned to baseline values 48 hours postinjection and that application of the PPARγ activator into areas adjacent to the confine of RVLM did not affect basal MSAP and HR suggest that the elicited cardiovascular responses were not the results of nonspecific neuro-cardiovascular toxicity. The elicitation of similar degree of pressor response by L-glutamate in RVLM before and after rosiglitazone treatment further indicates that the neural circuitry is functionally intact following transcriptional activation of UCP2. Because ICV Ang II infusion resulted in an increase in MSAP under the condition of UCP2 upregulation in RVLM, oxidative stress in other areas of brain may also participate in central Ang II–induced hypertension. In this regard, oxidative stress in the subfornical$^{34}$ and hypothalamic areas$^{35}$ in the forebrain also contributes to neural mechanism of Ang II–induced hypertension.

Our observation that transcriptional up- or downregulation of UCP2 exerted minimal effects on mitochondrial electron transport chain activity and tissue ATP content in RVLM implies that the primary function of UCP2 is not to promote gross thermogenesis or energetic inefficiency in the mitochondria of RVLM. This notion is in concordance with the consensus that a “mild” uncoupling caused by activation of UCP2 leads only to limited increases in proton conductance in the inner membrane of mitochondria, resulting in slightly increased oxidative phosphorylation rate but maintained production of ATP.$^{36,37}$ These observations also deemed unlikely the possibility that the regulatory actions of UCP2 on ROS level and hemodynamic functions are secondary to induced bioenergetic deficiency in RVLM. We reported recently$^{19}$ that impairment of mitochondrial electron transport chain activity increases O$_2^-$, leading to chronic oxidative stress in RVLM. Because UCP2 exerted minimal effects on electron transport chain activity, it is unlikely that this source of ROS is the target for its antioxidant effect. UCP2 is engaged in regulation of redox balance by decreasing proton electrochemical gradient across the inner mitochondrial membrane.$^{6,8}$ It follows that UCP2 may exert its antioxidant actions via this mode of mitochondrial action.

We recognize that results obtained from the mitochondrial fractions in this study depend on the purity of our preparations. In this regard, isolation of brain mitochondria by discontinuous Percoll density gradient$^{38}$ has been reported to yield approximately 90% recognizable mitochondria with little contamination of other organelles. We also confirmed the purity of the isolated mitochondria by showing a lack of cytosolic marker proteins in the mitochondrial fraction. We are also aware that acute injection of reagents such as tempol or DPI exerted effects on UCP2 expression 12 or 24 hours after administration. We interpret these seemingly long-lasting pharmacological effects by noting that the signaling cascade that interposes between stimulation of angiotensin type 1 receptor by Ang II, production of NADPH oxidase–derived O$_2^-$, activation of p38 MAPK, and transcriptional upregulation of UCP2 requires 12 to 24 hours to materialize. It follows that the same amount of time is required to manifest the effects of interrupting individual steps in this signaling cascade.
cascade by tempol (O$_2^{-}$) or DPI (NADPH oxidase) on Ang II--induced UCP2 expression.

In conclusion, the present study provided the first in vivo evidence for a direct link between brain mitochondrial UCP2 and central regulation of arterial pressure. Specifically, we demonstrated that transcriptional upregulation of mitochondrial UCP2 activated by an elevation in mitochondrial and NADPH oxidase--derived extramitochondrial O$_2^{-}$ plays an active role in feedback regulation of ROS production in RVLM and hypertension associated with chronic oxidative stress (Figure 8). Epigenetic studies reported a positive association between UCP2 gene polymorphism and increased oxidative stress in patients with hypertension. Because of the important role of mitochondrial oxidative stress in RVLM in neural mechanism of hypertension, our observed antioxidant action of mitochondrial UCP2 is of considerable importance in protection against neurogenic hypertension associated with brain oxidative stress. Our results also suggest the possibility of UCP2 as a target molecule for investigations on the etiology and treatment of neurogenic hypertension.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Transcriptional Upregulation of Mitochondrial Uncoupling Protein 2
Protects Against Oxidative Stress-associated Neurogenic Hypertension

