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Requirement for Rac1-Dependent NADPH Oxidase in the Cardiovascular and Dipsogenic Actions of Angiotensin II in the Brain

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Abstract—We have shown that intracellular superoxide ($O_2^{·-}$) production in CNS neurons plays a key role in the pressor, bradycardic, and dipsogenic actions of Ang II in the brain. In this study, we tested the hypothesis that a Rac1-dependent NADPH oxidase is a key source of $O_2^{·-}$ in Ang II–sensitive neurons and is involved in these central Ang II–dependent effects. We performed both in vitro and in vivo studies using adenoviral (Ad)-mediated expression of dominant-negative Rac1 (AdN17Rac1) to inhibit Ang II–stimulated Rac1 activation, an obligatory step in NADPH oxidase activation. Ang II induced a time-dependent increase in Rac1 activation and $O_2^{·-}$ production in Neuro-2A cells, and this was abolished by pretreatment with AdN17Rac1 or the NADPH oxidase inhibitors apocynin or diphenylene iodonium. AdN17Rac1 also inhibited Ang II–induced increases in NADPH oxidase activity in primary neurons cultured from central cardiovascular control regions. In contrast, overexpression of wild-type Rac1 (AdwtRac1) caused more robust NADPH oxidase-dependent $O_2^{·-}$ production to Ang II. To extend the in vitro studies, the pressor, bradycardic, and drinking responses to intracerebroventricularly (ICV) injected Ang II were measured in mice that had undergone gene transfer of AdN17Rac1 or AdwtRac1 to the brain. AdN17Rac1 abolished the increase in blood pressure, decrease in heart rate, and drinking response induced by ICV injection of Ang II, whereas AdwtRac1 enhanced these physiological effects. The exaggerated physiological responses in AdwtRac1-treated mice were abolished by $O_2^{·-}$ scavenging. These results, for the first time, identify a Rac1-dependent NADPH oxidase as the source of central Ang II–induced $O_2^{·-}$ production, and implicate this oxidase in cardiovascular diseases associated with dysregulation of brain Ang II signaling, including hypertension. (*Circ Res*. 2004;95:532-539.)

Key Words: reactive oxygen species ■ dominant-negative Rac1 ■ blood pressure ■ dipsogenic response ■ neurons

Angiotensin II (Ang II), the primary effector peptide of the renin-angiotensin system, acts in the central nervous system (CNS) to modulate neurohumoral pathways involved in water and salt appetite, vasopressin release, and sympathoexcitation.[1] Because dysregulation of central angiotensinergic systems is strongly implicated in cardiovascular diseases such as hypertension and heart failure,[1] elucidating the precise signaling mechanisms of Ang II in the CNS is critical in understanding the pathogenesis of these disorders.

We recently identified superoxide ($O_2^{·-}$) as a key signaling intermediate in Ang II–mediated actions in CNS neurons.[2] Ang II increased $O_2^{·-}$ in isolated cells derived from cardiovascular regions of the brain and overexpression of cytoplasmically-targeted superoxide dismutase (SOD) in the subfornical organ (SFO), a key central cardiovascular control region rich in Ang II type I (AT_1) receptors, markedly attenuated the classical pressor, bradycardic, and dipsogenic

response pattern elicited by Ang II administered intracerebroventricularly (ICV).[2] Although this previous study demonstrated that $O_2^{·-}$ plays a critical role in central Ang II–mediated cardiovascular responses, the cellular source(s) of $O_2^{·-}$ in Ang II–sensitive central neurons remains to be determined.

Extensive work during the past decade has identified reactive oxygen species (ROS), including $O_2^{·-}$ and hydrogen peroxide (H_2O_2), as novel molecules in the intracellular signaling mechanisms of Ang II in peripheral cell types, with an NADPH oxidase as the primary source of $O_2^{·-}$ and H_2O_2 generation in these cells.[3] NADPH oxidase is a membrane-bound heterodimeric subunit composed of a catalytic subunit from the Nox family homologues, p22phox, and several regulatory proteins including p47phox, p67phox, and Rac1.[4] Assembly and activation of the oxidase requires stimulation of the small GTPase Rac1, which involves guanine nucleotide

exchange factor–mediated replacement of bound GDP for GTP.[5] Formation and activation of NADPH oxidase allows electrons to be passed from cofactor NADPH to molecular oxygen to produce $O_2^{.-}$. Ang II–induced ROS production via activation of NADPH oxidase is known to be involved in peripheral cell growth, contraction, and inflammation,[6] and dysregulation of redox-mechanisms in the periphery are implicated in the pathogenesis of hypertension caused by systemic Ang II–infusion.[7,8]

Recent studies have identified NADPH oxidase components in CNS neuronal cultures,[9,10] and the subunits have been localized to brain tissue of rodents.[11,12] Interestingly, it has been suggested that NADPH oxidase-derived $O_2^{.-}$ in central neurons plays a role in neuronal apoptosis,[10] ischemic stroke,[13] and neurodegenerative diseases including Parkinson's and Alzheimer's disease.[14] In addition to its role in pathophysiological conditions, NADPH oxidase–derived $O_2^{.-}$ at low levels may also be involved in normal physiological processes in the CNS. Suzukawa et al[9] reported that a Rac1-activated system, possibly the NADPH oxidase, stimulates ROS production that mediates nerve growth factor–induced neuronal differentiation.

Although each of the NADPH oxidase subunits, including Rac1, have been identified in various regions of the rodent brain, the role of the oxidase in central cardiovascular regions is unknown. Furthermore, although NADPH oxidase–derived ROS is implicated in Ang II signaling in peripheral cells, the role of the oxidase in central Ang II signaling remains unknown. In this study, we sought to determine the role of the NADPH oxidase in Ang II–mediated $O_2^{.-}$ production in neurons isolated from key cardiovascular control regions of the brain. In addition, we tested the hypothesis that central Ang II–induced cardiovascular and dipsogenic actions are mediated by Ang II–stimulated NADPH oxidase activation. Using genetic approaches to modulate NADPH oxidase activity in a series of in vitro and in vivo experiments, our results demonstrate that Ang II stimulates a Rac1-dependent NADPH oxidase to increase $O_2^{.-}$ in CNS neurons, and this signaling mechanism plays a critical role in the pressor, bradycardic, and drinking responses to centrally administered Ang II.

