Transcriptional Upregulation of Brain-Derived Neurotrophic Factor in Rostral Ventrolateral Medulla by Angiotensin II
Significance in Superoxide Homeostasis and Neural Regulation of Arterial Pressure

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Rationale: 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. Emerging evidence suggests that brain-derived neurotrophic factor (BDNF) manifests “nontrophic” actions.

Objective: We assessed the hypothesis that BDNF plays an active role in oxidative stress–associated neurogenic hypertension by maintaining superoxide anion (O$_2^-$) homeostasis in RVLM.

Methods and Results: In Wistar–Kyoto rats, microinjection of angiotensin II (Ang II) bilaterally into RVLM upregulated BDNF mRNA and protein and induced cAMP response element binding protein (CREB) phosphorylation. The Ang II–induced BDNF upregulation in RVLM was attenuated by coadministration of the NADPH oxidase inhibitor apocynin; the superoxide dismutase mimetic tempol; or an antisense oligonucleotide against CREB. Intracisternal infusion of Ang II elicited phosphorylation of p47$^{phox}$ subunit of NADPH oxidase, suppression of mitochondrial electron coupling capacity, and augmentation in mitochondrial uncoupling protein (UCP)2 expression in RVLM. The former 2 cellular events were enhanced, whereas UCP2 upregulation was attenuated by gene knockdown of BDNF or depletion of tropomyosin receptor kinase (Trk)B ligands with recombinant human TrkB-Fc fusion protein. The same treatments also significantly potentiated both Ang II–induced O$_2^-$ production in RVLM and chronic pressor response.

Conclusions: Ang II induces O$_2^-$-dependent upregulation of BDNF in RVLM via phosphorylation of CREB. The Ang II–activated BDNF/TrkB signaling, in turn, exerts negative-feedback regulation on tissue O$_2^-$ level in RVLM through inhibition of p47$^{phox}$ phosphorylation, preservation of mitochondrial electron transport capacity, and upregulation of mitochondrial UCP2, resulting in protection against Ang II–induced oxidative stress and long-term pressor response. (Circ Res. 2010;107:1127-1139.)

Key Words: brain-derived neurotrophic factor ■ oxidative stress ■ angiotensin II ■ mitochondrial uncoupling protein ■ blood pressure

Oxidative stress resulting from an imbalance of production over degradation of the reactive oxygen species (ROS), particularly superoxide anion (O$_2^-$), is associated with hypertension.\cite{1,2} In the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of vasomotor tone are located,\cite{3} overproduction of O$_2^-$ plays a pivotal role in neural mechanism of hypertension by increasing sympathetic outflow to the peripheral blood vessels.\cite{1,2,4-6} Thus, NADPH oxidase–derived O$_2^-$ in RVLM mediates chronic pressor response induced by angiotensin II (Ang II).\cite{7}

A decrease in molecular synthesis and enzyme activity of superoxide dismutase (SOD) isoforms contributes to hyper-tensive phenotype in the spontaneously hypertensive rats (SHRs).\cite{8} On the other hand, transcriptional upregulation of mitochondrial uncoupling protein (UCP)2 abrogates Ang II–induced oxidative stress and alleviates oxidative stress–associated neurogenic hypertension via a negative-feedback mechanism.\cite{8}

One of the well-recognized protective mechanisms against stressfull insults in brain is expression of neurotrophic factors, in particular brain-derived neurotrophic factor (BDNF).\cite{9} Superimposed on its “classic” trophic functions in proliferation, differentiation, and survival of neurons in the peripheral and central nervous system during development,\cite{10} or in
superoxide dismutase
ROS reactive oxygen species
RVLM rostral ventrolateral medulla
SCCR succinate cytochrome c reductase
SOD superoxide dismutase
SON sense oligonucleotide
tempol 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
SHR spontaneously hypertensive rat
TrkB tropomyosin receptor kinase B
WKY Wistar–Kyoto
UCP uncoupling protein

Methods

All experimental procedures were carried out in compliance with the guidelines of our institutional animal care committee. Adult (12-week-old) male normotensive Wistar–Kyoto (WKY) rats or SHRs purchased from the Experimental Animal Center of the National Applied Research Laboratories, Taiwan, were used. Animals received intracisternal infusion or microinjection bilaterally into RVLM of Ang II, alone or with additional treatment with BDNF; a SOD mimetic, tempol; an inhibitor of NADPH oxidase assembly, apocynin; an angiotensin subtype 1 receptor (AT,R) antagonist, losartan; an antisense oligonucleotide (ASON) against p47phox subunit of NADPH oxidase, cAMP response element binding protein (CREB) or BDNF; or recombinant human TrkB-Fc chimera, which sequesters endogenously released TrkB ligands (BDNF and neurotrophin-4) and blocks the TrkB receptors, or TrkA-Fc chimera. The dose of molecular reagents used in this study was modified from previous studies that validated the selectivity/effectiveness of the chemicals. The key experimental procedures included measurement of mean arterial pressure (MAP) and heart rate by radiotelemetry under conscious conditions or in animals maintained under propofol anesthesia; determination of mRNA by reverse-transcription real-time PCR or protein expression by Western blot; determination of BDNF tissue level by a fluorometric 2-site enzyme immunoassay (ELISA); measurement of enzyme activity of nicotinamide-adenine dinucleotide cytochrome c reductase (NCCR) (marker for electron coupling capacity between complexes I and III), succinate cytochrome c reductase (SCCR) (marker for electron coupling capacity between complexes II and III), mitochondrial or cytosolic SOD; quantification of tissue level of O2 by lucigenin-enhanced chemiluminescence assay and immunofluorescence staining coupled with laser scanning confocal microscopy for location of BDNF in RVLM neurons.

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

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

Results

Ang II–Induced Increases in Protein Expression and Tissue Level of BDNF in RVLM Are Dependent on NADPH Oxidase–Derived Superoxide Anion

Our laboratory reported previously that Ang II induces oxidative stress by increasing O2 production in RVLM via activation of NADPH oxidase. We took advantage of this cellular event in our first series of experiments to investigate whether acute site-specific induction of oxidative stress by Ang II in RVLM affects BDNF expression in WKY rats. Microinjection bilaterally into RVLM of Ang II (100 pmol) induced a progressive increase in BDNF mRNA (Figure 1A) or protein (Figure 1B) expression that became significant 4 hours after application of the octapetide, reaching a maximum at 8 hours, and endured at least 24 hours. The maximal Ang II–induced upregulation of BDNF protein expression was significantly blunted on coadministration of an inhibitor of NADPH oxidase assembly, apocynin (2 mmol), or by gene knockdown with ASON against p47phox subunit of NADPH oxidase (200 pmol) (data not shown). The Ang II–induced BDNF upregulation was also inhibited by coadministration of an AT,R antagonist, losartan (2 nmol). Ang II, on the other hand, exerted no effect on baseline BDNF expression in RVLM of WKY rats (Online Figure I, A).

