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Superoxide Mediates Angiotensin II–Induced Influx of Extracellular Calcium in Neural Cells

Matthew C. Zimmerman, Ram V. Sharma, Robin L. Davisson

Abstract—We recently demonstrated that superoxide ($O_2^{\bullet-}$) is a key signaling intermediate in central angiotensin II (Ang II)-elicited blood pressure and drinking responses, and that hypertension caused by systemic Ang II infusion involves oxidative stress in cardiovascular nuclei of the brain. Intracellular Ca^{2+} is known to play an important role in Ang II signaling in neurons, and it is also linked to reactive oxygen species mechanisms in neurons and other cell types. However, the potential cross-talk between Ang II, $O_2^{\bullet-}$, and Ca^{2+} in neural cells remains unknown. Using mouse neuroblastoma Neuro-2A cells, we tested the hypothesis that $O_2^{\bullet-}$ radicals are involved in the Ang II–induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neurons. Ang II caused a rapid time-dependent increase in $[Ca^{2+}]_i$ that was abolished in cells bathed in Ca^{2+}-free medium or by pretreatment with the nonspecific voltage-gated Ca^{2+} channel blocker $CdCl_2$, suggesting that voltage-sensitive Ca^{2+} channels are the primary source of Ang II–induced increases in $[Ca^{2+}]_i$ in this cell type. Overexpression of cytoplasm-targeted $O_2^{\bullet-}$ dismutase via an adenoviral vector (AdCuZnSOD) efficiently scavenged Ang II–induced increases in intracellular $O_2^{\bullet-}$ and markedly attenuated the increase in $[Ca^{2+}]_i$ caused by this peptide. Furthermore, adenoviral-mediated expression of a dominant-negative isoform of Rac1 (AdN17Rac1), a critical component for NADPH oxidase activation and $O_2^{\bullet-}$ production, significantly inhibited the increase in $[Ca^{2+}]_i$ after Ang II stimulation. These data provide the first evidence that $O_2^{\bullet-}$ is involved in the Ang II–stimulated influx of extracellular Ca^{2+} in neural cells and suggest a potential intracellular signaling mechanism involved in Ang II–mediated oxidant regulation of central neural control of blood pressure. (*Hypertension*. 2005; 45[part 2]:717-723.)

Key Words: calcium channels ■ oxidative stress ■ imaging ■ central nervous system ■ renin-angiotensin system

The best-known physiological effect of angiotensin II (Ang II) acting in the central nervous system (CNS) is modulation of body fluid and cardiovascular homeostasis. Ang II, acting primarily via Ang II type-1 (AT_1) receptors located in central cardiovascular control regions, causes increases in blood pressure,[1] water intake,[2] vasopressin release,[3] and sympathoexcitation.[1] Dysregulation of central angiotensinergic signaling mechanisms is associated with numerous pathological cardiovascular conditions.[4] Therefore, elucidating the intracellular signaling mechanisms by which Ang II modulates neuronal activity is critical to our understanding of central Ang II–dependent cardiovascular diseases such as hypertension and heart failure.

We have recently identified reactive oxygen species (ROS), particularly superoxide anions ($O_2^{\bullet-}$), as key signaling intermediates in Ang II–stimulated activation of CNS neurons. Increased scavenging of cytoplasmic $O_2^{\bullet-}$ in key cardiovascular regulatory nuclei in the brain causes marked attenuation of the pressor effects elicited by Ang II administered either directly into the CNS[5] or via the systemic circulation.[6] More recently, our studies have shown that a Rac1-activated NADPH oxidase complex is the primary source of Ang II–induced $O_2^{\bullet-}$ production in neurons.[7] Although these studies have identified an important functional role of $O_2^{\bullet-}$ in central Ang II–mediated cardiovascular responses, the signaling mechanisms of $O_2^{\bullet-}$ in Ang II–mediated neuronal activation remain to be identified.

A potential downstream target of Ang II–induced $O_2^{\bullet-}$ production may involve Ca^{2+} signaling. It is well established that Ang II stimulates an increase in total Ca^{2+} current and a decrease in total K^+ current,[8] which leads to an increase in neuronal firing rate by controlling action potential generation.[9,10] Interestingly, in neurons, it has been shown that ROS induce an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that has been linked to cellular toxicity and neurodegenerative diseases. In fact, it is postulated that ROS may alter numerous proteins involved in Ca^{2+} signaling and neuronal firing, including voltage-gated K^+ and Ca^{2+} channels.[11–13] Whereas these studies have provided important evidence for a signaling cascade that involve both ROS and

Ca^{2+} in neurotoxicity, the cross-talk between these two important intermediates in Ang II signaling mechanisms involved in the central regulation of cardiovascular function remains to be investigated.