Samuel H.H. Chan, PhD; Chiung-Ai Wu, MS; Kay L.H. Wu, PhD;
Ying-Hao Ho, MD, Alice Y.W. Chang, PhD; Julie Y.H. Chan, PhD

From the Center for Translational Research in Biomedical Sciences, Chang Gung
Memorial Hospital-Kaohsiung Medical Center (S.H.H.C., A.Y.W.C.), Department of
Medical Education and Research (C.A.W., K.L.H.W., J.Y.H.C.), and Department of
Neurology (Y.H.H.), Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan,
Republic of China

Correspondence to Julie Y.H. Chan, PhD, Department of Medical Education and
Research, Kaohsiung Veterans General Hospital, Taiwan 813, Republic of China
Tel: 886-7-3422121 (ext. 1503), Fax: 886-7-3468056
E-mail: yhwa@isca.vghks.gov.tw
Expanded Methods

Animals
Adult, male Sprague-Dawley rats (10-11 week old, n = 345) 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-hours light-dark cycle (lights on during 08:00-20:00) and provided with standard chow and tap water ad libitum. All 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.

Measurement of Systemic Arterial Pressure and Heart Rate by Radiotelemetry
Systemic arterial pressure (SAP) and heart rate (HR) were measured in conscious rats using a radiotelemetry system (Data Sciences International, Minneapolis, MN). For implantation of radiotelemetry transmitter, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A flexible catheter attached to a telemetry transmitter (Data Sciences International) was inserted into the abdominal aorta immediate below the renal arteries and secured in place with surgical glue. The transmitter was secured to the abdominal muscle and remained in the abdominal cavity for the duration of the experiment. The skin was closed using non-absorbable suture, and rats were returned to individual cages positioned over an RLA-3000 radiotelemetry receiver (Data Sciences International). Animals routinely received procaine penicillin (1,000 IU, i.m.) injection postoperatively to prevent infection. Animals were allowed to recover from surgery for 3 days and only animals that showed progressive weight gain after the operation were used in subsequent experiments. SAP was recorded continuously for 24 hours after various treatments, or for 60 minutes every day between 1300 and 1500 for a maximum of 7 days.
Microinjections of Test Agents into RVLM

Microinjection bilaterally of test agents into RVLM was carried out with a glass micropipette (external tip diameter: 50-80 μm) connected to a 0.5-μl Hamilton microsyringe.1-3 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.4 As a routine, a total volume of 50 nl was delivered to each side of RVLM over 1-2 minutes to allow for complete diffusion of the test agents. 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 glutamate.5 Test agents used in this study included the activator of peroxisome proliferator-activated receptor γ (PPARγ), rosiglitazone (Cayman Chemical, Ann Arbour, MI); PPARγ antagonist, GW9662 (Cayman Chemical); angiotensin II (Ang II, Sigma-Aldrich, St. Louis, MO); angiotensin type 1 receptor antagonist, losartan (Merck Biosciences, Darmstadt, Germany); a flavoprotein inhibitor of NADPH oxidase, diphenyleneiodonium chloride (DPI, Calbiochem, San Diego, CA); a superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol; Calbiochem); a specific and cell-permeable inhibitor of p38 MAP kinase, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5- (4-pyridyl)1H-imidazole (SB203580; Calbiochem); or a potent and selective inhibitor of ERK1/2, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126; Calbiochem). Microinjection of artificial cerebrospinal fluid (aCSF) or 0.5% DMSO (solvent for GW9662, SB203580 or U0126) served as the vehicle and volume control. The composition of aCSF was (mM): NaCl 117, NaHCO3 25, Glucose 11, KCl 4.7, CaCl2 2.5, MgCl2 1.2 and NaH2PO4 1.2.

