Mitochondria-Derived Reactive Oxygen Species and Vascular MAP Kinases

Comparison of Angiotensin II and Diazoxide

Shoji Kimura, Guo-Xing Zhang, Akira Nishiyama, Takatomi Shokoji, Li Yao, Yu-Yan Fan, Matlubur Rahman, Youichi Abe

Abstract—Reactive oxygen species (ROS) are key mediators in signal transduction of angiotensin II (Ang II). However, roles of vascular mitochondria, a major intracellular ROS source, in response to Ang II stimuli have not been elucidated. This study aimed to examine the involvement of mitochondria-derived ROS in the signaling pathway and the vasoconstrictor mechanism of Ang II. Using 5-hydroxydecanoate (5-HD; a specific inhibitor of mitochondrial ATP-sensitive potassium [mitoK<sub>ATP</sub>] channels) and tempol (a superoxide dismutase mimetic), the effects of Ang II and diazoxide (a mitoK<sub>ATP</sub> channel opener) were compared on reduct-sensitive mitogen-activated protein (MAP) kinase activation in rat vascular smooth muscle cells (RVSMCs) in vitro and in rat aorta in vivo. Stimulation of RVSMCs by Ang II or diazoxide increased phosphorylated MAP kinases (ERK1/2, p38, and JNK), as well as superoxide production, which were then suppressed by 5-HD pretreatment in a dose-dependent manner, except for ERK1/2 activation by Ang II. The same events were reproduced in rat aorta in vivo. Ang II–like diazoxide depolarized the mitochondrial membrane potential (ΔΨ<sub>M</sub>) of RVSMCs determined by JC-1 fluorescence, which was inhibited by 5-HD. 5-HD did not modulate Ang II–induced calcium mobilization in RVSMCs and did not affect on the vasoconstrictor effect in either acute or chronic phases of Ang II–induced hypertension. These results reveal that Ang II stimulates mitochondrial ROS production through the opening of mitoK<sub>ATP</sub> channels in the vasculature-like diazoxide, leading to reduction of ΔΨ<sub>M</sub> and reduct-sensitive activation of MAP kinase; however, generated ROS from mitochondria do not contribute to Ang II–induced vasoconstriction. (Hypertension. 2005;45:438-444.)

Key Words: angiotensin ■ oxidative stress

Reactive oxygen species (ROS) are recognized as mediators of vascular signal transduction and are involved in activation of mitogen-activated protein (MAP) kinases. Participation of ROS in blood pressure regulation has been shown in Ang II–induced hypertension and other hypertensive models. We recently demonstrated time-dependent transition of ROS sensitivity of Ang II in rats, in which in the early phase of Ang II infusion, high blood pressure does not depend on ROS production, but thereafter it shifts to being ROS-sensitive. In this study, it was also found that in the acute phase of Ang II infusion, MAP kinases are stimulated through a ROS sensitive mechanism.

Recent reports have supported the hypothesis that the enzyme NAD(P)H oxidase plays a major role as the most important source of superoxide anions in vascular cells and contributes significantly to the functional and structural alterations present in hypertension or atherosclerosis. It has been further proposed that NAD(P)H oxidase is essential for production of superoxide in response to Ang II stimuli to vascular tissues. Mice deficient in the p47<sup>phox</sup> gene showed significantly lower arterial blood pressure elevation during chronic Ang II infusion. Moreover, endothelial cells and vascular smooth muscle cells from p47<sup>phox</sup>-deficient mice had less responsiveness to Ang II stimuli in the production of ROS. These findings clearly indicate that superoxide production by Ang II is regulated by the NAD(P)H oxidase-dependent mechanism. However, these results do not take into consideration mitochondria, which provide another major ROS source.