The goal of this study was to address the hypothesis that Ang II–stimulated increases in neuronal $[Ca^{2+}]_i$ involves ROS generation. Using the mouse neuroblastoma cell line Neuro-2A, recently shown to express high levels of AT_1 receptors,[14] we modulated $O_2^{\bullet-}$ levels with adenoviral vectors encoding either the cytoplasm-localized superoxide dismutase (AdCuZnSOD) or a dominant-negative isoform of an essential component of NADPH oxidase activation, Rac1 (AdN17Rac1). Measuring Ang II–induced increases in $[Ca^{2+}]_i$ via Fura-2 ratio fluorescence imaging, our results show that Ang II–mediated Ca^{2+} influx through voltage-sensitive Ca^{2+} channels involves NADPH oxidase-derived $O_2^{\bullet-}$ production.

Materials and Methods

### Adenoviral Vectors and Gene Transfer

Neuro-2A cells obtained from ATCC (Manassas, Va) were grown on 25-mm coverslips, as described previously.[7] Cells were infected with recombinant adenoviral vectors encoding β-galactosidase (AdLacZ), cytoplasm-targeted superoxide dismutase (AdCuZnSOD), or the dominant-negative mutant of Rac1 (AdN17Rac1) 24 hours before experimentation. Each of these viral vectors were constructed and characterized as described in detail previously.[7,15]

$[Ca^{2+}]_i$ Measurements

Ang II–stimulated changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were assessed by Fura-2 fluorescence ratio imaging using a microscopic digital imaging system (Photon Technology International) as described previously.[16–18] Briefly, subconfluent Neuro-2A cells grown on 25-mm coverslips were loaded with the Ca^{2+}-specific dye Fura-2 by incubating with 1 μmol/L Fura-2AM (Molecular Probes) at 37°C for 30 minutes. To examine the role of AT_1 receptors, Neuro2A cells were treated with the AT_1 receptor antagonist losartan (10 μmol/L) for 30 minutes before Ang II stimulation. Next, to determine the role of extracellular Ca^{2+} influx in the Ang II–stimulated increase in $[Ca^{2+}]_i$, separate subsets of cells were bathed in Ca^{2+}-free Hank's balanced salt solution containing the Ca^{2+} chelator EGTA (2 mmol/L) for 2 to 5 minutes or in normal Ca^{2+}-containing Hank's balanced salt solution containing $CaCl_2$ (1.26 mmol/L) and stimulated with Ang II prepared in the respective medium. Additionally, separate cultures were pretreated with the nonspecific voltage-sensitive Ca^{2+} channel blocker $CdCl_2$ (125 μmol/L; Fisher Scientific, New Lawn, NJ) for 5 minutes before Ang II stimulation to block Ca^{2+} influx from voltage-gated Ca^{2+} channels. To determine the patency of intracellular Ca^{2+} stores in cells incubated in Ca^{2+}-free medium or pretreated with $CdCl_2$, cultures were treated with thapsigargin (5 μg/mL; Sigma-Aldrich Co, St. Louis, Mo) after Ang II stimulation. Thapsigargin is known to release Ca^{2+} from the endoplasmic reticulum by inhibiting Ca^{2+} ATPase.[19] Finally, to examine the role of intracellular $O_2^{\bullet-}$ in Ang II–stimulated increases in $[Ca^{2+}]_i$ in neurons, we targeted $O_2^{\bullet-}$ levels by two different modes. First, to scavenge cytosolic $O_2^{\bullet-}$, Neuro-2A cells were infected with AdCuZnSOD (100 pfu/cell) 24 hours before Ang II stimulation and $[Ca^{2+}]_i$ measurements. In separate cultures, cells were transduced 24 hours before Ang II treatment with AdN17Rac1 (100 pfu/cell) to inhibit Rac1-mediated NADPH oxidase assembly, activation, and subsequent $O_2^{\bullet-}$ production. In both experiments, to control for possible effects of the adenoviral vector itself, cells were treated with AdLacZ (100 pfu/cell).