Materials and Methods

Rac1-dependent effects were manipulated using adenoviral vectors encoding a dominant-negative isoform (AdN17Rac1) or wild-type Rac1 (AdwtRac1). The effects of AdN17Rac1 and AdwtRac1 on Ang II–stimulated $O_2^{.-}$ production in cell culture and on Ang II–induced cardiovascular and dipsogenic actions in vivo were examined. What follows is a brief summary of the experimental protocols. A detailed description of all methods can be found in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

Rac1 Activity Assay

Rac1 activity after Ang II (1 μmol/L) stimulation for 1, 2, 5, or 15 minutes was measured in Neuro-2A cells using a Rac activation assay kit. In separate cultures, Rac1 activation was measured in cells pretreated with the Ang II type 1 receptor antagonist, losartan (10 μmol/L, 30 minutes), or infected with the control vector AdLacZ, AdN17Rac1, or AdwtRac1 (100 pfu/cell) 24 hours before Ang II stimulation (1 μmol/L, 1 minute).

Detection of $O_2^{.-}$ Production

Neuro-2A cells were infected with AdLacZ, AdN17Rac1, Adwt-Rac1, or AdCuZnSOD (100 pfu/cell) for 24 hours before loading the cells with dihydroethidium (DHE, 5 μmol/L) for 30 minutes. Additional cultures were treated with NADPH oxidase inhibitors diphenylene iodonium (DPI, 10 μmol/L, 60 minutes) or apocynin (100 μmol/L, 30 minutes). Separate cultures were pretreated with losartan (10 μmol/L) to inhibit AT$_1$ receptor activation. After collecting baseline images, cells were stimulated with Ang II (1 μmol/L) and the same cells were reimaged after 5 and 30 minutes. As a control, DHE fluorescence was measured in separate cultures incubated with vehicle. DHE fluorescence was quantified using Image J analysis software (version 1.31, NIH) and expressed relative to baseline fluorescence in individual cells.

Measurement of NADPH Oxidase Activity

Using $O_2^{.-}$-dependent lucigenin chemiluminescence, Ang II–induced NADPH oxidase activity was measured in primary neurons cultured from the lamina terminalis and infected with AdN17Rac1, Adwt-Rac1, or AdLacZ (100 pfu/cell). Separate cultures were stimulated with Ang II after 30 minutes of pretreatment with losartan (10 μmol/ L). NADPH (0.1 mmol/L) was added to cells after measurement of baseline lucigenin (5 μmol/L) chemiluminescence (relative light units, RLU), and RLU were recorded every 30 seconds for 5 minutes.

Physiological Studies

Adult C57BL/6 mice (Harlan, Indianapolis, Ind) were instrumented with intracerebroventricular (ICV) cannulae for central administration of adenoviruses and Ang II, and arterial catheters were implanted into the left carotid artery for direct measurement of mean arterial pressure (MAP) and heart rate (HR) as described.[2,15] Mice were injected ICV with either AdLacZ, AdN17Rac1, or AdwtRac1 (2×10^8 particles, 500 nL) on the day of surgery, and cardiovascular and dipsogenic responses to Ang II (200 ng, 200 nL) were recorded in conscious freely moving mice as described.[2,16] Separate groups of mice were coinfected (ICV) with AdwtRac1 and an adenovirus encoding either cytoplasm-targeted $O_2^{.-}$ dismutase (AdCuZnSOD) or AdLacZ. It should be noted that the total concentration and volume of virus given in the coinfection experiments were equal to that in the single viral infection studies. All procedures met or exceeded the guidelines set forth by the NIH and were approved by the University of Iowa Animal Care and Use Committee.

Western Blot Analysis of HA-Tagged Rac1 Constructs

To confirm adenovirus-mediated expression of the N17Rac1 and wtRac1 transgenes, brains were removed at the end of the physiological studies and micropunches from periventricular tissue, including Ang II–sensitive regions, were isolated and analyzed via Western blot analysis for the HA-tag expressed by both AdN17Rac1 and AdwtRac1.

Results

Ang II Stimulates Rac1 Activation in Neuro-2A Cells

We have recently identified $O_2^{.-}$ as an important signaling intermediate in central Ang II signaling[2]; however, the cellular source(s) of $O_2^{.-}$ production in Ang II–stimulated neurons remains unknown. Previous work in various peripheral cell types has identified a Rac1-dependent NADPH oxidase as a key source of ROS in response to Ang II stimulation.[3,17,18] In this study, we used a dominant-negative isoform of Rac1 (N17Rac1) to investigate the role of the NADPH oxidase in Ang II–mediated signaling in neurons. This strategy has been used extensively in other cell types to
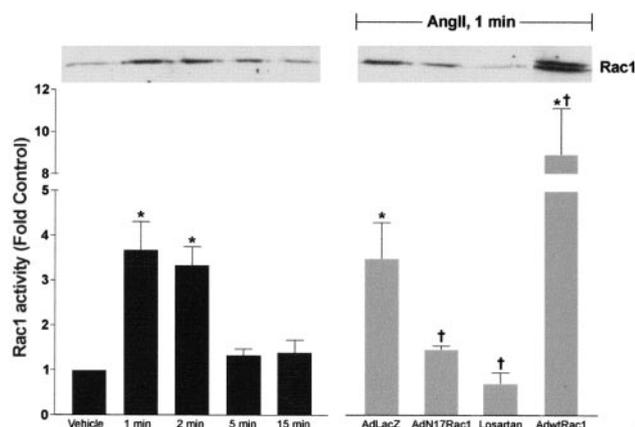
Figure 1. Dominant-negative Rac1 abolishes Ang II–induced Rac1 activation in Neuro-2A cells. Left, Representative Western blot and summary data showing time-dependent increases in Rac1 activation by Ang II (1 μmol/L) in cultured Neuro-2 cells. Vehicle-treated cells served as a control. Right, Representative Western blot and summary data showing the effects of adenovirus-mediated expression of dominant-negative (AdN17Rac1) or wild-type (AdwtRac1) isoforms of Rac1 on levels of activated Rac1 stimulated by 1 minute Ang II treatment. Noninfected cells were stimulated with Ang II alone (1 μmol/L, 1 minute) or subsequent to pretreatment with losartan (10 μmol/L, 30 minutes). Additional cultures were infected with control adenoviral vector encoding β-galactosidase (AdLacZ). Data are mean$\pm$SEM (n=3 to 6) and expressed relative to vehicle-treated cells. *P<0.05 vs vehicle, †P<0.05 vs Ang II (1 minute) and AdLacZ+Ang II.