Superoxide is produced in mitochondria by complexes I and III of the electron transport chain, and the rate of ROS synthesis can be modulated by mitochondrial inner membrane potential (ΔΨ<sub>M</sub>). Mitochondrial ATP-sensitive potassium (mitoK<sub>ATP</sub>) channel openers depolarize the ΔΨ<sub>M</sub> and stimulate mitochondrial ROS production. To examine the implication of mitochondria-derived ROS in Ang II–induced vasoconstriction and the vascular signaling pathway leading to MAP kinase activation, we examined the effects of 5-hydroxydecanoate (5-HD; a mitoK<sub>ATP</sub> channel inhibitor) on Ang II–induced vascular MAP (extracellular signal-regulated kinase [ERK]1/2, p38, and JNK) kinase activation, superoxide generation, and the ΔΨ<sub>M</sub>
as compared with diazoxide (a mitoK<sub>a</sub> channel opener). We further examined the effects of 5-HD and 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol; a membrane permeable radical scavenger)<sup>17,18</sup> on acutely and chronically Ang II–induced hypertension in in vitro conscious rats.

**Methods**

**Cell Culture**

Rat vascular smooth muscle cells (RVSMCs) prepared by the explant method from descending thoracic aortas of 4-week-old male Sprague–Dawley rats (CLEA; Osaka, Japan) were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum. RVSMCs were authenticated using immunohistochemical staining for smooth muscle α-actin (Sigma). Cultures showing 95% staining for α-actin between passages 5 and 7 were used. Before the experiments, cells were starved for 48 hours.

**Measurements of Superoxide Generation of RVSMCs**

Superoxide generation of RVSMCs was measured by the lucigenin chemiluminescence method and also visualized by dihydroethidium (DHE) fluorescence images. Methods are detailed in the online data supplement (available online at http://www.hypertensionaha.org).

**Visualization of Changes in ΔΨ<sub>m</sub> of RVSMCs**

Imaging study for ΔΨ<sub>m</sub> of RVSMC by JC-1 fluorescence is detailed in the online data supplement.

**Intracellular Calcium Measurements of RVSMCs**

Measurement of intracellular calcium of RVSMCs is detailed in the online data supplement.

**Measurements of Phosphorylation of ERK1/2, p38, and JNK MAP Kinases**

Measurements of phosphorylated levels of MAP kinases (ERK1/2, p38, and JNK) in the aorta and cultured RVSMCs were conducted as previously described.<sup>19</sup> As controls for protein loading and transfer to membrane, the total amounts of each MAP kinase in all groups were essentially constant, as determined by direct immunoblotting (data not shown).

**Animal Preparation**

Ten-week-old male Sprague–Dawley rats were used. Preparation of acutely and chronically Ang II–infused rats was described previously, and all hemodynamic measurements were performed on conscious rats.<sup>4,19</sup> In acute experiments, Ang II and diazoxide were given intravenously at a rate of 200 and 10 ng·kg<sup>−1</sup> per minute, respectively. Tempol (Sigma) was given at a priming dose of 30 mg·kg<sup>−1</sup>, followed by infusion at a rate of 0.5 mg·kg<sup>−1</sup> per minute. 5-HD (Sigma) was given at a dose of 10 mg·kg<sup>−1</sup> 5 minutes before the start of Ang II or diazoxide infusion. Arterial blood pressure was monitored continuously, and after 30 minutes rats were euthanized and the aorta taken, quickly frozen in liquid nitrogen (LN<sub>2</sub>), and then stored at −80°C. The effects of 5-HD and tempol on chronically Ang II–infused rats (for 14 days, 200 ng·kg<sup>−1</sup> per minute subcutaneous; by Alzet osmotic mini-pump) were examined similarly to the acute experiments. Measurements of lipid peroxidation levels in plasma were described previously. All the surgical and experimental procedures were performed according to the guidelines for the care and use of animals as established by Kagawa University.

**Statistical Analysis**

Values are expressed as the mean±SEM. Statistical significance between 2 groups was tested using 2-way ANOVA followed by Newman–Keuls test or unpaired 2-tailed Student t test as appropri-
ate, and values of \( P < 0.05 \) were considered to indicate statistical significance.