Detection of $O_2^{\bullet-}$ Generation

Changes in $O_2^{\bullet-}$ generation in Neuro-2A cells after Ang II (5 μmol/L) were measured using the fluorogenic probe dihydro-
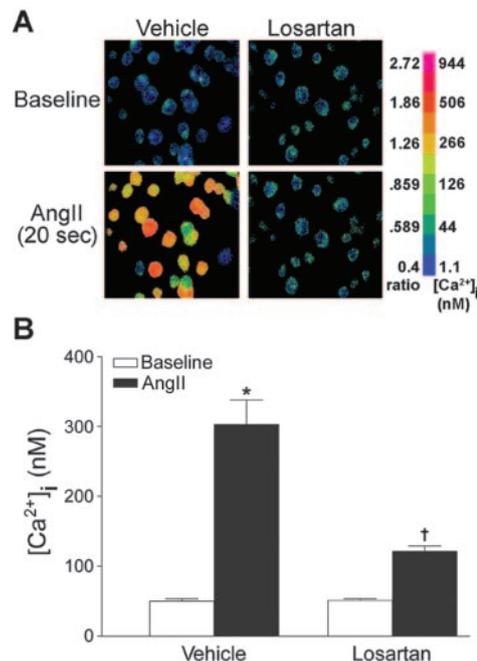
Figure 1. Ang II stimulates an increase in $[Ca^{2+}]_i$ in Neuro-2A cells. A, Representative pseudocolor 340/380-nm ratio images of Fura-2 fluorescence with computed $[Ca^{2+}]_i$ from coverslips pretreated for 30 minutes with either vehicle (n=29 cells) or losartan (10 μmol/L, n=17 cells) before (baseline) and 20 seconds after Ang II stimulation. The blue in the scale bar indicates low levels of $[Ca^{2+}]_i$, whereas the red depicts high levels of $[Ca^{2+}]_i$. B, Summary data (mean±SEM) of the basal and peak increase in $[Ca^{2+}]_i$ in cells stimulated with Ang II after 30 minutes of pretreatment with vehicle (n=90 cells from 4 coverslips) or losartan (n=124 cells from 5 coverslips). $[Ca^{2+}]_i$ values over the entire cell were averaged to obtain the changes in the whole cell Ca^{2+} concentration. $*P<0.05$ vs baseline, $\dagger P<0.05$ vs vehicle plus Ang II.

ethidium (DHE; 5 μmol/L, Molecular Probes, Inc) as described.[7] To confirm the fidelity of the assay, separate cultures were transfected with AdCuZnSOD (100 pfu/cell) 24 hours before DHE loading.

Statistics

Data were analyzed by Student t test when comparing only 2 groups and by ANOVA, followed by Newman–Keuls correction for multiple comparisons when comparing >2 groups. Data were expressed as mean±SEM and differences were considered significant at $P<0.05$.

Results

Ang II Stimulates an Increase in $[Ca^{2+}]_i$ in Neuro-2A Cells

A number of studies have shown that Ang II produces an increase in $[Ca^{2+}]_i$ in primary neurons cultured from the CNS.[8,16,17,20] Our initial experiments were designed to confirm this effect of Ang II in Neuro-2A cells. The rationale for choosing this cell line was 2-fold. First, these cells have been shown to express high levels of AT_1 receptors through rigorous expression quantification methods.[14] Second, our recent studies showed that Ang II induces an AT_1 receptor-dependent increase in $O_2^{\bullet-}$ generation in this cell line via NADPH oxidase activation.[7] Data presented in Figure 1A show the Ang II–elicited response of all cells in the field of view from a representative coverslip. Similar to the response

described in primary neurons cultured from the brain,[16,17,20] Ang II caused a time-dependent increase in $[Ca^{2+}]_i$ in Neuro-2A cells, which peaked between 20 to 30 seconds after stimulation and then returned to near basal levels by 60 seconds. This response was virtually abolished in cells pretreated with losartan (Figure 1A), suggesting that similar to primary neurons, the Ang II–induced increase in $[Ca^{2+}]_i$ in Neuro-2A cells is mediated by the AT_1 receptor. Summary data given in Figure 1B show that Ang II caused ≈3-fold increase in $[Ca^{2+}]_i$ (n=90 cells from 4 coverslips) in vehicle-treated cells. This response was significantly attenuated in cells pretreated with losartan (n=124 cells from 5 coverslips; $P<0.05$ versus Ang II alone). It should be noted that although basal $[Ca^{2+}]_i$ appears to be slightly different between the representative coverslips shown in Figure 1A, the average basal $[Ca^{2+}]_i$ from the 90 and 124 cells analyzed was similar in both groups of cells (50±4 nmol/L in vehicle-treated cells; 51±2 in losartan-treated cells, $P>0.05$, Figure 1B).

Ang II Increases Cytosolic Ca^{2+} by Stimulating an Influx of Extracellular Ca^{2+}