study the role of NADPH oxidase.[17,19,20] First, to demonstrate that Ang II stimulates Rac1 activation in neurons, and that N17Rac1 is capable of inhibiting this response, Rac1 activation was measured in Neuro-2A cells that were left untreated (n=5) or infected with AdN17Rac1 (n=3), AdwtRac1 (n=3), or control vector AdLacZ (n=4) 24 hours earlier. Ang II induced a robust and time-dependent increase in Rac1 activation, with the maximum response occurring after 1 minute of stimulation (Figure 1, left panel). This Ang II–induced increase in Rac1 activation at 1 minute was markedly attenuated in cells infected with AdN17Rac1 compared with untreated or AdLacZ-infected cells, and the level of inhibition was similar to that produced by preincubation with the specific Ang II type 1 receptor antagonist, losartan (Figure 1, right panel). On the other hand, overexpression of wild-type Rac1 caused increased levels of the activated form of this small GTPase on Ang II stimulation. Importantly, cells treated with the control vector AdLacZ showed similar Ang II–induced levels of Rac1 activation compared with noninfected cells, thus confirming that the adenovirus itself does not alter Rac1 activation. Together, these data demonstrate that Ang II activates Rac1 in neural cells, and validate the use of AdN71Rac1 and AdwtRac1 to study Rac1 activation.
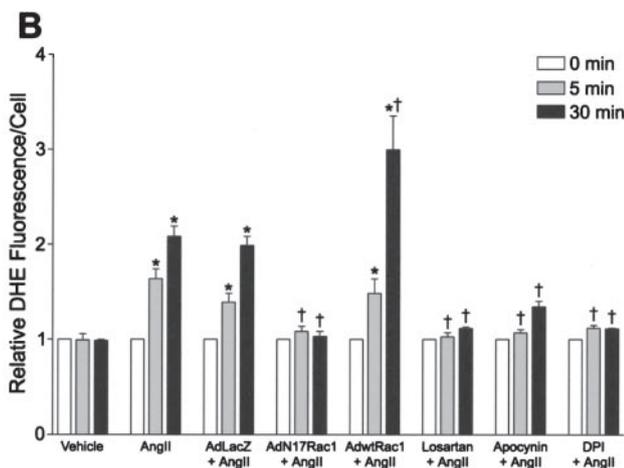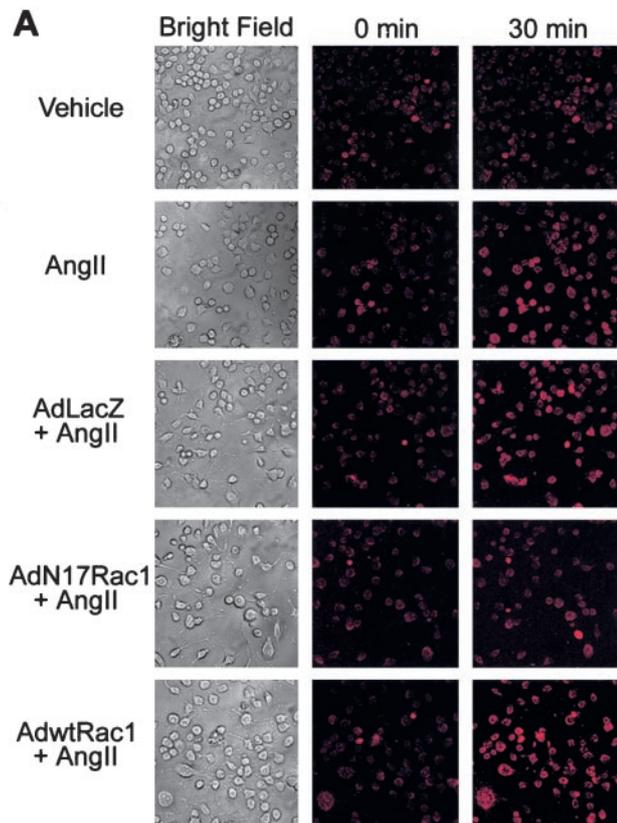
Rac1 Activation Is Essential for Ang II–Induced $O_2^{\cdot-}$ Production in Neuro-2A Cells

To link Ang II–stimulated Rac1 activation to $O_2^{\cdot-}$ production in Neuro-2A cells, we next examined the effect of AdN17Rac1 and AdwtRac1 on Ang II–induced dihydroethidium (DHE) staining. DHE is an oxidant-sensitive fluorogenic probe that is commonly used for monitoring intra-
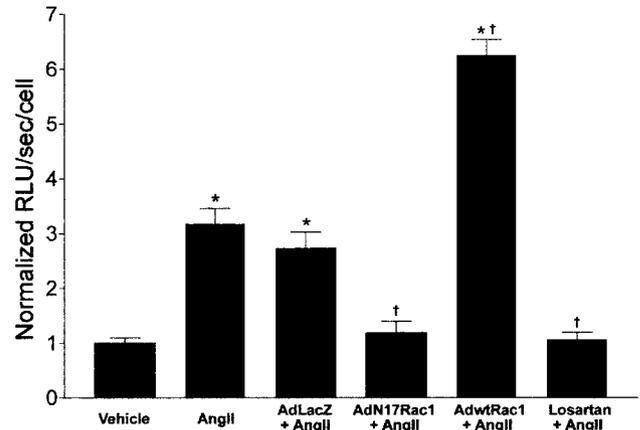
cellular $O_2^{\cdot-}$ levels.[2] Ang II caused a significant time-dependent increase in DHE fluorescence, indicating an increase in $O_2^{\cdot-}$ production in these neural cells (Figure 2). Summary data from 3 to 4 separate experiments show that DHE fluorescence was increased 1.6-fold after 5 minutes and 2.1-fold after 30 minutes of Ang II stimulation compared with baseline (0 minutes) fluorescence (n=163 cells, P<0.05 versus 0 minutes; Figure 2B). This response was abolished at both time-points in cells expressing N17Rac1 (n=116 cells), suggesting that Ang II–stimulated $O_2^{\cdot-}$ production in Neuro-2A cells is mediated by a Rac1-dependent mechanism, possibly NADPH oxidase activation. To provide further evidence for this, cells were also pretreated with the NADPH oxidase inhibitors apocynin (n=139 cells) or DPI (n=146 cells). As shown in Figure 2B, both of these reagents attenuated Ang II–stimulated increases in DHE fluorescence to a similar extent as N17Rac1-infected cells (P<0.05 versus Ang II alone). The Ang II–stimulated increase in $O_2^{\cdot-}$ production was mediated by the AT_1 receptor, as pretreatment with losartan (n=118 cells) also abolished the increases in DHE fluorescence at both time points (Figure 2B). Additionally, in cells overexpressing wild-type Rac1 (n=133 cells), the Ang II–stimulated increase in $O_2^{\cdot-}$ production was further augmented at 30 minutes compared with untreated or AdLacZ-treated cells (n=115 cells, P<0.05, Figure 2B). Importantly, DHE fluorescence in vehicle-treated cells (n=87 cells) did not change over the course of the experiment. Furthermore, the Ang II–induced increases in DHE fluorescence were virtually abolished after 5 minutes (1.08$\pm$0.01-fold increase versus 0 minutes) and 30 minutes (1.11$\pm$0.01-fold increase versus 0 minutes) in AdCuZnSOD-treated cells (n=167 cells, P<0.05), demonstrating the specificity of DHE for detecting $O_2^{\cdot-}$ levels.