**Results**

5-HD and Tempol Suppress MAP Kinase Activation by Ang II or Diazoxide in RVSMCs

As shown in Figure 1A, a 30-minute exposure to 100 nmol/L Ang II increased phosphorylation of ERK1/2, p38, and JNK MAP kinases in RVSMCs. Among these, Ang II–induced augmentation of phosphorylated p38 and JNK MAP kinases was suppressed by 5-HD dose dependently. However, 5-HD suppressed that of ERK1/2 with less intensity, and only the highest dose of 5-HD showed significance. Similarly to 5-HD, tempol suppressed Ang II–induced augmentation of phosphorylated p38 and JNK MAP kinases, and that of phosphorylated ERK1/2 kinase less effectively. As shown in Figure 1B, a 30-minute exposure to 200 nmol/L diazoxide augmented phosphorylation of all MAP kinases in RVSMCs, and the magnitude of induction by this dose of diazoxide was similar to 100 nmol/L Ang II. It is of note that both 5-HD and tempol suppressed all MAP kinase phosphorylation augmented by diazoxide more effectively than that of Ang II. Treatment with 5-HD or tempol at the highest dose alone had no effect on basal MAP kinase activities in RVSMCs (data not shown).

5-HD Suppresses Stimulated Superoxide Generation by Ang II or Diazoxide in RVSMCs

To gain insights into the relation between mitochondrial ROS and vascular MAP kinase activation, we studied the effects of 5-HD on superoxide generation using RVSMCs. As shown in Figure 2A, Ang II and diazoxide increased superoxide generation during the incubation time determined by the lucigenin chemiluminescence method with a lucigenin concentration of 10 \( \mu \)mol/L. The enhanced superoxide generation by Ang II or diazoxide was suppressed by pretreatment with 5-HD in a dose-dependent manner. DHE fluorescent images of RVSMCs also support 5-HD–sensitive superoxide generation by Ang II or diazoxide in RVSMCs (Figure 2 B).

Ang II and Diazoxide Depolarize \( \Delta \Psi_M \) in RVSMCs

To address the possibility that increases of phosphorylated MAP kinase and superoxide generation by Ang II and diazoxide are related to mitochondrial functions, the changes in \( \Delta \Psi_M \) of living RVSMCs by exposure to Ang II or diazoxide were visualized using dual simultaneous detection confocal microscopy (Figure 3). Before stimulation, a single cell included both green (JC-1 monomer) and red (J-aggregate) mitochondria, indicating that they were composed of a wide range of \( \Delta \Psi_M \) for different mitochondria.20 The number of red mitochondria decreased after the exposure to 300 nmol/L Ang II and 200 \( \mu \)mol/L diazoxide. These changes were blunted by pretreatment with 5-HD, indicating that the depolarization of \( \Delta \Psi_M \) by Ang II stimuli was mediated through the opening of mitoK\(_{\text{ATP}}\) channels.

Effects of 5-HD on Intracellular Calcium Mobilization by Ang II in RVSMCs

The interaction between mitochondrial and calcium mobilization is an important issue under some pathological circumstance, such as hypoxia.21 We examined the effect of 5-HD on Ang II–induced calcium mobilization in RVSMCs (Figure 4). Cultured RVSMCs revealed 75±8 nmol/L as a baseline of \( [Ca^{2+}]_i \); 100 nmol/L Ang II induced a rapid increase of \( [Ca^{2+}]_i \), to
450±54 nmol/L, which was followed by a slow return toward baseline. Pretreatment with 5-HD (30 and 300 nmol/L), added 5 minutes before Ang II stimuli, had no significant effect on baseline or Ang II–induced rapid increase of [Ca\(^{2+}\)].

**5-HD and Tempol Suppress Vascular MAP Kinase Activation by Ang II and Diazoxide in Conscious Rats**

Using catheterized conscious rats, the effects of 5-HD and tempol on aortic MAP kinase activation by acutely administered Ang II or diazoxide were examined. As shown in the Table, arterial blood pressure was increased by 50 mm Hg soon after intravenous infusion of Ang II (200 ng·kg\(^{-1}\) per minute) and was decreased by 15 mm Hg during diazoxide infusion (10 ng·kg\(^{-1}\) per minute). Pretreatments of 5-HD (10 mg·kg\(^{-1}\)) and tempol (30 mg·kg\(^{-1}\)) did not modify any hemodynamic changes elicited by Ang II or diazoxide.

A 30-minute infusion of Ang II or diazoxide increased phosphorylation of aortic ERK1/2, p38, and JNK MAP kinases. As shown in Figure 5, augmentation of p38 and JNK MAP kinase phosphorylation by Ang II in the aorta was significantly suppressed by 5-HD, whereas that of ERK1/2 was not affected by 5-HD treatment. The augmented phosphorylation of these MAP kinases by diazoxide was completely normalized by treatment with 5-HD. Tempol effectively suppressed Ang II–induced and diazoxide-induced aortic MAP kinase activation.