To further investigate the effect of chronic oxidative stress on BDNF expression in RVLM, Ang II was microinfused into the cisterna magna via an osmotic minipump for 7 days. Intracisternal infusion of Ang II (100 ng·g−1·h−1) increased BDNF protein expression (Figure 1C) and tissue level (Figure 1D) of BDNF detected at 12, 24, 48, or 72 hours or days 5 and 7 (data not shown) after infusion. This induced increase, detected on 48 or 72 hours after infusion, was attenuated by...
microinjection bilaterally into the RVLM of p47phox ASON (200 pmol) 24 hours after Ang II infusion. Immunofluorescent staining coupled with laser confocal microscopy further revealed an increase in BDNF immunoreactivity in RVLM neurons 48 hours after intracisternal infusion of Ang II (200 pmol), alone or with additional treatment of apocynin (2 nmol), losartan (2 nmol), or ASON against p47phox mRNA (200 pmol). E, Representative laser-scanning confocal microscopic images showing cells in RVLM that were immunoreactive to BDNF (green fluorescence) and additionally stained positively for a neuronal marker, neuron-specific nuclear protein (NeuN) (red fluorescence), 72 hours after intracisternal infusion of Ang II, alone or with additional microinjection bilaterally into RVLM of p47phox ASON. Scale bar, 10 μm. Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs saline control group (designated as C); #P<0.05 vs Ang II group in the Scheffé multiple-range analysis. Arrow depicts the BDNF immunoreactivity in RVLM neurons.

Figure 1. Ang II induces upregulation of BDNF in RVLM via activation of NADPH-oxidase-derived \( \text{O}_2^- \). Fold changes in BDNF mRNA (A) or representative gels (inset) or densitometric analysis of results from Western blots showing changes in expression of BDNF protein (B and C) or tissue level of BDNF (D) in RVLM of WKY rats after microinjection bilaterally into RVLM (A and B) or intracisternal infusion (C and D) of Ang II (100 pmol), alone or with additional treatment of apocynin (2 nmol), losartan (2 nmol), or ASON against p47phox mRNA (200 pmol). E, Representative laser-scanning confocal microscopic images showing cells in RVLM that were immunoreactive to BDNF (green fluorescence) and additionally stained positively for a neuronal marker, neuron-specific nuclear protein (NeuN) (red fluorescence), 72 hours after intracisternal infusion of Ang II, alone or with additional microinjection bilaterally into RVLM of p47phox ASON. Scale bar, 10 μm. Values are means±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs saline control group (designated as C); #P<0.05 vs Ang II group in the Scheffé multiple-range analysis. Arrow depicts the BDNF immunoreactivity in RVLM neurons.

Our second series of experiments therefore examined the involvement of CREB in Ang II–induced BDNF upregulation in RVLM of WKY rats. Microinjection bilaterally into RVLM of Ang II (100 pmol) induced phosphorylation of CREB at Ser133, detected 30, 60, or 120 minutes after injection (Figure 2A). The Ang II–induced CREB phosphorylation, when determined 60 minutes after infection, was significantly blunted by coadministration of a SOD mimetic, tempol (100 pmol), or apocynin (2 nmol). Gene knockdown of CREB expression by microinjection bilaterally into RVLM of CREB ASON (100 or 200 pmol), 24 hours before octapeptide administration, effectively suppressed CREB mRNA expression (Online Figure II, A) and significantly reduced the BDNF mRNA upregulation in RVLM detected 8 hours after Ang II administration (Figure 2B). Control microin-
jections of CREB sense oligonucleotide (SON) (100 or 200 pmol) were ineffective.

**BDNF Is Involved in Regulation of Chronic O$_2^-$ Production in RVLM After Intracisternal Infusion of Ang II**

We next delineated whether BDNF plays a role in ROS homeostasis at RVLM of WKY rats based on gene knockdown of BDNF or blockade of TrkB. Microinjection bilaterally into RVLM of a BDNF ASON (100 or 200 pmol) that effectively reduced BDNF mRNA and protein expression (Online Figure II, B) or recombinant human TrkB-Fc (1 or 5 nmol), but not TrkA-Fc (5 nmol), fusion protein that sequesters endogenously released TrkB ligands and blocks TrkB,20 24 hours or 1 hour, respectively, before O$_2^-$ measurement, significantly potentiated the increase in O$_2^-$ production in RVLM measured 24 hours after intracisternal infusion of the octapeptide (100 ng·μL$^{-1}$·h$^{-1}$) (Figure 3A). Similar to our previous observations,5 the Ang II–induced O$_2^-$ production was reversed by apocynin (2 nmol) or tempol (100 pmol), microinjected bilaterally into RVLM 1 hour before O$_2^-$ measurement. BDNF ASON or TrkB-Fc fusion protein, on the other hand, had no effect on basal levels of O$_2^-$ in RVLM (data not shown).

**BDNF Attenuates Ang II–Induced Phosphorylation of p47phox Subunit of NADPH Oxidase in RVLM**

We reported previously5 that phosphorylation of serine residue of p47phox subunit of NADPH oxidase is an important step for the AT1R-dependent O$_2^-$ production induced by Ang II in RVLM. Our fifth series of experiments determined whether BDNF exerts protection against the Ang II–evoked...
oxidative stress in RVLM of WKY rats by targeting this cellular event. Microinjection bilaterally into RVLM of BDNF (100 or 250 ng) significantly attenuated the Ang II–induced (100 pmol) serine phosphorylation of p47phox subunit, detected 30 minutes after infection, with minimal effects on total p47phox protein expression (Figure 3B). On the other hand, gene knockdown (24 hours before Ang II) or receptor blockade (1 hour before Ang II) by microinjection bilaterally into RVLM of BDNF ASON (100 or 200 pmol) or recombinant human TrkB-Fc fusion protein (5 nmol), but not BDNF SON or TrkA-Fc fusion protein, significantly enhanced the Ang II–induced serine phosphorylation of p47phox subunit. Intriguingly, Ang II–induced p47phox phosphorylation in RVLM was again inhibited by exogenous BDNF in rats treated with BDNF ASON (Online Figure III). Of note was that BDNF, its ASON, or TrkB-Fc fusion protein exerted minimal effect on basal protein expression of p40phox, p67phox, gp91phox, or p22phox subunit of NADPH oxidase in RVLM (Online Figure IV). Furthermore, phosphorylation of serine residue of p40phox or p67phox subunit of NADPH oxidase in RVLM of WKY rats was under detection limit under basal condition or after Ang II (100 pmol) treatment (data not shown).