Ang II Activates a Rac1-Dependent NADPH Oxidase in Neurons

To provide additional evidence that Ang II activates a Rac1-regulated NADPH oxidase in neurons, lucigenin-enhanced chemiluminescence was used to measure NADPH oxidase activity in primary neurons cultured from the lamina terminalis. This brain region is dense with AT_1 receptors and is known to be involved in cardiovascular regulation.[21] Ang II (n=11) caused a 3.2-fold increase in NADPH oxidase activity compared with vehicle-treated cells (n=16, P<0.05; Figure 3). Expression of N17Rac1 in these primary neurons (n=10) virtually abolished the Ang II–stimulated increases in NADPH oxidase activity, and this level of inhibition was similar to that induced by pretreatment with losartan (n=10). These data suggest that Ang II causes AT_1-dependent stimulation of NADPH oxidase in neurons, and that Rac1 activation is an obligatory step in this process. This is further supported by the finding that AdwtRac1 (n=9) caused a marked exaggeration of the Ang II effect, resulting in a 6.2-fold increase in NADPH oxidase activity (Figure 3). Cells infected with the control vector AdLacZ (n=10) showed a similar increase in oxidase activity compared with noninfected cells in response to Ang II (2.8-fold increase, P<0.05 versus vehicle), suggesting the viral vector itself cannot explain the results. Taken together with the results presented

Figure 3. Ang II increases Rac1-dependent NADPH oxidase activity in neurons. NADPH oxidase activity was measured using $O_2^{·-}$-dependent lucigenin chemiluminescence in primary neurons cultured from the lamina terminalis. Cells that were either left untreated or infected with AdLacZ, AdN17Rac1, or AdwtRac1 24 hours earlier were incubated with vehicle or Ang II (500 nmol/L, 2 minutes) and lucigenin chemiluminescence (relative light units, RLU) was measured after the addition of NADPH (0.1 mmol/L). Additional cultures were preincubated with losartan (10 μmol/L) for 30 minutes before Ang II stimulation. Data are expressed as mean±SEM (n=9 to 16) and normalized to RLU/second per cell in vehicle-treated cells. *$P<0.05$ vs vehicle; †$P<0.05$ vs Ang II and AdLacZ+Ang II.

in Figure 2, these data support the hypothesis that in neurons, Ang II–stimulated $O_2^{·-}$ production involves a Rac1-dependent NADPH oxidase.

Rac1-Activated NADPH Oxidase Is a Key Element of Central Ang II–Induced Cardiovascular and Dipsogenic Responses

Our in vitro studies using the Rac1 viruses suggest that Ang II–mediated activation of Rac1 is an obligatory step in the activation of the NADPH oxidase and subsequent $O_2^{·-}$ production in neurons. To determine the role of the oxidase in the central actions of Ang II in vivo, mice underwent brain gene transfer (AdN17Rac1, n=11; AdwtRac1, n=5; AdLacZ, n=7; saline, n=8) and 3 days later the pressor and bradycardic responses to ICV administered Ang II were recorded in conscious mice. As seen in the representative recordings, mice treated with saline or AdLacZ exhibited the characteristic pressor and bradycardic responses to ICV Ang II (Figure 4A). These cardiovascular effects were virtually abolished in mice overexpressing N17Rac1 in the brain (Figure 4A), whereas they were enhanced in mice overexpressing wtRac1 centrally. The peak changes in mean arterial pressure (MAP) and HR are summarized in Figure 4B, again demonstrating that expression of N17Rac1 in the brain completely abolished the pressor and bradycardic responses to ICV Ang II, whereas wtRac1 caused an augmentation of these cardiovascular responses compared with ICV saline or AdLacZ-treated mice. Taken together, these data suggest that central Ang II–mediated cardiovascular responses involve a Rac1-dependent signaling mechanism.

Although we have previously shown that $O_2^{·-}$ production in the brain is involved in the actions of central Ang II,[2] we wanted to provide more direct evidence linking Rac1-