**5-HD Does Not Affect Blood Pressure of Chronically Ang II-Induced Hypertension**

Previously, we demonstrated that hypertension of chronically Ang II–infused hypertensive rats is ROS dependent and that it can be normalized by tempol administration.\(^8\) To assess whether mitochondrial ROS is involved in the maintenance of chronic phase of Ang II hypertension, the effects of 5-HD were tested in chronically Ang II–infused hypertensive rats in which high arterial blood pressure (153±5 mm Hg) and
Figure 4. The effect of 5-HD on Ang II–induced intracellular calcium mobilization in RVSMCs. RVSMCs were incubated in normal Hanks solution (1 mmol/L Ca\(^{2+}\)). Different doses of 5-HD were given 5 minutes before exposure to Ang II (100 nmol/L). Closed and open columns indicate before and after Ang II exposure, respectively. Upper, Representative traces of experiments are shown. Data are presented as the mean±SEM obtained from 4 different experiments.

Figure 5. The effect of 5-HD and tempol on Ang II–induced or diazoxide-induced MAP kinase activation in the aorta of conscious rats. Aortas were taken 30 minutes after the start of infusion of Ang II (200 ng·kg\(^{-1}\)·per minute) or diazoxide (10 ng·kg\(^{-1}\)·per minute), 5-HD (10 mg·kg\(^{-1}\) intravenous) or tempol (30 mg·kg\(^{-1}\) intravenous) was administered 5 minutes before Ang II or diazoxide infusion. Top, Representative blots are shown. Bottom, Densitometric analysis of the phosphorylated forms of the MAP kinases. The mean value of each phosphorylated protein in control rats is represented as 1. Data are presented as the mean±SEM (n = 4 to 6). *P<0.05 vs control rats. †P<0.05 vs Ang II–infused or diazoxide–infused rats.

Table. Effects of Ang II and Diazoxide on Mean Blood Pressure Under Tempol or 5-HD Treatment

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control</th>
<th>Ang II</th>
<th>Diazoxide</th>
<th>Tempol + Ang II</th>
<th>Diazoxide + Ang II</th>
<th>5-HD</th>
<th>Ang II + 5-HD</th>
<th>Diazoxide + 5-HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>103±2</td>
<td>105±4</td>
<td>102±3</td>
<td>104±3</td>
<td>104±3</td>
<td>102±4</td>
<td>102±4</td>
<td>102±4</td>
</tr>
<tr>
<td>10</td>
<td>103±4</td>
<td>105±3</td>
<td>103±4</td>
<td>104±3</td>
<td>104±3</td>
<td>103±3</td>
<td>103±4</td>
<td>103±4</td>
</tr>
</tbody>
</table>

Discussion

The significant finding of the present study is that 5-HD, a specific inhibitor of mitoK\(_{ATP}\) channels, suppressed Ang II–induced redox-sensitive vascular MAP kinase activation, in particular, p38 and JNK MAP kinases in rat aorta in vivo and in cultured RVSMCs in vitro. These observations resemble the results of diazoxide treatment under both in vivo and in vitro conditions, indicating that acutely administered Ang II may activate mitoK\(_{ATP}\) channels and contribute to superoxide production in the vascular tissue. In support of this finding, we clearly demonstrated that Ang II reduced the ΔΨ\(_{M}\) of RVSMCs through the opening of mitoK\(_{ATP}\) channels in a similar manner to diazoxide.