BDNF Preserves Mitochondrial Electron Coupling Capacity and Upregulates Mitochondrial Uncoupling Protein Expression in RVLM After Intracisternal Infusion of Ang II

We reported recently that NADPH oxidase–derived \( \text{O}_2^\bullet^- \) produced by Ang II inhibits the activity of NCCR and SCCR. We further showed that mitochondrial UCP2 acts as an antioxidant under chronic oxidative stress in RVLM via feedback regulation of \( \text{O}_2^\bullet^- \) production. It follows that BDNF may chronically regulate the Ang II–evoked \( \text{O}_2^\bullet^- \) production in...
RVLM by preserving mitochondrial electron transport chain functions and/or by upregulating mitochondrial UCP2 expression. Our sixth series of experiments evaluated these 2 possibilities. Intracisternal infusion of Ang II (100 ng·μL⁻¹·h⁻¹ for 7 days) via osmotic minipump in WKY rats resulted in a significant reduction of NCCR or SCCR activity in RVLM, detected on day 7 after infusion (Figure 4A). The Ang II–induced reduction in mitochondrial electron coupling...
BNDF Regulates ROS Homeostasis in RVLM

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BNDF in RVLM Negatively Regulates the Chronic Pressor Response Induced by Intracisternal Infusion of Ang II

Our ninth series of experiments established the functional significance of BNDF at RVLM in cardiovascular phenotype induced by Ang II. Compared with its SON, microinjection bilaterally into RVLM of BNDF ASON (200 pmol) on day 3 after intracisternal infusion of Ang II (100 ng μL⁻¹ h⁻¹ for 7 days) significantly potentiated the induced long-term pressor response (Figure 6A), measured by radiotelemetry in conscious WKY rats. The Ang II–induced hypertension, detected under anesthetized condition on day 7 after intracisternal infusion, was significantly potentiated in animals that received microinjection bilaterally into RVLM of recombinant human TrkB-Fc, but not TrkA-Fc fusion protein (5 nmol) (Figure 6B). Coinfusion of BNDF (0.1, 0.5 or 1 ng μL⁻¹ h⁻¹) for 7 days into the cisterna magna of WKY rats, on the other hand, attenuated the Ang II–induced long-term pressor response (Figure 6C). Intracisternal infusion of BNDF alone promoted no significant change in basal MAP.

BNDF in RVLM Promotes Antihypertension in SHRs

Sustained oxidative stress in RVLM contributes to neurogenic hypertension in SHRs. To establish that depression of BNDF/TrkB signaling in the RVLM is involved in manifestation of neurogenic hypertension, we determined the cardiovascular responses to intracisternal infusion of BNDF (0.1 or 0.5 ng μL⁻¹ h⁻¹ for 7 days). Compared with vehicle control, BNDF infusion resulted in a gradual decrease in MAP, monitored by radiotelemetry in conscious SHRs (Figure 7A). This antihypertensive effect of BNDF was accompanied by a slight decrease in heart rate (data not shown). The BNDF-induced (0.5 ng μL⁻¹ h⁻¹) hypotension, measured on day 7 in the anesthetized SHRs following intracisternal infusion of the trophic factor, was antagonized by microinjection bilaterally into RVLM of recombinant human TrkB-Fc (1 or 5 nmol) fusion protein (Figure 7B).

Endogenous BNDF Is Not Involved in Tonic Regulation of Tissue Level of O₂⁻ in RVLM or MAP

Our final series of experiments investigated whether the endogenous BNDF/TrkB signaling is involved in regulation of MAP by modulating basal level of O₂⁻ in RVLM. Compared with artificial cerebrospinal fluid (aCSF) controls, microinjection bilaterally into RVLM of BNDF ASON (100 or 200 pmol) or recombinant human TrkB-Fc chimera (1 or 5 pmol) did not affect the upregulated SOD1 or SOD2 expression detected 2 hours after Ang II (100 pmol) treatment (Online Figure V, A and B), nor the minimal effect of the octapeptide on SOD3 expression (Online Figure V, C). In a separate series of experiments, cytosolic SOD1 or mitochondrial SOD2 enzyme activity was similarly unaffected in WKY rats by microinjection bilaterally into RVLM of BNDF (100 or 250 ng), TrkB-Fc fusion protein (5 nmol), or BNDF ASON (200 pmol) or SON (200 pmol) (Online Figure V, D).

homeostasis can be further substantiated in RVLM. Com-
paired by an elevated tissue level of O₂⁻. We therefore investigated whether SOD plays a role in the slow wave of the ventricular–RA pacemaker complex (Figure 5C) and an increase in tissue O₂⁻ level (Figure 5D). Increase in mitochondrial UCP2 protein expression, detected 12 hours after injection (Figure 4B). This Ang II–induced upregulation in mitochondrial UCP2 was attenuated by coadministration of BNDF ASON (200 pmol) or recombinant human TrkB-Fc fusion protein (5 nmol). Microinjection bilaterally into RVLM of BNDF (100 or 250 ng), on the other hand, resulted in an increase in mitochondrial UCP2 expression, detected 12 hours after infection. Immunoblot results further demonstrated that both full-length and truncated TrkB receptor proteins were expressed in the mitochondrial fraction of RVLM (Figure 4C), although they were minimally affected by BNDF (250 ng) or its ASON (200 pmol).

BNDF Restores the Reduced Mitochondrial Electron Coupling Capacity and Upregulates Mitochondrial UCP2 Expression in RVLM of Spontaneously Hypertensive Rats

We reported previously that mitochondrial dysfunction because of reduced enzyme activities in NCCR and SCCR contributes to chronic oxidative stress in RVLM of SHRs. We reasoned that a role for BDNF-TrkB signaling in the slow wave of the ventricular–RA pacemaker complex (Figure 5C) and an increase in tissue O₂⁻ level (Figure 5D). Increase in mitochondrial UCP2 protein expression, detected 12 hours after injection (Figure 4B). This Ang II–induced upregulation in mitochondrial UCP2 was attenuated by coadministration of BNDF ASON (200 pmol) or recombinant human TrkB-Fc fusion protein (5 nmol). Microinjection bilaterally into RVLM of BNDF (100 or 250 ng), on the other hand, resulted in an increase in mitochondrial UCP2 expression, detected 12 hours after infection. Immunoblot results further demonstrated that both full-length and truncated TrkB receptor proteins were expressed in the mitochondrial fraction of RVLM (Figure 4C), although they were minimally affected by BNDF (250 ng) or its ASON (200 pmol).