In some experiments, animals received microinjection bilaterally into RVLM of an antisense oligonucleotide (ASON) that targets against human p47phox or p22phox subunit of NADPH oxidase mRNA or human UCP2 mRNA (Genemed
Biotechnologies), or its sense (SON) sequence (Genemed Biotechnologies). The sequences used were: p22\textsuperscript{phox} ASON: 5’-CGCCAGCGCCTGCTCGTTGGC- 3’, p22\textsuperscript{phox} SON: 5’-GCGGTCGCGGACGAGCAACCG-3’; p47\textsuperscript{phox} ASON: 5’-TTTGTTCTGGTTGTCTGTGGG-3’, p47\textsuperscript{phox} SON: 5’-CCCACAGACACCAGACAAA-3’; UCP2 ASON: 5’-CCTTGAACCAACCATTGATTC-3’, UCP2 SON: 5’-GAATCATGGTTGGTTCAAGG-3’. The oligonucleotide was synthesized with phosphorothioate-modified bases to limit degradation and was high performance liquid chromatography purified to limit contamination by incomplete synthesis products. In animals that received intracerebroventricular (i.c.v.) infusion of Ang II for 7 days, pretreatment of UCP2 ASON or SON by microinjection bilaterally into RVLM of rats was delivered on day 4 after central infusion of Ang II. This treatment scheme was adopted based on previous publications\textsuperscript{5,7} or preliminary results, which showed an effective inhibition of mRNA expression for 72 h after treatment with the individual ASON. The wound was closed and animals were allowed to recover in individual cages. Microinjection of aCSF served as the vehicle and volume control.

Collection of Tissue Samples from Ventrolateral Medulla
At various time intervals after experimental treatment, rats were killed with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardiacly with 150 ml of warm (37°C) saline containing heparin (100 U/mL). The brain was rapidly removed and placed on dry ice, blocked in the coronal plane, and sectioned at 300-μm thickness in a cryostat. Both sides of the ventrolateral medulla covering RVLM were collected by micropunches made with a stainless steel bore (1 mm i.d.).\textsuperscript{1-3,5} Medullary tissues collected from animals under anesthesia but without treatment served as the sham control.

Isolation of Mitochondrial, cytosolic or membranous Fractions
Samples of the ventrolateral medulla were minced and disrupted in an ice-cold
isolation buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM NaN₃, 50 mM NaF and 250 mM sucrose. Aprotinin (10 μg/mL), phenylmethylsulfonyl fluoride (20 μg/mL) and trypsin inhibitor (10 μg/mL) were included in the isolation buffer to prevent protein degradation. Isolation of mitochondrial fraction was carried out by discontinuous Percoll gradient centrifugation according to procedures described previously. This procedure reportedly yields 10-15% of the total mitochondria, and enriches the mitochondrial fraction by at least 10-fold when compared with tissue homogenates. The morphology, integrity and function of the mitochondria isolated by this methods are also shown to be well preserved. In brief, tissue samples were gently homogenized with a glass-glass homogenizer. The homogenates were centrifuged at 1400g for 5 minutes at 25°C to remove nuclei and unbroken cell debris, and the supernatant was collected and centrifuged at 10,000g for another 20 minutes at 4°C to pellet the mitochondria. The supernatant was further centrifuged at 50,000g (4°C) for 60 minutes, and the resulting pellet contains total membranous protein, and the supernatant is referred to as the cytosolic fraction. The mitochondrial pellet was resuspended in 1 ml of isolation medium. This was layered on a discontinuous gradient consisting of 3 ml of 12% Percoll, 4 ml of 26% Percoll, and 4 ml of 40% Percoll and centrifuged 20,000g for 25 minutes. The dense band of material at the interface between the Percoll layers was collected, diluted 1:4 in fresh isolation buffer and centrifuged at 16,700g for 10 minutes. The pellet was again suspended in isolation buffer with 10 mg/ml of fatty acid-free bovine serum albumin and centrifuged again at 6900g for 10 minutes. The mitochondria pellet thus obtained was resuspended in a medium composed of 25 mM sucrose, 75 mM mannitol, 10 mM Hepes-Tris (pH7.2), and 0.05 mM EDTA. The purity of mitochondrial, cytosolic or membranous fraction was further verified by the selective expression of Complex IV (cytochrome c oxidase, COX) of the mitochondrial electron transport chain, α-tubulin or plasma membrane Ca²⁺-ATPase (PMCA), respectively. The amount of protein was determined by the method of Bradford with a protein assay kit (Bio-Rad, Hercules, CA).
Measurement of Mitochondrial H₂O₂ Levels