We compared 2 stimulants, Ang II and diazoxide, because the latter is a mitochondrial-related compound and has been implicated in redox-sensitive phosphorylation and activation of ERK.\(^{22}\) Although Ang II and diazoxide have opposite actions for vascular tone, i.e., as a vasoconstrictor and a vasodilator, both can activate aortic MAP kinases. The action of diazoxide is nonspecific to mitoK\(_{ATP}\) channels, and it also activates the sarcolemmal KATP channel. In our in vivo experiments, diazoxide decreased arterial blood pressure, which was caused by the opening of sarcolemmal KATP channels. However, the increases of MAP kinase activation by diazoxide may be caused by its action on mitoK\(_{ATP}\) but not on sarcolemmal KATP channels, because the vasodilator effect of diazoxide was not affected by pretreatment with 5-HD, a specific inhibitor of mitoK\(_{ATP}\) channels. The
opening of mitoK_{ATP} channels by diazoxide increases superoxide generation in the heart.\textsuperscript{23} In this study, we confirmed that diazoxide did increase superoxide generation in RVSMCs, and that 5-HD suppressed the enhancing signals of superoxide by diazoxide, determined by lucigenin chemiluminescence and DHE fluorescence. In a related study, we found that 5-HD did not possess any radical scavenging activity (not shown), nor did it affect on activation of vascular NAD(P)H oxidase, a well-known target of Ang II (see the online data supplement). Thus, it seems likely that vascular mitoK_{ATP} channels are responsible for the production of superoxide by diazoxide and Ang II in vasculature, at least during the early phases of their actions. There have been few reports concerning the effects of Ang II on mitochondrial function; however, in this study we found a decrease in ΔΨ_{M} and augmented superoxide generation in response to Ang II stimuli of RVSMCs in a manner similar to that of diazoxide, supporting the hypothesis that the intracellular signaling of Ang II passes through mitochondrial functions, reaching redox-sensitive MAP kinase activation.\textsuperscript{1}

In regard to the sensitivity of MAP kinase activation to mitochondrial ROS, a difference was seen in the case of ERK1/2 as activated by either Ang II or diazoxide. Previously, we have shown that the increase of phosphorylated MAP kinases in rat aorta by Ang II was eliminated by tempol.\textsuperscript{19} In this study, diazoxide-induced activation of ERK, p38, and JNK MAP kinases was also tempol-quenchable in the in vivo experiment, indicating a critical role of ROS for the activation of these 3 MAP kinases in vasculature. Furthermore, we found that 5-HD suppressed the increase of all the phosphorylated MAP kinases by diazoxide, and those of p38 and JNK MAP kinases by Ang II, but not that of ERK1/2 by Ang II. Although we used a high dose of tempol to suppress Ang II–induced ERK1/2 activation in this study, the resistance of ERK1/2 to tempol treatment was also observed in the in vivo study.\textsuperscript{19} Taken together, these results suggest that mitochondrial-derived ROS could be responsible for the Ang II–induced stimuli of all MAP kinases, including ERK1/2. However, another pathway independent of mitochondrial ROS might be involved in the redox-sensitive activation of vascular ERK1/2.

Inappropriate production of ROS in vasculature contributes to the maintenance of hypertension. To assess the possibility that Ang II–induced enhancement of mitochondrial ROS generation contributes to Ang II–mediated vasoconstriction, we examined the effects of 5-HD on blood pressure of acutely and chronically Ang II–infused rats. We previously demonstrated that tempol normalized blood pressure of chronically Ang II–infused hypertensive rats, but this is not the case for acute vasoconstrictor effect of Ang II.\textsuperscript{8} However, the data from this study clearly demonstrated that 5-HD does not affect on vasoconstriction in either acutely or chronically administered Ang II, indicating that mitochondrial ROS do not participate in the vasoconstrictor mechanisms of Ang II–induced hypertension. Because a rapid increase of intracellular calcium is a determinant of vasoconstrictor effects of Ang II,\textsuperscript{24} we also tested the effects of 5-HD on intracellular calcium induction of RVSMCs by Ang II. To support our in vivo observation, we confirmed that the calcium mobilization in RVSMCs by Ang II was not affected by mitoK_{ATP} channel inhibition. Torrecillas et al suggested the contribution of H_{2}O_{2} in vasoconstrictor mechanisms of Ang II by showing that catalase prevented Ang II–induced myosin light chain phosphorylation and intracellular calcium mobilization of RVSMCs, and also inhibited Ang II–induced constriction of rat aortic rings.\textsuperscript{25} Waypa et al also have shown mitochondria-mediated calcium increases in hypoxia in pulmonary arterial myocytes through ROS generation from the electron transport chain.\textsuperscript{21} However, in our in vivo and in vitro data arising from this study indicated that mitochondria-derived ROS could not influence contractility of vasculature during the chronic phase of Ang II–induced hypertension.