BNDF Does Not Affect Protein Expression or Enzyme Activity of SOD Isoforms in RVLM

An increase in antioxidant SOD expression in RVLM has been demonstrated to significantly reduce tissue O₂⁻ levels.5,6 We therefore investigated whether SOD plays a role in the BNDF-promoted protection against Ang II–induced oxidative stress in RVLM. Microinjection bilaterally into RVLM of BNDF (100 or 250 ng) did not alter basal expression of cytosolic SOD1, mitochondrial SOD2 or extracellular SOD3 (Online Figure V, A through C) in WKY rats. BNDF (250 ng) or BNDF ASON (200 pmol) also did not affect the upregulated SOD1 or SOD2 expression detected 2 hours after Ang II (100 pmol) treatment (Online Figure V, A and B), nor the minimal effect of the octapeptide on SOD3 expression (Online Figure V, C). In a separate series of experiments, cytosolic SOD1 or mitochondrial SOD2 enzyme activity was similarly unaffected in WKY rats by microinjection bilaterally into RVLM of BNDF (100 or 250 ng), TrkB-Fc fusion protein (5 nmol), or BNDF ASON (200 pmol) or SON (200 pmol) (Online Figure V, D).
nmol) elicited minimal effect on basal tissue levels of O₂ in RVLM of WKY rats, detected at 1 or 24 hours after infection (Online Figure VI). The same treatments also did not significantly affect basal MAP (Online Figure VII) or heart rate (data not shown) over 24 hours.

**Discussion**

A dysregulation of ROS production and/or elimination in brain plays a pivotal role in the pathophysiology of a number of cardiovascular diseases, including stroke, heart failure, and hypertension. Given its significance in the regulation of cardiovascular hemodynamics, there is a clear need to gain better insights into mechanisms for the maintenance of ROS homeostasis. Toward this end, the present study unveils a novel role for BDNF/TrkB signaling in O₂ homeostasis in RVLM and the associated cardiovascular phenotype. To our knowledge, ours is the first report on active engagement of BDNF in oxidative stress–associated neurogenic hypertension via negative-feedback regulation of tissue O₂ levels in RVLM (Figure 8). Our results also indicated that disruption of BDNF/TrkB signaling is involved in the manifestation of oxidative stress–associated neurogenic hypertension in SHRs.

Apart from its well-characterized actions as a trophic factor, accumulating evidence suggests that BDNF exhibits “nontrophic” neuroprotective actions. Whether BDNF protects the brain against oxidative stress, however, is controversial. The present study provided in vivo data to support an

**Figure 5. BDNF restores the reduced mitochondrial electron transport capacity and upregulates mitochondrial UCP2 expression in RVLM of SHR.** BDNF level (A), NCCR or SCCR enzyme activity (B), superoxide production (C), and representative gels (inset) or densitometric analysis of UCP2 protein (D) in mitochondrial fraction from RVLM of WKY or SHRs, which received intracisternal infusion of BDNF (0.1 or 0.5 ng·µL⁻¹·h⁻¹) for 7 days, alone or with additional microinjection bilaterally into RVLM of TrkB-Fc or TrkA-Fc (5 nmol) on day 7 after infusion. Values are means±SEM of quadruplicate analyses on samples pooled from 4 to 5 animals in each group.

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antioxidative role for BDNF against oxidative stress in RVLM by functioning as a component of the feedback loop that maintains O$_2$ homeostasis. NADPH oxidase has been demonstrated to be a major cellular source of Ang II–elicited O$_2$ production in RVLM. Intriguingly, we found that acute and chronic Ang II treatment induced an AT1R-dependent and NADPH oxidase–sensitive upregulation of BDNF mRNA and protein expression in RVLM, and gene knockdown of BDNF further augmented the Ang II–induced O$_2$ production in RVLM. Confocal microscopic images further indicate that the NADPH oxidase–sensitive increases in BDNF protein expression are distributed in RVLM neurons. We confirmed that the green fluorescence signals that were not colocalized with the neuronal marker NeuN (neuron-specific nuclear protein) were genuine BDNF immunoreactivity in RVLM because the signal was absent in control brainstem sections in which anti-BDNF antiserum was omitted from the incubation medium. Because a recent study reported the expression of BDNF in primary rat astrocytes, antioxidative role for BDNF against oxidative stress in RVLM by functioning as a component of the feedback loop that maintains O$_2$ homeostasis. NADPH oxidase has been demonstrated to be a major cellular source of Ang II–elicited O$_2$ production in RVLM. Intriguingly, we found that acute and chronic Ang II treatment induced an AT1R-dependent and NADPH oxidase–sensitive upregulation of BDNF mRNA and protein expression in RVLM, and gene knockdown of BDNF further augmented the Ang II–induced O$_2$ production in RVLM. Confocal microscopic images further indicate that the NADPH oxidase–sensitive increases in BDNF protein expression are distributed in RVLM neurons. We confirmed that the green fluorescence signals that were not colocalized with the neuronal marker NeuN (neuron-specific nuclear protein) were genuine BDNF immunoreactivity in RVLM because the signal was absent in control brainstem sections in which anti-BDNF antiserum was omitted from the incubation medium. Because a recent study reported the expression of BDNF in primary rat astrocytes,
the implicated presence of BDNF in glial cells in RVLM cannot be excluded. Our differential results from treatments with TrkB-Fc and TrkA-Fc further suggest that the antioxidant effect of BDNF against Ang II–induced oxidative stress in RVLM is mediated via TrkB. Activation of TrkB has been demonstrated to be engaged in neuroprotection against cerebrovascular disease by BDNF. The negligible effect of ROS inhibition on basal BDNF expression in RVLM argues for activation of the BDNF/TrkB signaling when tissues are exposed to oxidative insults. However, the design of the present study does not allow us to preclude the possibility that the Ang II–induced BDNF upregulation might take place in other brain sites. A recent study based on microarray analysis of human adrenocortical cells showed that bdnf gene is one of the most upregulated by Ang II.

The precise mechanisms that underlie the redox-sensitive transcriptional regulation of BDNF in brain are not fully elucidated. We identified in the present study that one signaling molecule that interposes between Ang II–induced oxidative stress and transcriptional activation of BDNF in RVLM is CREB. We reported previously that the Ang II–induced increase in O$_2^*$ production in RVLM occurs within 10 minutes after infection. The temporal profiles demonstrated in the present study revealed that phosphorylation of CREB at Ser133 in RVLM took place 30 minutes after application of Ang II, followed by upregulation of BDNF mRNA transcription 4 hours later. A permissive role for CREB phosphorylation in mediating the redox-sensitive up-regulation of BDNF by Ang II was further ascertained by observations that treatment with NADPH oxidase inhibitor, p47phox ASGN or SOD mimetic attenuated the Ang II–induced phosphorylation of CREB. Moreover, gene knockdown of CREB expression significantly blunted the Ang II–promoted upregulation of BDNF mRNA. CREB is a key transcription factor for BDNF induction, and phosphorylation of Ser133 is a central event in the activation of CREB and CRE-dependent BDNF gene transcription. In transgenic mice in which CREB-mediated transcription is repressed, the neuroprotective effect of BDNF against ROS-mediated cytotoxicity is abrogated. Our findings of a minimal effect by CREB ASON treatment on basal BDNF expression again argues for a redox-sensitive activation of this transcription factor. In this regard, oxidants such as hydrogen peroxide initiates an influx of extracellular calcium that leads to CREB phosphorylation in lung epithelial cells.