Figure 2. Rac1 mediates Ang II–induced $O_2^{·-}$ production in Neuro-2A cells. A, Representative confocal images of DHE (5 μmol/L)-loaded Neuro-2A cells showing the effects of Ang II (1 μmol/L, 30 minutes) on $O_2^{·-}$ production in noninfected cells or cells infected with AdLacZ, AdN17Rac1, or AdwtRac1 (100 pfu/cell, 24 hours). As a control, DHE fluorescence was measured in cells incubated with vehicle only. B, Summary of relative DHE fluorescence in individual cells before Ang II stimulation (0 minutes) and after 5 and 30 minutes of Ang II treatment. Cells were stimulated with Ang II alone, with Ang II subsequent to preincubation with losartan (10 μmol/L), apocynin (100 μmol/L), or DPI (10 μmol/L), or with Ang II 24 hours after infection with AdLacZ, AdN17Rac1, or AdwtRac1. Vehicle-treated cells served as a control. Data are mean±SEM (n=87 to 163 cells from 3 to 4 separate experiments) and expressed relative to DHE fluorescence pre-Ang II (0 minutes). *$P<0.05$ vs 0 minutes; †$P<0.05$ vs Ang II and AdLacZ+Ang II at the respective time-point.
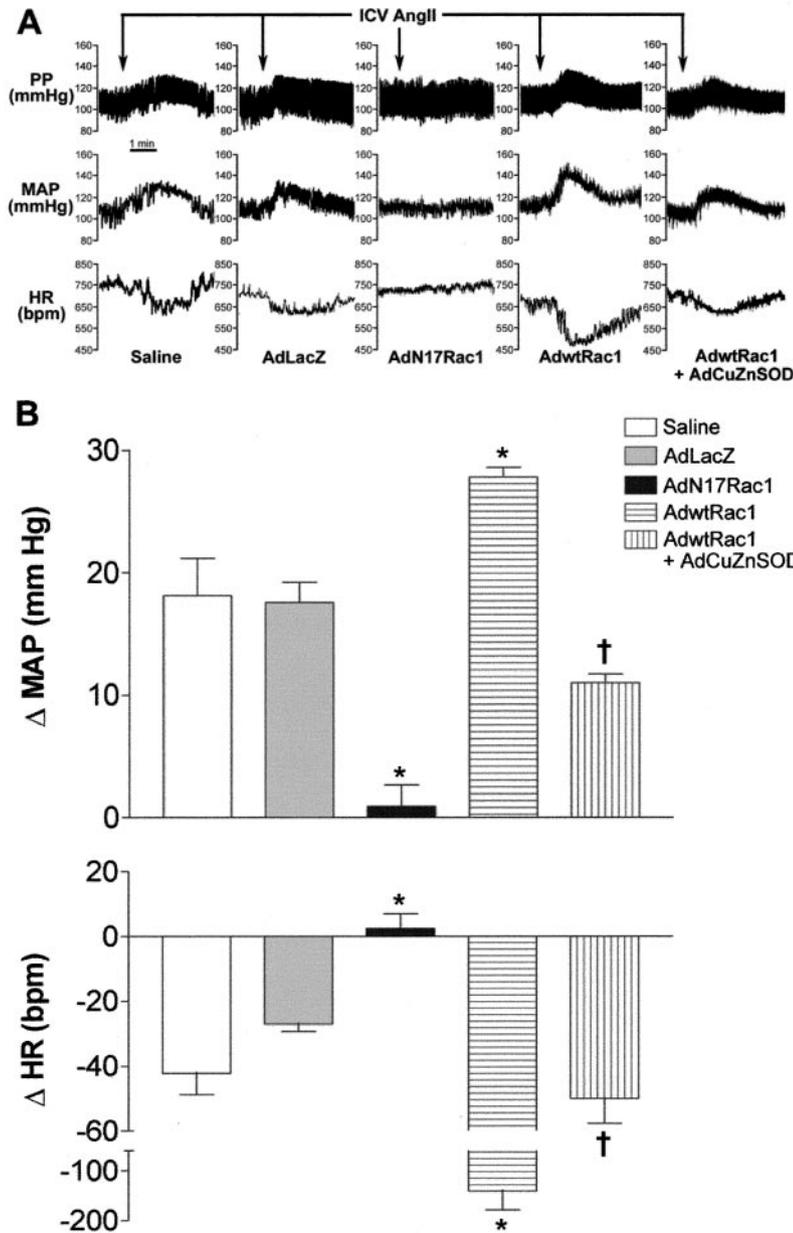
Figure 4. Rac1-activated NADPH oxidase is a key signaling component in the cardiovascular and dipsogenic actions central Ang II. A, Representative recordings of the effects of intracerebroventricular (ICV) Ang II (200 ng, 200 nL) on blood pressure and heart rate in mice that received saline, AdLacZ, AdN17Rac1, AdwtRac1, or AdwtRac1 plus AdCuZnSOD in the brain 3 days earlier. Arrows indicate Ang II injection. PP indicates pulsatile pressure; MAP, mean arterial pressure; HR, heart rate. B, Summary data of the peak change in MAP and HR in response to ICV Ang II in mice administered saline (n=8), AdLacZ (n=7), AdN17Rac1 (n=7), AdwtRac1 (n=5), or AdwtRac1 plus AdCuZnSOD (n=4) in the brain 3 days earlier. *$P<0.05$ vs saline and AdLacZ; †$P<0.05$ vs AdwtRac1.

dependent mechanisms to $O_2^{\cdot-}$ production in vivo. Therefore, Ang II–induced pressor and bradycardic responses were measured in mice (n=4) coinfected (ICV) with AdwtRac1 and AdCuZnSOD. As shown in Figure 4, the peak changes in MAP and HR following ICV Ang II administration were restored to normal in these mice compared with the augmented responses observed in mice treated with AdwtRac1 alone ($P<0.05$). To control for the coadministration of adenoviruses, a separate group of mice was infected with AdwtRac1 and AdLacZ. In these animals, the Ang II–induced pressor and bradycardic responses were not different from mice infected with AdwtRac1 alone (data not shown). These data suggest that the enhanced pressor and bradycardic response to ICV Ang II in mice infected with AdwtRac1 is, at least in part, attributable to $O_2^{\cdot-}$ production, and support the hypothesis that the central actions of Ang II involve Rac1-activated NADPH oxidase–derived $O_2^{\cdot-}$ generation.

In addition to its cardiovascular effects, centrally administered Ang II evokes a robust water intake response.[21] To determine the role of a Rac1-dependent NADPH oxidase in the potent dipsogenic actions of central Ang II, we also measured drinking behavior in a subset of the mice used for the cardiovascular studies (saline, n=8; AdLacZ, n=7; AdN17Rac1, n=7; AdwtRac1, n=5). Recorded for 30 minutes after Ang II injection (ICV), the total time spent drinking (seconds) is summarized in Figure 5. Saline- and AdLacZ-treated mice had a similar robust drinking response to central Ang II. However, in AdN7Rac1-treated mice, the ICV Ang II–evoked drinking response was significantly attenuated. On the other hand, overexpression of wild-type Rac1 in the brain caused an increase in the time spent drinking in response to Ang II compared with controls. Furthermore, similar to the cardiovascular responses, coinfection with AdwtRac1 and AdCuZnSOD significantly inhibited this augmented response
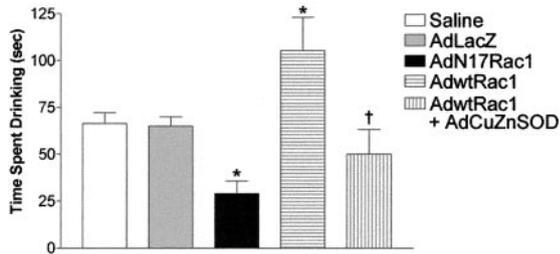
Figure 5. Central Ang II–mediated dipsogenic response involves Rac1-dependent $O_2^{.-}$ production. Summary of the drinking response to ICV Ang II (200 ng, 200 nL) in mice administered saline (n=8), AdLacZ (n=7), AdN17Rac1 (n=7), AdwtRac1 (n=5), or AdwtRac1 plus AdCuZnSOD (n=4) in the brain 3 days earlier. Data are expressed as the total time spent drinking (seconds) for 30 minutes after ICV injection of Ang II. *$P < 0.05$ vs saline and AdLacZ; †$P < 0.05$ vs AdwtRac1.