In conclusion, we studied the involvement of mitochondria-derived ROS in intracellular signal transduction and vasoconstriction by Ang II stimuli in vivo and in vitro. Our results reveal that Ang II stimulates mitochondrial ROS generation through the opening of mitoK_{ATP} channels in the vasculature, as does diazoxide, leading to the reduction of ΔΨ_{M} and redox-sensitive activation of MAP kinases, especially p38 and JNK, and that ROS generated from mitochondria do not contribute to Ang II–induced vasoconstriction.

Figure 6. The effect of 5-HD and tempol on blood pressure and plasma lipid peroxidation in acutely (open columns) and chronically (closed columns) Ang II–infused rats. 5-HD (10 mg · kg\textsuperscript{-1}) or tempol (30 mg · kg\textsuperscript{-1}) was administered intravenously, and blood pressure was continuously measured. A, Mean blood pressure at 30 minutes after the drug administration. B, The levels of plasma lipid peroxidation. The blood was collected 30 minutes after the drug administration for analysis of plasma lipid peroxidation. Data are presented as the mean±SEM (n=4 to ~6). *P<0.05 vs control rats or sham-operated rats in acute and chronic experiments, respectively. †P<0.05 vs acutely Ang II–infused rats or chronic Ang II–infused rats.
Perspectives
The findings of the current study indicate an important association between Ang II and mitochondrial function in ROS generation of vasculature. Inhibition of the renin-angiotensin system attenuated age-associated changes in mitochondrial function. Mitochondria are a major subcellular source of ROS, which would promote oxidative damage to cell structures and functions. In this respect, studies using materials such as mitochondrial-deficient (p38) cells or cells separated from aged population should provide further understanding of Ang II physiology and assessments of therapeutic intervention with renin-angiotensin system inhibitors. In this study, we have not examined inhibitors of mitochondrial respiratory chains to identify the site of superoxide generation. It has been shown that rotenone, an inhibitor of mitochondrial function.26 Mitochondria are a major subcellular source of ROS generation of vasculature. Inhibition of the renin-angiotensin system attenuated age-associated changes in mitochondrial function.26 Mitochondria are a major subcellular source of ROS, which would promote oxidative damage to cell structures and functions. In this respect, studies using materials such as mitochondrial-deficient (p38) cells or cells separated from aged population should provide further understanding of Ang II physiology and assessments of therapeutic intervention with renin-angiotensin system inhibitors. In this study, we have not examined inhibitors of mitochondrial respiratory chains to identify the site of superoxide generation. It has been shown that rotenone, an inhibitor of complex I, had no effect on Ang II-induced p38 and JNK activation in human vascular smooth muscle cells. Further studies will be necessary to clarify the site of superoxide generation for redox-sensitive MAP kinase activation in vasculature.

Acknowledgments
This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan.

References
Mitochondria-Derived Reactive Oxygen Species and Vascular MAP Kinases: Comparison of Angiotensin II and Diazoxide
Shoji Kimura, Guo-Xing Zhang, Akira Nishiyama, Takatomi Shokoji, Li Yao, Yu-Yan Fan, Matlubur Rahman and Youichi Abe

Hypertension. 2005;45:438-444; originally published online February 7, 2005;
doi: 10.1161/01.HYP.0000157169.27818.ae

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/45/3/438

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2005/02/17/45.3.438.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Methods

Measurements of superoxide generation of RVSMC

Superoxide generation of RVSMC was measured by a lucigenin chemiluminescence method with a lucigenin concentration of 10 μmol/L. Suspended RASMC (~10^6 cells) were transferred to a glass tube in D-MEM (without phenol red). The chemiluminescence of the cell suspension was recorded every 30 seconds for 1 hour with a luminescence reader (BLR-301; Aloka). 5-HD was added 5 minutes before application of Ang II or diazoxide. Lucigenin chemiluminescence was expressed as counts per min per 10^6 cells. After measurements, tiron (20 mmol/L) was added to confirm that increases of lucigenin chemiluminescence by Ang II or diazoxide reflected superoxide production. It is noteworthy that tiron did not significantly reduce basal lucigenin chemiluminescence before stimulation. We also used dihydroethidium (DHE; Molecular Probes), a specific dye for superoxide, to visualize the increased generation of superoxide in RVSMC. DHE is oxidized by superoxide to ethidium that stains nuclei bright fluorescent red. The cells were incubated with 5 μg/mL DHE for 5 minutes at 37 °C. After washing the cells with PBS, DHE fluorescent images of RVSMC were visualized by excitation at 488 nm and emission at 530 nm.