Inhibition of enzymatic production of O$_2^*$ and/or potentiation of cellular antioxidant defense mechanisms lessens tissue oxidative stress. We demonstrated in the present study that BDNF may alleviate Ang II–induced elevation in O$_2^*$ levels in RVLM by at least 3 mechanisms. First, BDNF suppresses...
Ang II–induced phosphorylation of p47phox subunit of NADPH oxidase, with minimal effect on phosphorylation of p40phox or p67phox subunit or protein expression of membrane (gp91phox or p22phox) or cytosolic (p40phox, p47phox or p67phox) subunits. NADPH oxidase is a major enzyme for Ang II–elicited O$_2$\textsuperscript{−} production in RVLM,\textsuperscript{1,8} and its activation is a multistep process that is initiated by serine phosphorylation of the cytosolic regulatory p47phox subunit.\textsuperscript{8,37} We noted, however, that those in vivo observations contradict earlier in vitro studies\textsuperscript{15,38} in which BDNF reportedly activates NADPH oxidase. Differences in experimental design (in vivo against in vitro studies) and dose or duration of BDNF treatment may account for these discrepancies. Second, BDNF preserves the mitochondrial electron coupling capacity in RVLM. During normal cellular respiration, O$_2$\textsuperscript{−} is produced by a reaction between O$_2$ and electrons leaked from the mitochondrial electron transport chain. A reduction in the electron coupling capacity between complexes I and III or complexes II and III thus results in ROS generation,\textsuperscript{39} and contributes to chronic oxidative stress in RVLM that is associated with neurogenic hypertension.\textsuperscript{24} By preserving NCCR and SCCR activities in RVLM, the upregulated BDNF may thus exert a feedback regulation on cellular ROS level by decreasing mitochondrial O$_2$\textsuperscript{−} production. This notion is further supported by observations that there was a reduction in BDNF expression and NCCR and SCCR activity, alongside an augmented tissue level of O$_2$\textsuperscript{−} in RVLM of SHRs. Importantly, replacement of BDNF restored the reduced mitochondrial electron coupling capacity, resulting in amelioration of oxidative stress in RVLM of SHRs. BDNF has been demonstrated to increase respiratory coupling at complex I in pure rat brain mitochondrial preparations.\textsuperscript{40}

Third, BDNF upregulates the expression of mitochondrial antioxidant UCP2. UCP2 is a homolog of 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 a decrease in proton electrochemical gradient across the inner mitochondrial membrane and the resultant mitigation in the production of mitochondria-derived ROS, in particular O$_2$\textsuperscript{−}. We reported recently\textsuperscript{8} that transcriptional upregulation of mitochondrial UCP2 in response to oxidative stress plays an active role in feedback regulation of ROS production in RVLM. By potentiating the Ang II–induced upregulation of UCP2 expression, it is conceivable that BDNF may regulate ROS homeostasis by potentiating this antioxidant mechanism in RVLM. This notion is further supported by observations that an increase of BDNF upregulated UCP2 expression and ameliorated the augmented tissue level of O$_2$\textsuperscript{−} in RVLM of SHRs. The cellular mechanisms underlying BDNF-induced UCP2 upregulation await further elucidation. Our finding that both full-length and truncated TrkB receptor proteins are expressed in the mitochondrial fraction of tissue samples from RVLM suggests that BDNF may regulate UCP2 expression as a signaling molecule that targets the mitochondria. Our data, on the other hand, indicate that SODs may not be the targets of BDNF in the maintenance of ROS homeostasis because basal and Ang II–induced increases in protein expression and enzyme activity of SOD isoforms were not affected by the neurotrophin.

Accumulating evidence\textsuperscript{5–8,24} indicates that Ang II–induced oxidative stress in RVLM plays a pivotal role in neural mechanism of hypertension. We demonstrated in the present study that feedback regulation of ROS by the Ang II–induced upregulation of BDNF is functionally significant in central regulation of arterial pressure. Confusion of BDNF attenuated the long-term pressor response evoked by intracisternal infusion of Ang II. Gene knockdown of BDNF or blockade of TrkB, on the other hand, augmented the Ang II–induced hypertension. In RVLM of SHRs, which exhibits heightened tissue level of O$_2$\textsuperscript{−}\textsuperscript{6,24} BDNF that reached a tissue level comparable to that in RVLM of WKY rats on intracisternal infusion ameliorates chronic oxidative stress and promotes TrkB-dependent antihypertension. Our results thus provide novel evidence to support an ameliorative action of BDNF in RVLM on oxidative stress–associated neurogenic hypertension. The negligible effect of endogenous BDNF on basal arterial pressure further ascertains that the BDNF-evoked amelioration of neurogenic hypertension is not secondary to perturbation of basal hemodynamic parameters by the neurotrophin. We noted that whereas exogenous infusion of BDNF reduced the heightened tissue level of O$_2$\textsuperscript{−} in RVLM of SHRs to a level comparable to WKY rats, the same treatment promoted only a partial antihypertensive effect. These results are interpreted to suggest that the hypertensive phenotype in SHRs cannot be explained solely by a defect in BDNF-regulated ROS homeostasis in RVLM. In addition, whether BDNF participates in central cardiovascular regulation via actions on other brain regions also remains to be investigated. In this regard, BDNF is distributed in the nucleus tractus solitarii and is involved in postdevelopmental modification at first-order synapses in arterial baroreceptor pathways.\textsuperscript{41} BDNF also attenuates Ang II–induced and oxidative stress–associated facilitation of calcium channel\textsuperscript{42} in this nucleus.\textsuperscript{43}

We recognize that results obtained from experiments with exogenously administered Ang II are not necessarily applied to the observation in SHRs. In this regard, instead of an anticipated elevation of BDNF level in RVLM of SHRs, which exhibits an increase in tissue level of O$_2$\textsuperscript{−}\textsuperscript{6,26,27} we found that the protein expression of BDNF was comparable to that in WKY rats and was not affected by ROS inhibition; tissue level of BDNF was significantly less. These results are interpreted to imply a potential defect in oxidative stress–associated transcriptional upregulation of BDNF in RVLM of SHRs. Functional single nucleotide polymorphism of BDNF gene was recently reported to be associated with the development of cardiovascular disease.\textsuperscript{44,45} It is thus tempting to speculate that an inherited variations in BDNF gene in SHRs may render transcriptional regulation of this neurotrophic factor nonresponsive to oxidative stress. This speculation, however, awaits further validation.