compared with mice infected with AdwtRac1 alone ($P < 0.05$), again providing a link between Rac1, NADPH oxidase, and Ang II signaling. It should be noted that spontaneous water drinking is virtually absent during daylight when the experiments were performed. Furthermore, adenoviral expression did not affect this baseline drinking behavior (data not shown).

Efficient Adenovirus-Mediated N17Rac1 and wtRac1 Transgene Expression in the Brain

Finally, to confirm efficient adenoviral-mediated expression of N17Rac1 and wtRac1 in the brains of mice administered the respective viral vectors, Western blot analysis for the HA-epitope tag expressed by both AdN17Rac1 and AdwtRac1 was performed. As shown in the representative blot in Figure 6, brain tissue from AdN17Rac1- and AdwtRac1-treated mice revealed robust expression of the HA tag, thus demonstrating adenoviral-mediated transgene expression. As expected, no HA expression was detected in tissue from ICV saline and AdLacZ-treated mice. To ensure equal protein loading, β-actin expression was measured (Figure 6).

Discussion

We previously identified $O_2^{.-}$ as a novel intermediate in central Ang II signaling, and demonstrated that $O_2^{.-}$ scaveng-
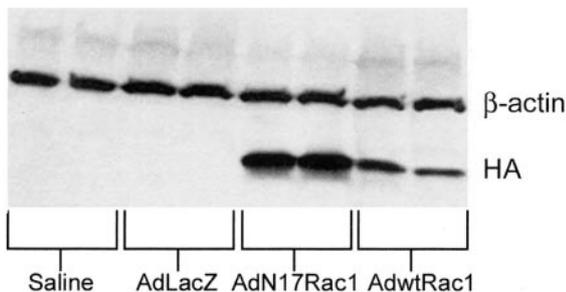
Figure 6. Adenovirus-mediated expression of N17Rac1 and wtRac1 in periventricular brain tissue. Representative Western blot showing adenoviral mediated expression of the HA epitope tag, inserted into the AdN17Rac1 and AdwtRac1 genome, in periventricular brain tissue microdissected from mice ICV administered saline, AdLacZ, AdN17Rac1, or AdwtRac1 three days earlier. To ensure equal loading, expression of β-actin was simultaneously measured.

ing via adenovirus-mediated SOD expression in the brain, predominantly in the subfornical organ (SFO), markedly attenuates the cardiovascular and dipsogenic action of ICV-administered Ang II.[2] In the present study, we provide the first direct evidence that a Rac1-dependent NADPH oxidase is a key source of Ang II–stimulated $O_2^{.-}$ production in neurons. By using an adenoviral vector encoding a dominant-negative isoform of Rac1, we have shown that inhibition of NADPH oxidase activity inhibits Ang II–stimulated $O_2^{.-}$ production in neural cells and abolishes central Ang II–mediated pressor, bradycardic, and dipsogenic responses in vivo. Furthermore, these responses were enhanced when NADPH oxidase activity was increased by ectopically expressing wild-type Rac1. Taken together, our data suggest that a Rac1-dependent NADPH oxidase is a key component of central Ang II–signaling involved in regulating cardiovascular function and body fluid homeostasis.

In the present study, we used a dominant-negative Rac1 strategy to tease out the role of a Rac1-stimulated NADPH oxidase in the signaling mechanism of Ang II in the CNS. A similar strategy has been used in other cell types to determine the role of this small GTPase in Ang II signaling pathways and Ang II–mediated physiological responses.[17,22] For example, in cardiac fibroblasts, N17Rac1 abolished the Ang II–induced activation of c-Jun NH_2-terminal kinase (JNK).[22] Most recently, Gorin et al[17] demonstrated that Ang II activates Rac1 in a rapid, time-dependent fashion in mesangial cells, which was abolished by expression of N17Rac1. These studies, taken together with our current results showing that N17Rac1 attenuates Ang II–induced Rac1 activation in Neuro-2A cells, support the use of N17Rac1 to determine the role of Rac1 in Ang II–stimulated signaling mechanisms.

Our choice of the Neuro-2A cell line for a subset of studies was based on a recent report by Hoffmann and Cool[23] that used quantitative analyses to demonstrate that these cells express both of the subtypes of the AT_1 receptor (AT_{1A} and AT_{1B}) along with AT2 receptors. Although there are a number of other neuronal cell lines that have been used to study Ang II signaling in neurons, eg, PC12, N1E-115, and NG108–15, there are controversies and limitations associated with each of them. For example, PC12 cells express only AT_2 receptors,[24] whereas in N1E-115 and NG108–15 there are conflicting reports concerning the expression of both AT_1 and AT_2.[25,26] To our knowledge, analysis of the AT_1 receptor subtypes has not been performed in these other cell lines and given the importance of both AT_{1A} and AT_{1B} in central Ang II actions,[16] we thought it important to use a cell line in which both subtypes have been identified. As such, the Neuro-2A cell line provides an excellent model system to study Ang II signaling in neurons.

Although it is known that Rac1 is involved in numerous cell signaling pathways, the primary function of Rac1 in Ang II–induced ROS generation is believed to be through the activation of the NADPH oxidase complex.[4] Studies in cell-free systems have demonstrated that Rac1 is an obligatory subunit of NADPH oxidase activation,[27] and N17Rac1 was shown to attenuate Ang II–induced ROS generation in mesangial and vascular smooth muscle cells (VSMCs) through the inhibition of a Rac1-regulated NADPH oxi-

dase.[6,17] In neurons, a role for Rac1-stimulated NADPH oxidase-derived ROS production has been demonstrated by the N17Rac1-mediated attenuation of ROS generation in nerve growth factor–stimulated PC12 cells.[9] In this study, we have shown a similar effect of N17Rac1 on Ang II–induced $O_2^{.-}$ production in neurons. Further converging evidence that this oxidase is involved is the finding that Ang II–stimulated $O_2^{.-}$ production was also markedly attenuated by apocycin or DPI, two commonly used NADPH oxidase inhibitors. In addition, $O_2^{.-}$ scavenging with AdCuZnSOD prevented the enhanced cardiovascular and dipsogenic responses to Ang II observed in AdwtRac1-treated mice, providing a further link between Rac1, the oxidase, and the actions of central Ang II in vivo.

