Brainstem Phosphorylated Extracellular Signal-Regulated Kinase 1/2-Nitric-Oxide Synthase Signaling Mediates the Adenosine A2A-Dependent Hypotensive Action of Clonidine in Conscious Aortic Barodenervated Rats

Noha Nassar and Abdel A. Abdel-Rahman

Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, North Carolina

Received August 3, 2007; accepted October 11, 2007

ABSTRACT

The cellular mechanisms that underlie the enhancement of clonidine-evoked hypotension in aortic barodenervated (ABD) rats and its dependence on central adenosine A2A receptor (A2A) are not known. We tested the hypothesis that A2A-mediated phosphorylation of extracellular signal-regulated kinase (pERK)1/2 in the rostral ventrolateral medulla (RVLM) and its downstream activation of nitric-oxide synthase (NOS)-NO signaling underlie the centrally (clonidine)-mediated hypotension. We first demonstrated an up-regulation of the molecular targets for clonidine [imidazoline I1 and A2A adrenergic receptors (A2AR)] in the RVLM of ABD compared with sham-operated (SO) rats; this finding might explain the enhanced clonidine hypotension in ABD rats. A similar anatomical up-regulation of the RVLM A2AR was evident and was complemented with enhanced central A2AR [2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine; CGS21680]-mediated hypotension in ABD rats. The hypotension produced by intracisternal CGS21680 or clonidine, in conscious ABD rats, was associated with a significant increase in pERK1/2 level in the RVLM. Whereas selective A2AR blockage [5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; SCH58261] or NOS inhibition (N-v-nitro-L-arginine methyl ester) virtually abolished clonidine-evoked hypotension, clonidine-evoked enhancement of RVLM pERK1/2 production was only abrogated by SCH58261 pretreatment. These findings suggest that interventions that act centrally to increase RVLM neuronal pERK1/2 production elicit hypotension via the activation of downstream NOS-NO signaling. The findings also yield insight into a cellular mechanism that might explain the dependence of centrally (clonidine)-mediated hypotension on central A2AR signaling in the ABD rat.

This study was supported in part by National Institutes of Health Grant 2R01 AA07839. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.107.129692.

ABBREVIATIONS: ABD, aortic barodenervated; SO, sham-operated; A2AR, adenosine subtype A2A receptor; RVLM, rostral ventrolateral medulla; pERK, phosphorylated extracellular signal-regulated kinase; NOS, nitric-oxide synthase; CGS21680, 2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine; SCH58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; i.c., intracisternal; L-NAME, N-v-nitro-L-arginine methyl ester; BP, blood pressure; HR, heart rate; aCSF, artificial cerebrospinal fluid; MAP, mean arterial pressure; ANOVA, analysis of variance; PD98059, 2'-amino-3'-methoxyflavone; ir, immunoreactive.
pERK1/2 generation (Schulte and Fredholm, 2003), and clonidine-like drugs, whose hypotensive action is dependent on central adenosine A2aR (Nassar and Abdel-Rahman, 2006a,b), enhance the production of pERK1/2 in the RVLM (Zhang and Abdel-Rahman, 2005). The possibility has not been investigated that pERK1/2 generation in the RVLM contributes to the A2aR-dependent hypotension caused by clonidine. Furthermore, the mechanism by which pERK1/2 generation in the RVLM leads to hypotension is not known. It is imperative to note that 1) pERK1/2 is formed within minutes in brainstem nuclei (Springell et al., 2005), and cell culture studies show that pERK1/2 enhances NOS-NO signaling (Wyatt et al., 2002; Vásquez et al., 2004); and 2) NO generation in the brainstem nuclei causes reduction in blood pressure (Chan et al., 2001). Whether pERK1/2 activation of NOS-NO signaling occurs in vivo and contributes to a biological response has not been investigated.

In the present study, we tested the hypothesis that an enhanced pERK1/2-NOS signaling in the RVLM plays a critical role in the central adenosine A2aR-dependent hypotensive action of clonidine. As a first step, it was important to determine whether the enhanced A2aR-dependent hypotensive action of clonidine in ABD rats, in our recent study (Nassar and Abdel-Rahman, 2006a), could be explained by up-regulated A2aR signaling in ABD rats. Therefore, we conducted immunohistochemical and dose-response [intracerebral (i.c.) CGS21680] studies in ABD and SO rats to establish whether 1) the adenosine A2aR is overexpressed along with clonidine molecular targets, the imidazoline I1R and α2AR, in the RVLM of ABD rats; and 2) the adenosine A2aR is functionally up-regulated in ABD rats and that central A2aR activation (i.c. CGS21680) enhances pERK1/2 production in the RVLM. Additional studies were conducted in conscious ABD rats to elucidate the role of pERK1/2-NOS signaling in the RVLM in the A2aR-dependent hypotensive action of clonidine. To this end, pharmacological and neurochemical studies were undertaken to investigate the effect of clonidine on RVLM pERK1/2 level and blood pressure in the absence or presence of central adenosine A2aR blockade (SCH58261) or NOS inhibition (Nω-nitro-L-arginine methyl ester; l-NAME). These studies were conducted in conscious rats to circumvent potential confounding effects of anesthetics.