H₂O₂ production in the mitochondrial fraction was assessed using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probe, Inc., Eugene, OR).² Reaction mixtures containing 50 μM Amplex Red reagent, 0.1 units/mL peroxidase and mitochondrial fraction (1 mg protein/mL) were incubated at room temperature for 30 minutes. H₂O₂ levels were determined by measuring the absorbance at 570 nm, and expressed as pmol/min/mg protein, using a standard curve.

Western Blot Analysis

Proteins from the mitochondrial (6 μg), cytosolic (50 μg) or membranous (80 μg) fraction were separated by using 10-12% SDS-PAGE and transferred to PVDF membrane. The primary antiserum used for Western blot analysis included a rabbit polyclonal antiserum against UCP2 (1:1000; Calbiochem) or PGC-1α (1:1000; Cell Signaling, Danvers, MA), a mouse monoclonal antiserum against cytochrome c oxidase (COX, 1:1000; Invitrogen, Carlsbad, CA), a mouse monoclonal against PMCA (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit monoclonal antiserum against PPARγ (1:1000; Cell Signaling), or α-tubulin (1:5000; Sigma-Aldrich). This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (UCP2, PGC-1α) or goat anti-mouse IgG (cytochrome c oxidase) (Jackson ImmunoResearch, 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 COX protein.

Isolation of Nuclear Fractions and Immunoprecipitation

Tissues obtained from ventrolateral medulla of 5 to 6 rats were homogenized, centrifuged and pellets were collected to prepare purified nuclear fraction.¹³⁵ Nuclear
proteins were extracted using commercially available nuclear and cytoplasmic extraction kits (Pierce, Rockford, IL), and were stored at -85°C until further use. Protein concentrations were determined by the Bradford assay.

PGC-1α in the nuclear fraction was immunoprecipitated with affinity-purified goat polyclonal antiserum coupled to protein G-agarose beads. Immunoprecipitation was performed at 4°C overnight and the precipitated beads were washed with ice-cold lysis buffer followed by kinase buffer containing 20 mM Tris pH 7.4, 10 mM MnCl₂, 1 mM dithiothreitol. PGC-1α phosphorylation was assessed by immunobloting with a rabbit polyclonal anti-phosphothreonine antiserum (1:1000; Santa Cruz Biotechnology), and formation of PGC-1α/PPARγ complex was assessed using a rabbit monoclonal antiserum against PPARγ (1:1000; Cell Signaling).

RNA Isolation and Reverse Transcription Real-Time Polymerase Chain Reaction

For UCP2 ASON-, or SON-treated animals, total RNA from RVLM was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. All RNA isolated was quantified by spectrophotometry and the optical density 260/280 nm ratio was determined. Reverse transcriptase (RT) reaction was performed using a SuperScript Preamplification System (Invitrogen) for the first-strand cDNA synthesis. Real-time polymerase chain reaction (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’-CAGTGACCTGCCTGGTGCAA-3’ for the reverse. Primer pairs for GAPDH cDNA (GenBank accession no. NM017008) were 5’-GCCAAAAGGGTCAATCATCTC-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^{-ΔΔCt}, where ΔΔCt = (CtUCP2 - CtGAPDH_{oligonucleotide treatment}) - (CtUCP2 - CtGAPDH_{control}). Note that Ct value is the cycle number at which fluorescence signal crosses the threshold.