Although numerous studies have demonstrated that Ang II causes an increase in $[Ca^{2+}]_i$ in neurons cultured from the central nervous system, the Ca^{2+} pools that are mobilized by Ang II appear to vary.[8,16,17,20] To determine the source of $[Ca^{2+}]_i$ in Ang II–stimulated Neuro-2A cells, cells were bathed either in Ca^{2+}-free medium containing 2 mmol/L EGTA or in normal Ca^{2+}-containing medium. Similar to the data shown in Figure 1, Ang II caused a robust time-dependent increase in $[Ca^{2+}]_i$ between 20 and 30 seconds after incubation in Ca^{2+}-containing medium (n=25 cells from 1 representative coverslip; Figure 2A). This response was completely abolished in cells bathed in Ca^{2+}-free medium (n=37 cells from 1 representative coverslip; Figure 2A), suggesting that the Ang II–induced increase in $[Ca^{2+}]_i$ is caused by an influx of extracellular Ca^{2+}. To confirm that intracellular Ca^{2+} stores remained intact in cells bathed in Ca^{2+}-free medium, cells were treated with thapsigargin to release Ca^{2+} from internal stores after Ang II stimulation. Regardless of bathing medium, thapsigargin caused a similar increase in $[Ca^{2+}]_i$ (Figure 2A), demonstrating that Neuro-2A cells bathed in Ca^{2+}-free medium retain functional intracellular Ca^{2+} stores. Data summarized from 4 to 6 separate coverslips show that Ca^{2+}-free medium did not alter either the basal Ca^{2+} values or the thapsigargin-mediated increase in $[Ca^{2+}]_i$ (Figure 2B). In contrast, the Ang II–stimulated increase in peak $[Ca^{2+}]_i$ was completely abolished in Ca^{2+}-free medium compared with Ca^{2+}-containing medium (Figure 2B).
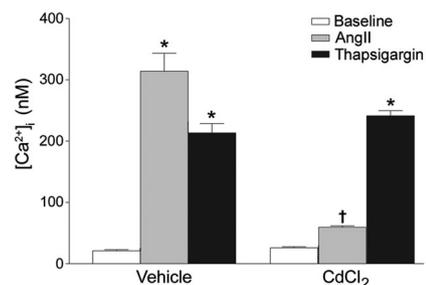
Ang II–Induced Influx of Extracellular Ca^{2+} Involves Voltage-Sensitive Channels

Although data presented in Figure 2 demonstrate that the Ang II–induced increase in $[Ca^{2+}]_i$ is extracellular Ca^{2+}-dependent, we sought to extend these studies and examine the role of voltage-gated Ca^{2+} channels in the Ang II–induced Ca^{2+} influx. It has been shown in neurons that Ang II–activated Ca^{2+} channels are inhibited by the nonspecific voltage-sensitive Ca^{2+} channel blocker Cd^{2+}.[9] As such, cultures were pretreated for 5 minutes with $CdCl_2$ (125 μmol/L) before Ang
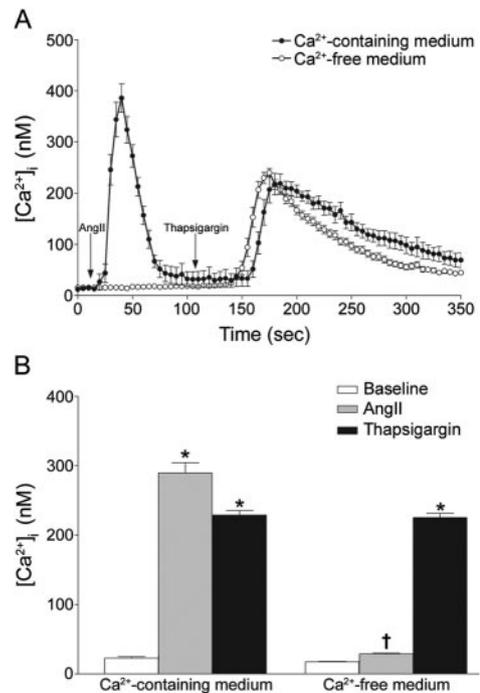

Figure 2. Ang II–stimulated increase in $[Ca^{2+}]_i$ in Neuro-2A cells is dependent on extracellular Ca^{2+}. A, Average data from one representative coverslip in each treatment group demonstrating the changes in $[Ca^{2+}]_i$ vs time in normal Ca^{2+}-containing medium (n=25 cells) or Ca^{2+}-free medium containing 2 mmol/L EGTA (n=37 cells) in response to Ang II stimulation. After $[Ca^{2+}]_i$ returned to baseline, cultures were treated with thapsigargin to confirm the functional integrity of intracellular Ca^{2+} stores in both treatment groups. B, Summary data (mean±SEM) of the basal and the peak increase in $[Ca^{2+}]_i$ after Ang II or thapsigargin stimulation in cells bathed in Ca^{2+}-containing (n=80 cells from 4 coverslips) or Ca^{2+}-free medium (n=138 cells from 6 coverslips). *$P<0.05$ vs baseline; †$P<0.05$ vs Ca^{2+}-containing medium plus Ang II.

II stimulation and $[Ca^{2+}]_i$ measurements. Summary data presented in Figure 3 show that the mean peak Ang II–induced increase in $[Ca^{2+}]_i$ was markedly attenuated by pretreatment with $CdCl_2$ (n=148 cells on 5 coverslips) compared with vehicle-treated cells (n=59 cells on 3 cover-

Figure 3. Ang II–stimulated influx of extracellular Ca^{2+} involves voltage-sensitive Ca^{2+} channels. Cells were treated with vehicle or $CdCl_2$ (125 μmol/L) for 5 minutes before Ang II stimulation. After the resolution of the Ang II response, cells were treated with thapsigargin to verify the integrity of intracellular Ca^{2+} stores in both groups. Summary data (mean±SEM) of the baseline $[Ca^{2+}]_i$ and the peak increase in $[Ca^{2+}]_i$ after Ang II or thapsigargin stimulation in cells pretreated with vehicle (n=59 cells on 3 coverslips) or $CdCl_2$ (n=148 cells on 5 coverslips). *$P<0.05$ vs baseline; †$P<0.05$ vs vehicle plus Ang II.