**Materials and Methods**

**Animals**

In total, 53 male Sprague-Dawley rats (11–12 weeks; Harlan, Indianapolis, IN) weighing 310 ± 10 g were used in the present study. All rats were housed in a room with controlled environment at a constant temperature of 23 ± 1°C, humidity of 50 ± 10%, and a 12-h light/dark cycle. Food (Prolab RMH300; Granville Milling, Creedmoor, NC) and water were available ad libitum. Surgical procedures and postoperative cycle. Food (Prolab RMH300; Granville Milling, Creedmoor, NC) and water were available ad libitum. Surgical procedures and postoperative care were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee and in accordance with the Institute of Laboratory Animal Resources (1996) Guide for the Care and Use of Laboratory Animals 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.

**Aortic Barodenervation, Intracisternal Cannulation, and Intravascular Catheterization**

These surgical procedures were performed as in our previous studies (el-Mas and Abdel-Rahman, 1995, 1997; Nassar and Abdel-Rahman, 2006a). In brief, 5 days before starting the experiment, a stainless steel guide cannula was implanted into the cisterna magna under pentobarbital anesthesia (60 mg/kg i.p.). The head was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The dura matter covering the foramen magnum was exposed. A hole was drilled 1 to 1.5 mm distal to the caudal edge of the occipital bone (Granata et al., 1985). A stainless steel cannula (23-gauge; Small Parts, Miami, FL) was passed between the occipital bone and the cerebellum so that its tip protruded into the cisterna magna. The cannula was secured in place with small metal screws and dental acrylic cement (Durelon; Thompson Dental Supply, Raleigh, NC). The guide cannula was considered patent when spontaneous outflow of cerebrospinal fluid was observed and by gross post-mortem histological verification after injection of 5 μl of fast green dye (EM Science, Cherry Hill, NJ). Catheters (polyethylene-50) were placed in the abdominal aorta and vena cava via the femoral artery and vein for measurement of blood pressure and intravenous injections, respectively. The catheters were advanced 5 cm into the femoral vessels and secured with sutures. Aortic barodenervation was accomplished by bilateral transection of the aortic depressor nerves following a midline incision in the cervical region (Abdel-Rahman, 1992; el-Mas and Abdel-Rahman, 1995, 1997; Nassar and Abdel-Rahman, 2006a). Finally, the catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. The catheters were flushed with heparin in saline (200 U/ml) and plugged by stainless steel pins. Incisions were closed by surgical staples and swabbed with povidone-iodine solution. Each rat received an intramuscular injection of 30,000 U of penicillin G benzathine and penicillin G procaine in aqueous suspension (Durapen; GC Hanford, New York, NY) and a subcutaneous injection of buprenorphine hydrochloride (30 μg/kg Buprenex; Hospira, Inc., Lake Forest, IL), and then it was housed in a separate cage. On the day of the experiment, the arterial catheter was connected to a Gould-Statham (Oxnard, CA) pressure transducer, and BP was displayed on a polygraph (model 7D; Grass Instruments, Quincy, MA). Heart rate (HR) was computed from blood pressure waveforms by a Grass tachograph and displayed on another channel of the polygraph. In all groups, a period of 30 min was allowed at the beginning of the experiment for stabilization of BP and HR at baseline before i.c. drug or vehicle (aCSF) administration.