**Intracerebroventricular Infusion with Osmotic Minipump**

After obtaining baseline SAP for at least 3 days using radiotelemetry, animals were again anesthetized with pentobarbital sodium (50 mg/kg, i.p.) for implantation of osmotic minipump. A 25-gauge stainless steel cannula was implanted stereotaxically into the lateral cerebral ventricle on the right side at coordinates 0 to 0.5 mm posterior to bregma, 1.3 to 1.5 mm lateral to midline and 3.2 to 3.5 mm below dorsal surface of cerebral cortex. This cannula was connected, via PE-60 tubing, to an osmotic minipump (Alzet 2001, Alzet Corp., Palo Alto, CA), which was placed under the skin in the neck region, for infusion of Ang II at 1 μL/h for 7 days. Implantation was permanently fixed to the skull with surgical glue. Animals received procaine penicillin (1,000 IU, i.m.) injection postoperatively, and only animals that showed progressive weight gain after the operation were used in subsequent experiments. Control infusion of aCSF served as volume and vehicle control.
Intracisternal Infusion with Osmotic Minipump

Some rats received surgery for chronic infusion into the cisterna magna. Under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), a midline dorsal neck incision was made, and the dura mater between the foramen magnum and the C1 lamina of spinal cord was exposed after dissection of overlying muscles. An osmotic minipump (Alzet 2001) filled with 0.5% DMSO or rosiglitazone (1 or 5 μg·μL⁻¹·h⁻¹), was implanted subcutaneously in the back and connected to a polyethylene tube (PE 10). A small hole was then made in the atlantooccipital membrane that covers the dorsal surface of the medulla, and the tip of the tube was placed intracisternally and fixed in place with tissue glue. SAP was recorded by telemetry for 60 minutes every day between 1300 and 1500 for 7 days.

Construction and Purification of Adenovirus Vectors

To generate adenovirus encoding SOD1 (AdSOD1) or SOD2 (AdSOD2), human SOD1 or SOD2 cDNA was subcloned into HindIII and XbaI site of adenovirus transfer vector pCA13 to yield pCA13-SOD1 or pCA13-SOD2, in which the transgene was driven by early promoter from the cytomegalovirus and flanked by polyadenylation sequences from SV40. Similarly, human catalase (CAT) cDNA was subcloned into EcoRI site of adenovirus transfer vector pCA13 to yield pCA13-CAT for generation of AdCAT. Recombinant adenovirus was then generated by cotransfection of the transfer vectors with pJM17 vector (Microbix; Toronto, Canada), a plasmid containing the entire type 5 Ad genome with E1-insertion and E3-deletion, into 293 cells. After homologous recombination, the virus plaques were verified by PCR and Western blot analyses. The virus was subsequently amplified by two rounds of cesium chloride ultracentrifugation and desalted by G-25 gel-filtration chromatography. The titer of virus solution was determined by measuring optical density at 260 nm and plaque-forming assay on 293 cells before storage at -80°C.
In Vivo Gene Transfer into RVLM

Microinjection bilaterally of adenoviral vectors encoding green fluorescence protein (AdGFP), AdSOD1, AdSOD2, or AdCAT was carried out stereotaxically and sequentially into RVLM sites.\textsuperscript{3,5,15} An adenoviral suspension containing $1 \times 10^8$ plaque-forming units (pfu)/100 nL was administered into each injection site over 10-15 minutes using a glass micropipette. A total of eight injections (4 on each side) were made at stereotaxic coordinates of 4.5-5 mm posterior to lambda, 1.8-2.1 mm lateral to the midline, and 8.0-8.5 mm below the dorsal surface of cerebellum. Microinjection of aCSF served as the volume control. Animals were allowed to recover in their home cages with free access to food and water.

Assays for Activity of Individual Mitochondrial Electron Transport Chain Enzyme

Activity of individual mitochondrial ETC complex enzymes was determined immediately after isolation of mitochondrial fraction, using a thermostatically regulated ThermoSpectronic spectrophotometer (Fisher Scientific, Loughborough, UK). All enzyme assays were performed at 30°C according to procedures reported previously.\textsuperscript{1,2} At least quadruplicate determinations were carried out for each tissue sample in all enzyme activity assays, and the activity is expressed as nmol/min/mg protein.