In conclusion, the present study unveiled a novel role for BDNF in central control of arterial pressure in the form of negative-feedback alleviation of Ang II–induced sustained oxidative stress in RVLM and long-term pressor response. It has been shown that BDNF deficiency plays a role in the etiology of cardiovascular diseases.\textsuperscript{46} Our observations in
SHRs thus suggest the possibility that disruption of BDNF/TrkB signaling in RVLM may be an important cellular event in disease-dependent increases in oxidative stress. At the same time, our results raise the prospect that strategies focusing on augmentation of BDNF/TrkB cassette may prove clinically beneficial against oxidative stress–associated cardiovascular disorders.

Sources of Funding

This study was supported by National Science Council, Taiwan, Republic of China research grants NSC98-2321-B-182A-003 and NSC98-2321-B-182A-005 (to S.H.H.C.); NSC98-2811-B-075B-003 (to C.-W.J.W.); NSC98-2321-B-182A-006 (to A.Y.W.C.); NSC-98-2321-B-006-008 (to K.-S.H.); and NSC98-2321-B-075B-001 (to J.Y.H.C.).

Disclosures

None.

References

BDNF Regulates ROS Homeostasis in RVLM

What Is Known?

- The rostral ventrolateral medulla (RVLM) is intimately related to the maintenance of arterial pressure. In RVLM, oxidative stress elicited by superoxide anion (O$_2^-$) plays a pivotal role in neural regulation of hypertension by increasing sympathetic outflow to the peripheral blood vessels.
- Oxidative stress in RVLM is attributable to an increase in NADPH oxidase activity, reduction in mitochondrial electron coupling capacity, and downregulation of the mitochondrial uncoupling protein (UCP).
- One of the nontrophic actions of brain-derived neurotrophic factor (BDNF) exists in the form of antioxidant.

What New Information Does This Article Contribute?

- Activation of NADPH oxidase–derived O$_2^-$ by angiotensin II (Ang II) transcriptionally upregulates BDNF in RVLM via phosphorylation of the redox-sensitive cAMP response element binding protein (CREB).
- In RVLM, BDNF elicited by intracisternal infusion of Ang II attenuates the phosphorylation of NADPH oxidase, preserves mitochondrial electron coupling capacity, and upregulates UCP.
- BDNF confers protection against Ang II–induced long-term pressor response and promotes antihypertension in spontaneously hypertensive rats (SHRs).

Given the pivotal role of oxidative stress in RVLM in the pathophysiology of hypertension, a better understanding of mechanisms for the maintenance of O$_2^-$ homeostasis is of clinical significance. We report for the first time an active engagement of BDNF in oxidative stress–associated neurogenic hypertension via negative-feedback regulation of tissue O$_2^-$ levels in RVLM. In normotensive rats, we found that acute and chronic Ang II treatment induced a NADPH oxidase–sensitive upregulation of BDNF in RVLM via the transcription factor CREB. Gene knockdown of BDNF further augmented the Ang II–induced O$_2^-$ level in RVLM, indicating a feedback mechanism. We also found that that the upregulated BDNF could alleviate Ang II–induced oxidative stress in RVLM by at least 3 mechanisms: (1) suppression of Ang II–induced phosphorylation of p47phox subunit of NADPH oxidase; (2) preservation of the mitochondrial electron coupling capacity in RVLM; and (3) upregulation of UCP. Finally, we established that BDNF in RVLM protects against Ang II–induced long-term pressor response and exerts an antihypertensive effects in SHRs. Our results suggest that strategies to augment BDNF may prove clinically beneficial against oxidative stress–associated cardiovascular disorders, including hypertension.
Transcriptional Upregulation of Brain-Derived Neurotrophic Factor in Rostral Ventrolateral Medulla by Angiotensin II: Significance in Superoxide Homeostasis and Neural Regulation of Arterial Pressure
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Circ Res. 2010;107:1127-1139; originally published online September 2, 2010; doi: 10.1161/CIRCRESAHA.110.225573

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Transcriptional Upregulation of Brain-Derived Neurotrophic Factor in Rostral Ventrolateral Medulla by Angiotensin II: Role in Superoxide Homeostasis and Neural Regulation of Arterial Pressure

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Expanded Methods

Animals
Adult, male normotensive Wistar-Kyoto (WKY, n = 214) rats or spontaneously hypertensive (SHR, n = 67) 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.

Measurement of Systemic Arterial Pressure and Heart Rate by Radiotelemetry
Systemic arterial pressure (SAP) and heart rate (HR) were measured in rats under conscious conditions using a radiotelemetry system (Data Sciences International, Minneapolis, MN). For implantation of radiotelemetry receiver, rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). 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, IM) injection postoperatively to prevent infection. Only animals that showed progressive weight gain after the operation were used in subsequent experiments. For recording that continued for at least 7 days, the averaged mean SAP (MSAP) recorded 60 minutes every day between 13:00 and 15:00 was used as the daily value. For recording that continued for 24 hours, the MSAP recorded every 3 hours was averaged.
Microinjections of Test Agents into RVLM

Microinjection bilaterally of test agents into RVLM was carried out according to procedures described previously. For acute experiments that lasted for 60 minutes, animals were maintained under propofol (20 mg·kg⁻¹·h⁻¹) 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 (20-25 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. For animals that received two injections into RVLM during the course of the experiments, the wound was closed in layers following the completion of the first microinjection and animals were allowed to recover in individual cages. At various postinjection intervals, the animals were anesthetized again with pentobarbital sodium (50 mg/kg, IP), and the above procedures for microinjection were repeated. Only animals that showed steady gain in body weight were used in the subsequent experiments. SAP and HR were measured for various postinjection intervals under conscious condition using radiotelemetry.

The chemicals used included L-glutamate (Sigma-Aldrich, St. Louis, MO); angiotensin II (Ang II; Sigma-Aldrich); Ang II subtype 1 receptor antagonist, losartan
(Merck, Darmstadt, Germany); a NADPH oxidase inhibitor, apocynin (Calbiochem-Novabiochem, San Diego, CA); a stable, metal-independent, membrane permeable SOD mimetic that scavenges $O_2^-$, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol; Calbiochem-Novabiochem); recombinant human BDNF (Sigma-Aldrich); recombinant human tyrosine kinase A-(TrkA) or TrkB-Fc chimera (R&D Systems, Minneapolis, MN); and antisense (ASON) which sequester endogenously released TrkB putative ligands (BDNF or neurotrophin-4) and block the TrkB receptor, or sense (SON) oligonucleotide against cyclic AMP-response element binding protein (CREB) or BDNF (Genemed Biotechnologies, San Francisco, CA). Microinjection of artificial cerebrospinal fluid (aCSF) or 0.2% DMSO served as the vehicle and volume control.