**Immunohistochemistry**

The procedure reported in Current Protocols in Neuroscience for immunohistochemistry for light microscopy (Gerfen, 1997) was followed. In brief, brains were fixed by transcardiac perfusion with 4% paraformaldehyde in Tris-buffered saline (TBS) following a lethal dose of sodium pentobarbital. Brains were then transferred into 30% sucrose in TBS for infiltration until they sank. Brain sections that contained the RVLM were cut serially at −24°C with a microtome cryostat (HM 505 E; Microm International GmbH, Walldorf, Germany) in accordance with Paxinos and Watson (1982) as in our previous study (Zhang and Abdel-Rahman, 2005). Brain sections (16 μm each) were collected in each well of a cell culture plate (12 wells; BD Biosciences, San Jose, CA) containing ice-cold TBS. The avidin-biotin complex method was used following the manufacturer’s instruction (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). For immunohistochemical detection/quantification of the three receptor proteins (α2a, I1, and α2aR) in the RVLM, sections from vehicle-treated (aCSF) ABD and SO rats were simultaneously incubated with the primary antibody, rabbit anti-canine adenosine A2a receptor antibody, affinity pure (Alpha Diagnostic International, San Antonio, TX) (1:400), rabbit anti-adrenergic α1a receptor antibody (Calbiochem, San Diego, CA) (1:1000), or rabbit anti imidazoline I1 developed by Zhang and Abdel-Rahman (2005) (1:1000). For the pERK1/2 immunohistochemistry studies, brain sections containing the RVLM, obtained from treatment and control groups, were simultaneously incubated with the primary antibody (phospho-p42/44 mitogen-activated protein kinase; Thr202/Tyr204; E10) mouse mAb.
in adenosine A2AR-mediated hypotension, pERK1/2 expression was received aCSF represented basal expression of pERK1/2 in the RVLM. The expression of pERK1/2 in the RVLM of ABD and SO rats that received either aCSF or CGS21680, the expression of pERK1/2 was as detailed under Materials and Methods.

**Experiment 1: Effect of Aortic Barodenervation on the Expression of Adenosine A2AR, Adrenergic α2A, and Imidazole I1 Receptors.** Conscious ABD and SO rats (n = 4–6), which served as vehicle controls, received aCSF (i.c.) of the following composition: 123 mM NaCl, 0.86 mM CaCl2, 3 mM KCl, 0.89 mM MgCl2, 25 mM NaHCO3, 0.5 mM NaH2PO4, and 0.25 mM Na2HPO4, pH 7.4. Baseline BP and heart rate for each animal were obtained after the stabilization period. Brains were removed and used for immunohistochemical detection/quantification of the adenosine A2AR, imidazole I1, and adrenergic α2A receptors in the RVLM as detailed under Materials and Methods.

**Experiment 2: Effect of Central Adenosine A2AR Activation on Blood Pressure and the Expression of pERK1/2 in the RVLM of Conscious ABD and SO Rats.** In this experiment, we tested the hypothesis that an enhanced pERK1/2 production in the RVLM contributes to the hypotensive response elicited by the activation of the central adenosine A2AR. After stabilization of BP and HR at baseline, dose-hypotensive response curves were constructed with CGS21680 (1, 2, 4, and 8 µg i.c.) in ABD (n = 8) and SO (n = 7) rats; the doses of CGS21480 were based on a pilot study. Recovery from a previous response was allowed before the administration of the subsequent dose of CGS21680. Control (aCSF treated) animals were also monitored for blood pressure and heart rate (ABD, n = 7 and SO, n = 7). At the end of the experiment, brains were removed as detailed under Materials and Methods. In ABD or SO rats that received either aCSF or CGS21680, the expression of pERK1/2 was quantified by immunohistochemistry as described under methods. The expression of pERK1/2 in the RVLM of ABD and SO rats that received aCSF represented basal expression of pERK1/2 in the RVLM neurons. To determine whether VRM pERK1/2 is implicated in adenosine A2AR-mediated hypotension, pERK1/2 expression was measured in the RVLM of the ABD and SO rats, sacrificed during the hypotensive response elicited by the last dose of CGS21680, and the data were compared with their corresponding control (aCSF) values.

**Experiment 3: Effect of Central Adenosine A2AR Blockade or NOS Inhibition on RVLM pERK1/2 Expression and Blood Pressure Responses Elicited by Intracisternal Clonidine.** In this experiment, we investigated the role of central pERK1/2/NOS signaling in the A2AR-dependent hypotensive action of clonidine in conscious ABD rats. In the first part of this experiment, we investigated the effect of intracisternal clonidine (0.6 µg i.c.) on pERK1/2 expression in the RVLM of ABD rats pretreated with the selective adenosine A2AR antagonist SCH58261 (150 µg; n = 3) or its vehicle aCSF (n = 4); the hypotensive response elicited by clonidine was virtually abolished by central A2AR blockade in our recent study (Nassar and Abdel-Rahman, 2006a). To determine whether pERK1/2 generated in the RVLM contributed to clonidine-evoked hypotension by activating downstream NOS, conscious ABD rats received i.c. l-NAME (4 µg; n = 3) 30 min before i.c. clonidine (0.6 µg). The chosen dose of l-NAME had no effect on baseline BP and heart rate, but it attenuated moxonidine (selective I1 receptor agonist)-evoked hypotension when administered centrally (i.c.v.) (Moreira et al., 2004). pERK1/2 was measured in the RVLM in all groups of rats sacrificed at a time that coincides with the maximal hypertensive response elicited by i.c. clonidine in our previous study (Nassar and Abdel-Rahman, 2006a).