The activity of complex I (NADH:ubiquinone oxidoreductase) was determined by monitoring the oxidation of NADH at 340 nm. The assay medium contains a mixture of 25 mM potassium phosphate (pH 7.2 at 20°C), 5 mM MgCl\textsubscript{2}, 2.5 mg/ml bovine serum albumin, and 2 mM KCN. Baseline activity was established for 1 minute after the addition of 0.13 mM NADH, 65 μM ubiquinone, and 2 μg/mL antimycin A. The reaction was initiated by addition of mitochondria (25 μg of protein), and the rate of oxidation of NADH was monitored by the decrease in absorbance at 340 nm and was recorded for 3 minutes. Rotenone (2 μg/mL) was then added, and the rate of change in absorbance was measured for an additional 3 minutes. Complex I activity was
determined by subtracting the rotenone insensitive activity from the total activity.

The activity of complex II (succinate:ubiquinone oxidoreductase) was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. The assay medium is the same as for complex I assay. Mitochondria (25 μg of protein) were incubated at 30°C for 20 minutes in an assay buffer containing 20 mM succinate and 0.2 mM ATP. The reaction was initiated by addition of 2 μg/mL antimycin A, 2 μg/mL rotenone, 2 mM KCN, 50 μM 2,6-dichloroindophenolate, and 65 μM ubiquinone. The rate of reduction of 2,6-dichloroindophenolate was recorded for 3 minutes. 10 mM malonate was then added to inhibit the enzymatic activity. Complex II activity was determined by subtracting the malonate insensitive activity from the total activity.

The activity of complex III (ubiquinol:cytochrome c oxidoreductase) was determined by monitoring the reduction of ferrocytochrome c at 550 nm. The assay medium is the same as for complex I. 2 mM KCN was included in the assay media to prevent the reoxidation of the product, ferrocytochrome c, by cytochrome c oxidase. Nonenzymatic activity was recorded for 1 minute after the addition of 15 μM ferrocytochrome c, 2 μg/mL rotenone, 0.6 mM dodecyl-β-D-maltoside, and 35 μM ubiquinol. The complex III activity was initiated by addition of mitochondrial fraction (10 μg of protein), and the rate of reduction of ferrocytochrome c was recorded for 1 minute. Specific complex III activity was calculated by subtracting the antimycin A (2 μg/mL) insensitive activity from the total activity.

Complex IV (cytochrome c oxidase) activity was assessed by following the oxidation of reduced cytochrome c (90 μM) at 550 nm at 30°C in the assay buffer containing 10 mM Tris-HCl and 120 mM KCl (pH7.0), antimycin A (2 μg/mL) in the presence and absence of KCN (2 mM). The nonenzymatic rate was recorded for 1 minute after the addition of 2 μg/mL antimycin A, 0.45 mM dodecyl-β-D-maltoside and mitochondria (10 μg of protein). The reaction was initiated by the addition of 11 μM ferrocytochrome c, and the rate of oxidation of ferrocytochrome c was measured for 3 minutes. Specific complex IV activity was calculated by subtracting the KCN-insensitive activity from the total activity.
Complex V (ATP synthase) activity was measured at 340 nm in a reaction mixture containing assay buffer, 25 units of pyruvate kinase, 25 units of lactate dehydrogenase, 20 μM rotenone, 2 mM KCN, 5 mM phosphoenolpyruvate, 150 μM NADH and mitochondria (25 μg of protein). The reaction was initiated by the addition of 2.5 mM ATP and the enzymatic activity was measured for 3 minutes. Oligomycin (15 μM) was added and the rate of change in absorbance was measured for an additional 3 minutes. Specific complex V activity was calculated by subtracting the oligomycin-insensitive activity from the total activity.