Although the present study supports the hypothesis that a Rac1-activated NADPH oxidase is involved in the intracellular signaling mechanism of Ang II in neurons, the precise mechanism by which oxidase activation leads to the physiological effects of central Ang II remains unclear. We speculate that NADPH oxidase-derived $O_2^{.-}$ stimulates an increase in neuronal activity in important central neural networks, which in turn leads to the cardiovascular and dipsogenic actions of central Ang II. It is well established that Ang II–mediated activation of central neurons is, at least in part, attributable to the influx of extracellular calcium though voltage-gated calcium channels.[28,29] We have recently demonstrated that Ang II–stimulated influx of extracellular calcium in neural cells is mediated by NADPH oxidase-derived $O_2^{.-}$ production[30] (unpublished data, 2004). Further support of this comes from recent studies by Wang et al,[31] demonstrating that inhibition of NADPH oxidase assembly attenuates Ang II–mediated potentiation of L-type calcium currents in nucleus tractus solitarius neurons. In addition, Sun et al[32] recently reported that either $O_2^{.-}$ scavenging or NADPH oxidase inhibition reduced Ang II–stimulated neuronal activity by ≈50%. Taken together, these studies support the notion that NADPH oxidase–derived $O_2^{.-}$ production is involved in the intracellular signaling mechanisms underlying Ang II–induced neuronal activation, and provide a possible explanation by which Rac1-dependent NADPH oxidase activation leads to the systemic effects of central Ang II.

Additional support for the importance of NADPH oxidase in the CNS comes from work on the role of ROS in neurotoxicity and neurodegenerative diseases such as Parkinson and Alzheimer disease.[14] Previous studies have reported oxidase subunit p22phox, gp91phox, p40phox, p47phox, and p67phox immunoreactivity in the mouse brain, and Rac1 has been identified in central neurons.[11,12] Furthermore, recent studies have implicated a neuronal NADPH oxidase in causing neurotoxicity and neuronal cell death.[10] Neurons deficient in gp91phox have been shown to be resistant to nerve growth factor deprivation–induced apoptosis.[10] Walder et al[13] demonstrated that ischemia-reperfusion injury in the brain was reduced in mice lacking a functional gp91phox. Together, these studies support the notion that NADPH oxidase in the central nervous system is involved in the pathogenesis of neurodegenerative diseases.

These findings, along with results from the current studies lead us to speculate about the importance of NADPH oxidase in the pathophysiology of central Ang II–dependent cardiovascular diseases. Our previous work demonstrated a link between Ang II and $O_2^{.-}$ production in the acute cardiovascular actions of Ang II in the brain, and this study suggests an important role for NADPH oxidase in those responses. However, additional recent work using models of chronic hypertension and heart failure also implicates oxidative stress in the brain as a key mechanism in these diseases. For example, we showed recently that increased intracellular $O_2^{.-}$ production in brain regions lacking the blood-brain barrier is involved in the development of hypertension caused by chronic infusion of Ang II in the systemic circulation.[33] Furthermore, oxidative stress in central cardiovascular regulatory regions is implicated in the pathogenesis of heart failure because overexpression of SOD in these brain areas decreases sympathoexcitation and improves cardiac function and survival in mice 2 to 4 weeks after myocardial infarction.[34,35] Given the findings here linking a Rac1-activated NADPH oxidase to $O_2^{.-}$ production and short-term cardiovascular effects of Ang II in the brain, it will be interesting to determine whether this oxidase plays a pivotal role in the generation of oxidative stress and neurocardiovascular dysregulation that underlies these diseases.

In summary, we have identified a Rac1-dependent NADPH oxidase as a key source of Ang II–stimulated $O_2^{.-}$ production in the brain, and we speculate that dysregulation of the oxidase in Ang II–sensitive brain regions may be involved in the pathogenesis of brain-dependent cardiovascular diseases. Elucidating the exact composition of the oxidase, including identification of the Nox homologue(s) involved, and determining the downstream signaling events elicited by Ang II–induced $O_2^{.-}$ production in neurons is the subject of our ongoing investigations. Meanwhile, we speculate that targeting expression of the NADPH oxidase subunits, oxidase activation, or NADPH-derived reactive oxygen species in central cardiovascular networks may provide important new therapeutic strategies for some cardiovascular diseases.

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Material and Methods

Animals

Adult C57BL/6 mice (20-25 g; Harlan, Indianapolis, IN) were used in all in vivo studies, and C57BL/6 pre-weanlings (3 days old) were used for primary cell cultures. Mice were fed standard chow (Harlan) and water ad libitum. All procedures met or exceeded the guidelines set forth by the NIH and were approved by the University of Iowa Animal Care and Use Committee.

Cell Culture

Neuro-2A cells obtained from American Type Culture Collection (Manassas, VA) were cultured in Minimum Essential Medium with Earle's salts containing 1.5 g/L Na_2HCO_3, 0.1 mmol/L non-essential amino acids, 1.0 mmol/L sodium pyruvate, 25 mmol/L HEPES, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS). Twenty-four hours prior to AngII stimulation, cells were serum-starved via incubation in the culture medium containing 0.1% FBS. It should be noted that it was recently reported that Neuro-2A cells express high levels of the AngII type 1 (AT_1) and type 2 (AT_2) receptors at the mRNA and protein level [1].

Primary neuronal cultures were established from the lamina terminalis of C57BL/6 pre-weanling pups (3 day old, 10-12 pups per culture) as previously described [2]. Cells were cultured for 4 days in Dulbecco's Modified Eagles Medium (DMEM):Ham's F12 medium (1:1) supplemented with 1% L-glutamine-penicillin-streptomycin and 10% FBS.

Neuronal cultures were serum-starved for 24 hours prior to AngII stimulation by decreasing FBS to 0.1% in the culture medium.