A

B
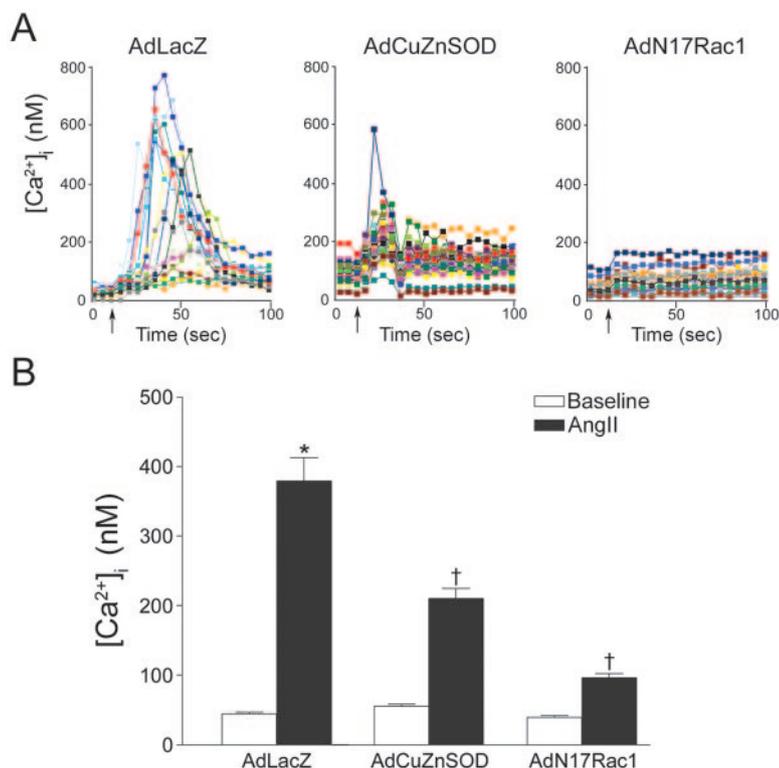
Figure 4. Superoxide is required for Ang II–stimulated influx of extracellular Ca^{2+} in Neuro-2A cells. A, Representative tracings of the changes in $[Ca^{2+}]_i$ vs time of all cells from one representative coverslip infected with AdLacZ (n=21), AdCuZnSOD (n=40), or AdN17Rac (n=29) 24 hours before Ang II stimulation. Each line represents the response of a single cell on the coverslip. Arrows indicate time of Ang II stimulation. B, Summary data (mean±SEM) showing baseline $[Ca^{2+}]_i$ and the peak increase in $[Ca^{2+}]_i$ after Ang II stimulation in cultures treated with AdLacZ (n=138 cells from 5 coverslips), AdCuZnSOD (n=146 cells from 5 coverslips), or AdN17Rac1 (n=136 cells from 5 coverslips) 24 hours earlier. *$P<0.05$ vs baseline; †$P<0.05$ vs AdLacZ plus Ang II.

slips; $P<0.05$). These data suggest that Ang II modulates voltage-sensitive Ca^{2+} channels, resulting in an influx of extracellular Ca^{2+} into Neuro-2A cells. To verify that $CdCl_2$ pretreatment does not affect internal Ca^{2+} stores, vehicle-treated or $CdCl_2$-treated cells were stimulated with thapsigargin after Ang II stimulation. Thapsigargin induced comparable increases in $[Ca^{2+}]_i$ in both groups of cells (Figure 3).

## Superoxide Mediates Ang II–Induced Influx of Ca^{2+} in Neuro-2A Cells

We have shown in primary neurons and in Neuro-2A cells that Ang II stimulates an increase in $O_2^{\bullet-}$ production.[5,7] Furthermore, a Rac1-activated NADPH oxidase plays an important role in this response in vitro, and is implicated in the in vivo effects of central Ang II on blood pressure.[7] To investigate whether there is a functional link between Ang II–stimulated increases in $O_2^{\bullet-}$ and $[Ca^{2+}]_i$ in neurons, Neuro-2A cells were infected with AdCuZnSOD to increase $O_2^{\bullet-}$ scavenging or AdN17Rac1 to inhibit NADPH oxidase activation and subsequent $O_2^{\bullet-}$ production. Overexpression of CuZnSOD caused a significant blunting of the Ang II–induced increase in $[Ca^{2+}]_i$ as seen in the tracings of all cells from one representative coverslip in Figure 4A (n=40 cells). Similarly, inhibition of NADPH oxidase activation by adenoviral expression of N17Rac1 nearly abolished Ang II–induced increases in $[Ca^{2+}]_i$ (n=29 cells on 1 coverslip; Figure 4A). Importantly, adenoviral infection itself did not affect the Ang II response, because AdLacZ-treated cells exhibited the same robust time-dependent increase in $[Ca^{2+}]_i$ (n=21 cells on 1 coverslip; Figure 4A), as seen in noninfected cells (Figure 1). Summary data in Figure 4B show that the peak Ang II–induced increase in $[Ca^{2+}]_i$ in control vector-infected cells (n=138 cells from 5 coverslips) was