**Intracisternal Implantation of Osmotic Minipump**

After obtaining baseline SAP for at least 2 days using radiotelemetry, animals were anesthetized with pentobarbital sodium (50 mg/kg, IP) for implantation of osmotic minipump into the cisterna magna. This procedure was performed under the guidance of a surgical microscope. A midline dorsal neck incision was made, and the dura mater between the foramen magnum and C1 lamina was exposed following dissection of muscles. The dura was perforated with a 22-gauge steel needle. After observation of CSF leakage from this hole, a PE-5 catheter (Clay Adams, Sparks, MD) was advanced for 5 mm into the cisterna magna. The catheter was sealed to the dura with tissue glue and incision was closed with layered sutures. The outer end of the catheter was connected to a micro-osmotic minipump (Alzet 1007D, DURECT Co., Cupertino, CA), which was placed under the skin in the neck region, for infusion of Ang II or BDNF at 0.5 $\mu$L/h for 7 days. Animals received procaine penicillin (1,000 IU, IM) 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.
Collection of Tissue Samples from RVLM

At various time intervals after experimental treatment, rats were killed with an overdose of pentobarbital sodium and perfused intracardially with warm saline. The brain was rapidly removed and immediately frozen on dry ice. Medulla oblongata covering RVLM was blocked between 0.5 and 1.5 rostral to the obex, which was adopted from the atlas of Watson and Paxinos\(^8\) and served as the anatomical landmark. 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) were collected by micropunches with a 1-mm inner diameter burr.\(^1\)\(^-\)\(^5\) Medullary 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 BDNF mRNA in the isolated RVLM was evaluated by RT real-time quantitative PCR.\(^5\) RT reaction was performed using a SuperScript Preamplification System (Invitrogen, Carlsbad, CA) 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 MgCl\(_2\) and 5 μM of each primer, together with 5 μL of purified DNA or negative control. The primer pairs for amplification of total BDNF cDNA (GenBank accession number M61178) were 5’-CCATAAAGGACGCGGACTTGTAC- 3’ for the forward primer, and 5’-AGACATGTTTGCZGGCATCCAGG’-3’ for the reverse.\(^9\) CREB cDNA (GenBank
accession number AB041522) were 5’-GCCTCTGGTGATGTACAAACATACC-3’ for the forward primer, and 5’-GGGAGGACGCCATAACAACCTC-3’ for the reverse. GAPDH cDNA (GenBank accession number 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 BDNF mRNA expression was determined by the fold-change analysis.

Isolation of Cytosolic and Mitochondrial Fractions

Tissue samples of RVLM were immediately placed in ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA 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. Solubilized proteins were centrifuged at 20000 g at 4°C for 15 minutes, and proteins in the supernatant were quantified by the Bradford assay with a protein assay kit (Bio-Rad, Hercules, CA, USA). Isolation of rat mitochondria was performed at 4°C and completed within 2 hours. Tissue samples were gently homogenized with a glass-glass homogenizer. Homogenates were centrifuged at 1400 g (25°C) for 5 minutes, and supernatants were collected and centrifuged at 11,000 g (4°C) for 20 minutes to pellet the mitochondria.
The supernatant was further centrifuged at 12,000 g (4°C) for 10 minutes to yield the cytosolic fraction. The purity of mitochondrial-rich fraction was verified by the expression of the mitochondrial inner membrane specific protein, cytochrome c oxidase (COX) or superoxide dismutase 2 (SOD2). SOD1, on the other hand, was mainly expressed in the cytosolic fraction. Protein in the mitochondrial or cytosolic extracts was estimated by the method of Bradford with a protein assay kit (Bio-Rad).

**Western Blot Analysis**

Total, cytosolic or mitochondrial protein extract from RVLM homogenates was used to analyze protein expression by Western blot. In brief, proteins (50 μg for total protein, 20 μg for cytosol, or 10 μg for mitochondria) 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 mouse monoclonal antiserum against cytochrome c oxidase (COX, 1:1000; Invitrogen, Carlsbad, CA), a rabbit polyclonal antiserum against BDNF (1:1000; Abcam, Cambridge, UK), full-length TrkB (1:1000; BD Biosciences, San Jose, CA) that avoids cross reactivity with TrkA or TrkC, or truncated TrkB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), CREB (1:2000; Calbiochem-Novabiochem), phosphorylated CREB (pCREB) (1:1000; Calbiochem-Novabiochem), SOD1-3 (1:1000; Cell Signaling, Danvers, MA), mitochondrial UCP2 (1:1000; Calbiochem-Novabiochem) or β-tubulin (1:1000; Cell Signaling). 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). Full-length and truncated TrkB proteins were identified based on its molecular size (full-length TrkB: 145 kDa; truncated TrkB: 95 kDa). 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 COX.
Immunoprecipitation of p47phox

Cytosolic fraction was isolated as described above. The NADPH oxidase subunit p47phox was immunoprecipitated with affinity-purified goat polyclonal antiserum coupled to protein G-agarose beads. 1 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. Western blot analysis of p47phox phosphorylation was assessed using an anti-phosphoserine antibody (1:1000; Sigma), and was carried out as described above.

Double Immunofluorescence Staining and Laser Confocal Microscopy

The procedures for double immunofluorescence staining were modified from those reported previously. 13,14 In brief, free-floating 30-μm sections of the medulla oblongata containing the RVLM were incubated with a rabbit polyclonal antiserum against BDNF (1:500; Abcam, Cambridge, UK), together with a mouse monoclonal antiserum against a specific neuron marker, 15 neuron-specific nuclear protein (NeuN) (1:1000; Chemicon, Temecula, CA). The sections were subsequently incubated concurrently with a goat anti-rabbit IgG conjugated with Alexa Fluor 488 for BDNF, or a goat anti-mouse IgG conjugated with Alexa Fluor 568 for NeuN. No specific immunoreactivity was observed in control sections, which were incubated without anti-BDNF antiserum or substituting anti-BDNF antiserum with normal rabbit serum. View under a Fluorview FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan), immunoreactivity for BDNF exhibited green fluorescence and NeuN manifested red fluorescence. The co-localization of red and green fluorescence on merged images indicated the presence of BDNF immunoreactivity in neurons.