Assays for Electron Coupling Capacity in Mitochondrial Electron Transport Chain

For nicotinamide adenine dinucleotide (NADH) cytochrome c reductase (NCCR; marker for electron coupling capacity between Complexes I and III) activity, the mitochondrial fraction (20 μg of protein) was incubated in a mixture containing 50 mM K2HPO4 buffer, pH7.4, 1.5 mM KCN, 1 mM β-NADH, 20 μM rotenone at 37°C for 2 minutes. After the addition of 0.1 mM cytochrome c, the reduction of oxidized cytochrome c was measured as the difference in the presence or absence of rotenone at 550 nm for 3 minutes at 37°C.1,2

Determination of succinate cytochrome c reductase (SCCR; marker for electron coupling capacity between Complexes II and III) activity in the mitochondrial fraction (30 μg) was performed in 40 mM K2HPO4 buffer (pH 7.4), 1.5 mM KCN, supplemented with 20 mM succinate. After a 5-minute equilibration at 37°C, 50 μM cytochrome c was added and the reaction was monitored at 550 nm for 3 minutes at 37°C.1,2

Measurement of ATP concentration

Samples of the ventrolateral medulla were homogenized in a protein extraction solution (Pierce). The supernatant after centrifugation at 10,000g for 10 minutes was subject to determination of ATP concentration, using an ATP bioluminescence assay.
Light emitted from a luciferase-mediated reaction and measured by a tube luminometer (Berthold Detection Systems, Pforzheim, Germany) was used to calculate the measured values.

**Histology**

With the exception of animals used for biochemical analyses, the brain stem was removed from animals after they were killed by an overdose of sodium pentobarbital (100 mg/kg, i.v.), and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥72 hours. Frozen 25-μm sections of the medulla oblongata were stained with Cresyl violet for histological verification of the location of microinjection sites.
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Online Figure I. Representative Western blot gels showing protein expression of p47phox, p22phox, COX, α-tubulin or PMCA in the cytosolic, mitochondrial or membranous fraction from RVLM 24 h after p47phox or p22phox ASON (100 pmol) or aCSF treatment.
Online Figure II. Representative gels (inset) or densitometric analysis of UCP2 protein level detected in RVLM on day 7 after i.c.v. infusion of Ang II (100 μg·μL⁻¹·h⁻¹), alone or with additional treatment with microinjection of losartan (2 nmol), AdSOD1 or AdSOD2 bilaterally into RVLM. Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P < 0.05 versus Ang II group in the Scheffé multiple range analysis.
Online Figure III. Representative gels (A) or densitometric analysis of UCP2 protein level (B) detected in the cytosolic or mitochondrial fraction from RVLM on day 7 after intracisternal infusion of 0.5% DMSO or rosiglitazone (5 μg·μL⁻¹·h⁻¹). Values are mean ± SEM of quadruplicate analyses on samples pooled from 4 animals in each group. *P < 0.05 versus DMSO group in the Scheffé multiple range analysis. ND, below detection limit.
A

![Graph A: Hydrogen Peroxide](image)

Hydrogen Peroxide

(mmol/mmol/mg protein)

C  6  12  24

UCP2 ASON

UCP2 SON

h

B

![Graph B: Mean Arterial Pressure](image)

Mean Arterial Pressure (mm Hg)

-6  0  6  12  18  24

POSTINJECTION TIME (h)

- aCSF
- UCP2 ASON
- UCP2 SON
Online Figure IV. Temporal changes in mitochondrial level of hydrogen peroxide (A), MSAP (B), UCP2 mRNA expression (C) and representative gels (inset) or densitometric analysis of UCP2 protein level (D) detected in RVLM in response to microinjection bilaterally into RVLM (at time 0) of UCP2 ASON or SON (100 pmole). Values are mean ± SEM, n = 5 to 7 animals in each group (B) or quadruplicate analyses on samples pooled from 5 to 6 animals in each group (A, C, D). Changes in UCP2 mRNA in (C) are presented in folds with reference to the sham group (designated 1). *P < 0.05 versus vehicle-control (C) group in the Scheffé multiple-range analysis.
Online Figure V. Enzyme activity of complexes I (CI) to V (CV) of mitochondrial electron transport chain (A), electron coupling capacity between complexes I and II or complexes I and III, as denoted by the activity of NCCR or SCCR (B) or tissue ATP concentration (C) detected from RVLM 12 or 24 hours after microinjection bilaterally into RVLM of rosiglitazone or UCP2 ASON. Control group received aCSF or SON. No significant difference ($P > 0.05$) was detected among different groups of animals by one-way ANOVA.