markedly attenuated in both AdCuZnSOD-infected (n=146 cells from 5 coverslips; $P<0.05$ versus AdLacZ) and AdN17Rac1-infected cells (n=136 cells from 5 coverslips; $P<0.05$ versus AdLacZ). These data suggest that $O_2^{\bullet-}$ plays a key signaling role in Ang II–induced increases in $[Ca^{2+}]_i$ in Neuro-2A cells.

Ang II–Induced $O_2^{\bullet-}$ Production Is Not Dependent on an Increase in $[Ca^{2+}]_i$

Although data presented in Figure 4 suggest that Ang II–induced $O_2^{\bullet-}$ production stimulates an increase in $[Ca^{2+}]_i$ in Neuro-2A cells, previous studies in other cell types have reported that an increase in $[Ca^{2+}]_i$ leads to the production of ROS.[21,22] To determine the role of Ca^{2+} in $O_2^{\bullet-}$ generation in neurons stimulated with Ang II, and to investigate a potential feed-forward loop of $O_2^{\bullet-}$-induced Ca^{2+} and Ca^{2+}-induced $O_2^{\bullet-}$ production, we measured $O_2^{\bullet-}$ generation in Ang II–stimulated Neuro-2A cells using the DHE. As we have shown previously,[7] Ang II caused a significant time-dependent increase in DHE fluorescence in cells bathed in normal Ca^{2+}-containing medium (Figure 5). Interestingly, despite complete abolition of Ang II–induced increases in $[Ca^{2+}]_i$ by Ca^{2+}-free medium, cells bathed in this medium showed a similar increase in $O_2^{\bullet-}$ formation as cells bathed in normal medium. These data suggest that Ang II–stimulated increases in $O_2^{\bullet-}$ production in Neuro-2A cells are independent of changes in cytosolic Ca^{2+}. It should be noted that Ang II–induced increases in DHE fluorescence were significantly inhibited in cells pretreated with losartan, as we have previously reported,[7] and in cells infected with AdCuZnSOD 24 hours earlier (Figure 5), thus corroborating the fidelity of the assay for measuring $O_2^{\bullet-}$ levels. In addition, there was no

A

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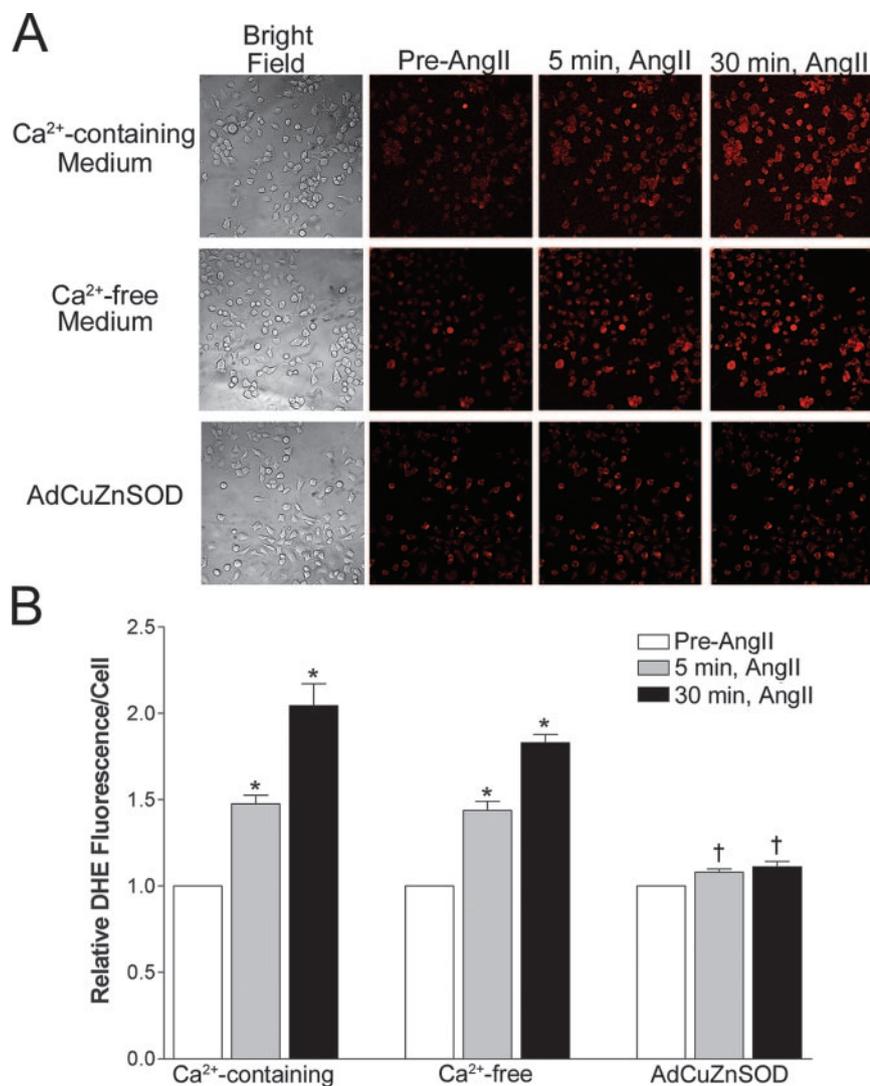
Figure 5. Ang II–induced O$_2$$^{•-}$ production in Neuro-2A cells is independent of changes in [Ca^{2+}]$_i$. A, Representative confocal images of DHE-loaded Neuro-2A cells, showing the effects of Ang II treatment after 5 and 30 minutes in cells bathed in normal Ca^{2+}-containing or Ca^{2+}-free medium. To demonstrate the specificity of DHE for O$_2$$^{•-}$ production, separate cultures were infected with AdCuZnSOD 24 hours before Ang II stimulation. B, Summary data of DHE fluorescence per cell before Ang II stimulation or after 5 and 30 minutes of Ang II treatment. Cells were bathed in Ca^{2+}-containing (n=142 cells, 5 plates) or Ca^{2+}-free medium (n=155 cells, 6 plates) during Ang II stimulation. As a control, additional cultures were treated with AdCuZnSOD (n=168 cells, 5 plates). Data are mean±SEM and expressed relative to DHE fluorescence before Ang II. *P<0.05 vs before Ang II; †P<0.05 vs Ca^{2+}-containing and Ca^{2+}-free medium at 5 or 30 minutes.