Measurement of Superoxide Anion

Production of O₂⁻ in the RVLM was measured by the lucigenin-enhanced chemiluminescence (ECL) assay according to previously described and validated methods. 16 The RVLM tissue was homogenized in a 20 mM sodium phosphate buffer,
pH 7.4 that contains 0.01 mM EDTA by a glass-to-glass homogenizer. The homogenate was subjected to low speed centrifugation at 1000g for 10 minutes at 4°C to remove nuclei and unbroken cell debris. The pellet was discarded and the supernatant was obtained immediately for \( \text{O}_2^{•−} \) measurement. Background chemiluminescence in a buffer (2 ml) that contains lucigenin (5 \( \mu \)M) was measured for 5 minutes. An aliquot of 100 \( \mu \)l of the supernatant was then added, and chemiluminescence measured for 10 minutes at room temperature (Sirius Luminometer, Berthold, Germany). \( \text{O}_2^{•−} \) production was calculated and expressed as \( \mu \)mol/min/mg protein. Specificity for \( \text{O}_2^{•−} \) was determined by adding SOD (350 U/ml) into the incubation medium.

**Measurement of superoxide dismutase activity**

The activity of SOD1 or 2 in cytosolic or mitochondrial fractions from the RVLM was measured using a SOD assay kit (Calbiochem-Novabiochem). This assay kit utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine as the substrate. This reagent undergoes alkaline autoxidation, which is accelerated by SOD, and yields a chromophore that absorbs maximally at 525 nm. Total activity of SOD was measured according to manufacturer's instructions. A 50% inhibition is defined as one unit of SOD and the specific activity was expressed as U/mg total protein.

**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 \( \mu \)g of protein) was incubated in a mixture containing 50 mM \( \text{K}_2\text{HPO}_4 \) buffer, pH7.4, 1.5 mM KCN, 1 mM \( \beta \)-NADH, 20 \( \mu \)M 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.
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.³

**BDNF Determination by ELISA**

RVLM tissue was homogenized and centrifuged at 15,000 g for 15 minutes, and the supernatant was collected. Protein concentration was determined and adjusted to 5 mg/ml before being applied to the microtiter plates for BDNF quantification. The concentration of BDNF protein was assessed using a fluorometric two-site enzyme immunoassay (ELISA) kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 96-well immunoplates were coated overnight at 4°C with monoclonal anti-BDNF antiserum (100 μl/well) in carbonate coating buffer. Wells were washed the next day with 0.1% Triton X-100 in Tris buffered saline (TBST), and incubated with blocking and sample buffer for 1 hour at room temperature. Serial dilutions of recombinant BDNF standard (0-500 pg/ml) were added to duplicate wells in each plate to generate a standard curve. The plates were then washed three times with wash buffer, and supernatants of RVLM tissue homogenates were incubated with shaking in the coated wells (100 μl each) for 2 hours at room temperature. After additional washes, wells were incubated in a solution containing HRP conjugated polyclonal BDNF antibody and washed five times with TBST. Plates were then incubated in a solution containing HRP conjugated anti-mouse IgG antibody for 2.5 hours at room temperature. After another wash, the plates were incubated with a 3,3′,5,5′-tetramethyl benzidine/peroxidase substrate solution for 30 minutes and 1 N HCl was then added to the wells to stop the reaction. The colorimetric reaction product was measured at 450 nm using a microplate reader. BDNF concentration was
determined from the regression line for the standard BDNF that incubated under similar conditions in each assay. BDNF concentration was expressed as picograms per milliliter and all assays were performed in triplicate.

**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 $\geq$ 72 hours. Frozen 25-μm sections of the medulla oblongata were stained with Cresyl violet for histological verification of the location of microinjection sites.
References


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**Online Figure I.** Representative gels (inset) or densitometric analysis of results from Western blots showing basal expression of BDNF or α-tubulin protein in RVLM of normotensive Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR), alone or after microinjection bilaterally into RVLM of apocynin (2 nmole), losartan (2 nmole), artificial cerebrospinal fluid (aCSF) or antisense oligonucleotide (ASON) against BDNF (200 pmol). Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. No significant difference ($P > 0.05$) among groups in one-way ANOVA.
**Online Figure II.** Fold changes in CREB (A) or BDNF (B) mRNA in RVLM, detected at 24 hours after microinjection bilaterally into RVLM of a CREB or BDNF ASON (200 pmole). Values are mean ± SEM of quadruplicate analyses on samples pooled from 3 animals in each group. *P < 0.05 versus control group in the Scheffé multiple-range analysis.
Online Figure III. Representative gels (insets) or densitometric analysis of results from immunoprecipitation (IP) followed by immunoblot (IB) assays on phosphorylated serine residues or total p47^phox NADPH oxidase subunit in RVLM after microinjection bilaterally into RVLM of Ang II (100 pmole), alone or with additional treatment with BDNF ASON or BDNF ASON plus BDNF. Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P < 0.05 versus control group, and #P < 0.05 versus Ang II+BDNF ASON group in the Scheffé multiple-range analysis.
**Online Figure IV.** Densitometric analysis of results from Western blots showing changes in expression of p40\textsuperscript{phox}, p67\textsuperscript{phox}, gp91\textsuperscript{phox}, or p22\textsuperscript{phox} subunit of NADPH oxidase in RVLM after microinjection bilaterally into RVLM of BDNF (250 ng), BDNF ASON (200 pmol) or TrkB-Fc (5 nmol). Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. No significant difference (\(P > 0.05\)) among groups in one-way ANOVA.
Online Figure V. Representative gels (inset) or densitometric analysis of changes in superoxide dismutase (SOD) 1, 2 or 3 protein level (A,B,C) or enzyme activity of SOD 1 or 2 (D) respectively in cytosolic or mitochondrial fraction from RVLM after microinjection bilaterally into RVLM of BDNF (100 or 250 ng) or BDNF ASON, SON (A,B,C,D) or TrkB-Fc (D) alone or with additional treatment of Ang II (100 pmole). Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P < 0.05 versus control group in the Scheffé multiple-range analysis.
Online Figure VI. Tissue level of superoxide anion in RVLM, measured 1 hour or 24 hours after microinjection bilaterally into RVLM of Ang II (100 pmole), alone or with additional treatment of BDNF ASON or TrkB-Fc. Values are mean ± SEM, n = 6 to 8 animals in each group. No significant difference among groups in two ANOVA. Arrows in B denote time during which drug treatment was executed.
Online Figure VII. Temporal changes in MSAP measured by radiotelemetry under conscious conditions after microinjection bilaterally into RVLM of BDNF ASON (200 pmole) or TrkB-Fc fusion protein (5 nmole). Values are mean ± SEM, n = 6 to 8 animals in each group. No significant difference among groups in two ANOVA. Arrows denote time during which drug treatment was executed.