Visualization of changes in [M] of RVSMC

For imaging study of [M], RVSMC plated on coverslips were incubated with 2 μg/mL JC-1 (Molecular Probes) for 2 minutes at 37°C. JC-1 enters mitochondria in proportion to the membrane potential and forms J-aggregates at the high intramitochondrial concentrations induced by higher [M] values. Formation of J-aggregates is associated with a Stoke’s shift in emission from 527 nm for the monomer to 590 nm for the J-aggregates. Fluorescent images were visualized by confocal microscopy (Bio-Rad) using an argon laser to excite JC-1 at 488 nm. Emissions were recorded simultaneously at 530 nm for JC-1 monomer and 590 nm for J-aggregates into independent detectors.1
Intracellular calcium measurements of RVSMC

RVSMC plated on coverslips were loaded with 2μmol/L fura-2 AM (Molecular Probes) for 20 minutes at 37°C in a Hank’s solution, then further incubated without fura-2 AM for 30 minutes. Intracellular calcium changes were monitored using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) at 510 nm with excitation wavelengths alternating between 340 and 380 nm. At the end of each experiment, the minimum and maximum ratios of fluorescence at 340 and 380 nm were determined by the addition of 50 μmol/L fura-2 in a Ca²⁺-free (2 m mol/L EGTA) buffer and a buffer containing 10 m mol/L [Ca²⁺], respectively. The intracellular calcium concentration was then calculated from the resulting data by ratio analysis according to the method of Grynkiewicz et al². All data were individually corrected for autofluorescence by ionic manganese (1 mmol/L Mn²⁺) quenching and each coverslip was calibrated separately.

Reference


Figure I  The effects of 5-HD and apocynin on membrane NADPH oxidase activity in the aorta of Ang II-infused rats. 5-HD was given intravenously 10 minutes before Ang II infusion (200ng·kg⁻¹ per minute) at a dose of 30 mg·kg⁻¹ over a period of 2 hours before Ang II. Apocynin was given at 10 mg·kg⁻¹ over a period of 2 hours before Ang II. The aorta was taken 30 minutes after Ang II infusion, and homogenized in ice-cold modified Krebs-HEPES buffer composed of (in mmol/L) NaCl 119, HEPES 20, KCl 4.6, CaCl₂ 1.2, KH₂PO₄ 0.4, MgSO₄ 1.0, Na₂HPO₄ 0.15, NaHCO₃ 25.0, glucose 5.5, Na₃VO₄ 0.1, and a protease inhibitor tablet (1 tablet per 25 mL of buffer) (pH 7.4). The post-mitochondrial fraction was further centrifuged at 100,000 g for 60 minutes at 4°C. The membrane fraction was incubated in Krebs-HEPES buffer including 10 µmol/L lucigenin at 37°C for 1 minute, then 100µmol/L NADPH was added to the reaction mixture. Chemiluminescence was measured with a luminescence reader (BLR-301; Aloka). Data are presented as the mean ± SEM (n=4 ~6). *: p<0.05 vs. control rats. †: p<0.05 vs. Ang II-infused rats.
**Figure II** The effect of 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron; Sigma) on blood pressure and plasma lipid peroxidation in acutely (open columns) and chronically (closed columns) Ang II-infused rats. Tiron (30 mg·kg⁻¹) was administered intravenously, and blood pressure was continuously measured. (a) Mean blood pressure at 30 minutes after drug administration. (b) Levels of plasma lipid peroxidation. Blood was collected 30 minutes after drug administration for analysis of plasma lipid peroxidation. Data are presented as the mean ± SEM (n=3 ~6). *: p<0.05 vs. control rats or sham operated rats in acute and chronic experiments, respectively. †: p<0.05 vs. acutely Ang II-infused rats or chronic Ang II-infused rats.