increase in DHE fluorescence over 30 minutes in vehicle-treated cells[7] (data not shown).

Discussion

We have previously identified O$_2$$^{•-}$ as a novel signaling intermediate in the actions of Ang II in CNS neurons.[5–7] Given the evidence that increased [Ca^{2+}]$_i$ plays a critical role in Ang II–stimulated neuronal activation,[23] we examined the cross-talk between O$_2$$^{•-}$ and Ca^{2+} in Neuro-2A cells stimulated with Ang II. Our results demonstrate that in this cell line, similar to what is seen in primary neurons from central cardiovascular regions, Ang II increases [Ca^{2+}]$_i$ by inducing an influx of extracellular Ca^{2+} through Cd^{2+}-sensitive Ca^{2+} channels. This response was markedly attenuated by gene transfer of either cytoplasm-targeted SOD or a dominant-negative isoform of Rac1 (AdN17Rac), an obligatory subunit for NADPH oxidase complex activation and O$_2$$^{•-}$ production. These data provide the first evidence that the Ang II–induced influx of extracellular Ca^{2+} in neuronal cells is mediated by increased O$_2$$^{•-}$ anion formation. A further important finding is that the increase in O$_2$$^{•-}$ production is independent of the associated Ca^{2+} entry. Together, these data suggest that O$_2$$^{•-}$

-induced modulation of Ca^{2+} influx is an important factor in Ang II signaling in neurons.

Our initial studies characterizing the Ang II–stimulated Ca^{2+} response in Neuro-2A cells were designed to establish the feasibility of using this cell line to study the interplay between Ang II, Ca^{2+}, and ROS in neurons. It has been shown by a number of investigators that Ang II stimulates an increase in [Ca^{2+}]$_i$ in primary neurons derived from key central cardiovascular control regions,[8] including the subfornical organ[17] and area postrema.[16] For example, Sumners et al have shown that Ang II stimulation of hypothalamic neurons leads to inositol 1,4,5-triphosphate generation and the subsequent increase in [Ca^{2+}]$_i$.[8] Gebke et al demonstrated that in neurons cultured from the subfornical organ or organum vasculosum of the lamina terminalis the Ang II–stimulated increase in [Ca^{2+}]$_i$ is dependent on the presence of extracellular Ca^{2+}.[20] In support of these findings, Sumners et al reported that Ang II–induced activation of neurons cultured from the hypothalamus and brain stem is, at least in part, caused by the stimulation of Cd^{2+}-sensitive voltage-gated Ca^{2+} channels.[8,9] These studies have been further extended with the identification of N-type Ca^{2+} channels as the primary

voltage-sensitive Ca^{2+} channel activated by Ang II in subfornical organ neurons.[24] Thus, there are striking similarities in the Ang II/Ca^{2+} mechanisms between primary neurons cultured from the CNS and the mouse neuroblastoma Neuro-2A cell line. This was important because our previous studies showing Ang II–stimulated ROS generation in neurons used mouse models, and we wanted to remain with this species for the current study. Although we have cultured primary neurons from cardiovascular control regions of mouse brain and we have shown Ang II–induced $O_2^{\bullet-}$ generation,[5] the limited number of cells obtained make this a difficult model system to employ for Ca^{2+} imaging studies. Moreover, the ease and efficiency of gene transfer is greater in this cell line compared with primary neurons. Therefore, Neuro-2A cells provide a good model for studying the role of $O_2^{\bullet-}$ in Ang II–induced Ca^{2+} responses.