Adenoviral Vectors

An E1-deleted adenovirus encoding β-galactosidase (AdLacZ) was purified as previously described and used as a control viral vector [3]. Adenoviral vectors encoding HA-epitope tagged dominant-negative Rac1 containing a substitution at position 17 (AdN17Rac1) or wild-type Rac1 (AdwtRac1) were constructed by PCR amplification of cDNA sequences using primers that incorporated an N-terminal HA-fusion to Rac1. The HA-tagged N17Rac1 or wtRac1 cDNA fragment was inserted into pAd.CMVLink containing the CMV promoter and an SV40 polyadenylation site for efficient transgene expression. Recombinant adenoviruses were generated by co-transfection of the *NheI-*cut pAd plasmid, containing N17Rac1 or wtRac1, with *Pac1*-digested Ad5.sub360 (E3-deleted) viral DNA [4]. The particle titers of adenoviral stocks were determined by A_{260} readings and were typically 10^{13} particles/ml. Functional viral titers were determined by assessing plaque forming units (pfu) on 293 cells and expression assays for encoded proteins [3;4].

Rac1 activity assay

Rac1 activity in response to AngII (1 μmol/L) was measured in Neuro-2A cells using a Rac activation assay kit (Upstate Biotechnology, NY) following manufacturer's instructions and as previously reported [5]. Following AngII stimulation for 1, 2, 5, or 15 minutes, cell lysates were incubated with agarose beads conjugated to the p21(Rac1)-

binding domain (PBD) to immunoprecipitate activated (GTP-bound) Rac1. The

immunoprecipitate was subjected to electophoresis followed by Western blot analysis

using a Rac1 antibody (1:1000, Upstate Biotechnology, NY). In separate cultures, Rac1

activation was measured in cells pre-treated with the specific AngII type 1 receptor

antagonist, losartan (10 μmol/L, 30 minutes) or infected with AdLacZ, AdN17Rac1 or

AdwtRac1 (100 pfu/cell) 24 hours prior to AngII stimulation (1 μmol/L, 1 minute). AngII-

induced Rac1 activation was quantified by densitometry (Quantity 1, Bio-Rad).

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

Neuro-2A cells were infected with AdLacZ, AdN17Rac1, or AdwtRac1 (100 pfu/cell) for

24 hours prior to loading the cells with dihydroethidium (DHE, 5 μmol/L) for 30 minutes.

Additional cultures were treated with NADPH oxidase inhibitors diphenylene iodonium

(DPI, 10 μmol/L, 60 min), a flavoprotein inhibitor, apocynin (100 μmol/L, 30 min), which

inhibits the assembly of NADPH oxidase [6], or the AT1 receptor antagonist losartan (10

μmol/L) during DHE loading. DHE fluorescence was imaged using confocal microscopy

(Zeiss LSM 510) as described [2]. After collecting baseline images, cells were stimulated

with AngII (1 μmol/L) and the same cells were re-imaged after 5 and 30 minutes. As a

control, DHE fluorescence was measured in separate cultures stimulated with vehicle.

DHE fluorescence was quantified using Image J analysis software (version 1.31, NIH)

and expressed relative to baseline fluorescence in individual cells.

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured in primary lamina terminalis cultures using

$O_2^{\bullet-}$-dependent lucigenin chemiluminescence. Cells were incubated with AdN17Rac1,

AdwtRac1, or AdLacZ (100 pfu/cell) for 24 hours and then stimulated with vehicle or

AngII (500 nmol/L, 2 minutes). Separate cultures were stimulated with AngII following

30 minutes of pretreatment with losartan (10 μmol/L). Culture dishes were incubated

with lucigenin (5 μmol/L) in a luminometer (model FB12: Zylux) and baseline

chemiluminescence (relative light units, RLU) was recorded every 30 seconds for 5

minutes. It should be noted that at this low concentration, lucigenin has been reported

to be suitable for $O_2^{\bullet-}$ detection with minimal redox cycling [7]. To measure NADPH

oxidase activity, NADPH (0.1 mmol/L) was added and RLU were again recorded every

30 seconds for 5 minutes. Mean background lucigenin RLU were subtracted from

NADPH-dependent RLU and NADPH oxidase activity was expressed relative to vehicle-

treated neurons.

Physiological Studies

Adult C57BL/6 mice were instrumented with intracerebroventricular (ICV) cannulae for

central administration of AngII or adenoviruses, and arterial catheters were implanted

into the left carotid artery for direct measurement of mean arterial pressure (MAP) and

heart rate (HR) as described previously [2;8]. Mice were injected ICV with either saline,

AdLacZ, AdN17Rac1, or AdwtRac1 ($2x10^8$ particles, 500 nL) on the day of surgery, and

cardiovascular and dipsogenic responses to AngII (200 ng, 200 nL) were recorded in

conscious freely moving mice as described by us in detail [2;9]. We have demonstrated

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previously that ICV injection of this concentration of adenovirus results in high level

transgene expression in forebrain circumventricular organs (CVOs), including the SFO

and the organum vasculosum of the lamina terminalis, by 3 days and for up to 4 weeks

without inflammatory effects [2;10;11]. To investigate a link between AngII, Rac1, and $O_2^{\bullet-}$

in the physiological effects of central AngII, additional groups of mice were ICV injected

with both AdwtRac1 and an adenovirus encoding either cytoplasm-targeted $O_2^{\bullet-}$

dismutase (AdCuZnSOD) or AdLacZ. In these co-infection experiments, mice were

injected with 250 nL of each virus, thus equaling the total concentration and volume

($2x10^8$ particles, 500 nL) of virus given to mice that received only a single viral injection.

Western blot analysis for Adenoviral Expression In Vivo

To confirm adenovirus-mediated expression of the N17Rac1 and wtRac1 transgenes,

brains were removed at the end of the physiological studies and micropunches from

periventicular tissue, including AngII-sensitive CVOs, were isolated and analyzed via

Western blot analysis for the the HA-tag expressed by both AdN17Rac1 and AdwtRac1

(see above). HA expression was detected by blotting the membrane with an HRP-

conjugated HA antibody (Roche, Indianapolis, IN) diluted 1:750, and all samples were

run in duplicate. To ensure equal loading, β-actin expression was measured

simultaneously.

Statistics

All data were expressed as mean ± SEM and analyzed by ANOVA followed by

Newman-Keuls correction for multiple comparisons. Differences were considered

significant at *P*<0.05.

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