Our previous evidence suggests that NADPH oxidase-derived intracellular $O_2^{\bullet-}$ anion generation in central cardiovascular sites is required for the blood pressure and dipsogenic effects of Ang II in the CNS.[5–7] Given the evidence that an increase in total Ca^{2+} current is involved in Ang II–stimulated neuronal activation,[8,9] we speculate that Ang II–induced $O_2^{\bullet-}$ production in central neurons is involved in Ang II–stimulated increases in $[Ca^{2+}]_i$ and the subsequent neuronal activation resulting in the physiological actions of Ang II in the brain. Recent studies by other groups lend support to this hypothesis. Sun et al showed that the $O_2^{\bullet-}$ scavenger Tempol or the NADPH oxidase inhibitor gp91ds-Tat markedly attenuated Ang II–mediated increases in neuronal firing rate of hypothalamic neurons.[25] In addition, Wang et al recently demonstrated that the Ang II–induced potentiation of L-type Ca^{2+} currents in neurons isolated from the nucleus of the solitary tract was blocked by $O_2^{\bullet-}$ scavenger MnTBAP, as well as by NADPH oxidase inhibitors gp91ds-Tat and apocynin.[26] These exciting new studies taken together with our current work strongly support the notion that NADPH oxidase-derived $O_2^{\bullet-}$ radicals are key signaling intermediates in the Ang II–induced increase in $[Ca^{2+}]_i$ and neuronal activation.

Although our present study demonstrates that Ang II–induced influx of extracellular Ca^{2+} in neurons involves $O_2^{\bullet-}$ production, we can only speculate about the mechanism by which this occurs. It is possible that $O_2^{\bullet-}$ itself acts on specific redox-sensitive amino acids of Ca^{2+} channels, thus altering either the opening or closing kinetics of the channels. ROS have been shown to increase neuronal Ca^{2+} current by regulating the opening of Ca^{2+} channels, possibly by oxidizing amino acid residues within the Ca^{2+} channel complex.[11] Moreover, Ciorba et al reported that the reversible oxidation of a methionine residue in a voltage-sensitive potassium channel modulated channel activity.[13] An alternative hypothesis is that $O_2^{\bullet-}$ and other ROS alter membrane phospholipids, thereby modifying the ionic conductance of channel proteins.[27,28] Further studies will be required to determine how ROS, particularly $O_2^{\bullet-}$, regulates Ang II–induced influx of extracellular Ca^{2+} and neuronal activation.

In addition to the notion that ROS induce increases in $[Ca^{2+}]_i$ in neurons, the reverse signaling mechanism has also been postulated, that is, ROS-induced Ca^{2+} leading to an increase in ROS in a feed-forward signaling loop. For example, Oyama et al demonstrated that H_2O_2 causes a dose-dependent increase in $[Ca^{2+}]_i$ in cerebellar neurons and that the Ca^{2+} ionophore ionomycin stimulated the production of ROS as measured by an increase in 2′,7′-dichlorofluorescin fluorescence.[21] Our DHE results demonstrating that Ang II stimulates an increase in $O_2^{\bullet-}$ production independent of Ang II–induced increases in $[Ca^{2+}]_i$ suggest this potential feed-forward loop does not operate in the context of Ang II responses in neurons.

Perspectives

This study provides direct evidence that Ang II–induced increases in $[Ca^{2+}]_i$ in neural cells are dependent on an increase in intracellular $O_2^{\bullet-}$ levels. Importantly, this increase in $O_2^{\bullet-}$ formation occurs independently of changes in cytosolic Ca^{2+} levels, suggesting that the radicals modulate Ca^{2+} entry. Although Neuro-2A cells provide an excellent model to study the intracellular signaling mechanisms of Ang II, $O_2^{\bullet-}$, and Ca^{2+} in neurons, future studies in primary neurons are needed to confirm and extend the results of the present study to help in translating these findings to CNS neurons in vivo. Studies are ongoing in our laboratory and others to determine the functional significance of this interplay between Ang II, ROS, and Ca^{2+} in neurons. Furthermore, experiments designed to explore the target(s) of superoxide in mediating the Ang II–induced increase in $[Ca^{2+}]_i$ in central neurons will be of particular interest. Nevertheless, $O_2^{\bullet-}$ production in the central nervous system may be an important new therapeutic target in cardiovascular diseases associated with increased central Ang II signaling and neuronal activation, including hypertension and heart failure.

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