**Results**

**Adenosine A2AR α2A Adrenergic, and Imidazole I1 Receptors Are Up-Regulated in the RVLM of ABD Rats.** In this experiment, the brains obtained 5 days after ABD or SO were processed for immunohistochemical detection of the adenosine A2AR, adrenergic α2A, and imidazole I1 receptors in the RVLM. Before sacrifice, the ABD and SO rats used in this experiment exhibited similar baseline MAP and HR (Table 1). Figure 1 shows representative examples of immunostaining for each of the three receptors in the RVLM. Compared with the corresponding brainstem area of the control (SO) rats, the RVLM of ABD rats exhibited significantly (P < 0.05) greater number of neurons that expressed each of the three receptors (Fig. 1).

**Hypotensive Responses and the Associated pERK1/2 Generation in the RVLM Elicited by Central A2AR Activation Are Enhanced in ABD Rats.** To determine whether the up-regulation of the A2AR in the RVLM of ABD rats (Fig. 1) was functionally relevant, dose-hypotensive response-curves were constructed with the selective adenosine A2AR agonist CGS21680 (1, 2, 4, and 8 µg i.c.) in conscious ABD and SO rats. Control ABD and SO rats received i.c. Drugs

CGS21680, dimethyl sulfoxide, l-NAME, and SCH58261 were purchased from Sigma-Aldrich (St. Louis, MO). Pentobarbital sodium was provided by Webster Veterinary Supplies (Vortech Pharmaceutical Ltd., Dearborn, MI).

**Statistical Analysis**

Mean arterial pressure (MAP) was calculated as diastolic pressure + one-third pulse pressure (systolic pressure – diastolic pressure). Mean arterial pressure and heart rate are expressed as mean ± S.E.M. change from their respective baselines. The dose-response curves were analyzed by repeated measures ANOVA using SPSS 13.0 statistical package for Windows (SPSS Inc., Chicago, IL), for differences in treatment trends. Contrasts based on the t test and the ANOVA error terms were used to compare differences at each dose level in the dose-response curve in each group using the error term of the within-subject effects. Student’s t test (unpaired, two-tailed) was used for comparing the mean of positive cell count data. A one-way ANOVA was used to evaluate the effect of SCH58261 or l-NAME pretreatment, compared with aCSF, on the pERK1/2 production in the RVLM and the hypotensive response elicited by clonidine in ABD rats. P < 0.05 was considered significant.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAP</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm Hg</td>
<td>beats/min</td>
</tr>
<tr>
<td>Aortic barodenervated rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF (n = 12)</td>
<td>116.0 ± 6.0</td>
<td>407 ± 11</td>
</tr>
<tr>
<td>CGS21680 (n = 8)</td>
<td>110.0 ± 3.0</td>
<td>406 ± 17</td>
</tr>
<tr>
<td>l-NAME (n = 7)</td>
<td>108.0 ± 2.0</td>
<td>408 ± 10</td>
</tr>
<tr>
<td>SCH58261 (n = 5)</td>
<td>114.3 ± 9.5</td>
<td>430 ± 12</td>
</tr>
<tr>
<td>Sham-operated rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF (n = 10)</td>
<td>110.0 ± 5.0</td>
<td>393 ± 13</td>
</tr>
<tr>
<td>CGS21680 (n = 7)</td>
<td>108.0 ± 3.0</td>
<td>370 ± 14</td>
</tr>
</tbody>
</table>

Downloaded from jpet.aspetjournals.org at ASPET Journals on January 8, 2018
aCSF. Baseline MAP and HR, measured 5 days after ABD or SO and before CGS21680 or aCSF administration, were similar (Table 1). aCSF caused no change in blood pressure in ABD or SO rats (data not shown), whereas CGS21680 elicited dose-related reductions in blood pressure that were more evident in ABD rats and reached statistical significance (P < 0.05) at the two higher doses (Fig. 2); pERK1/2 was measured in the RVLM of rat brains collected during the hypotensive response elicited by the last dose of CGS21680 in ABD and SO rats and compared with the level obtained from the corresponding aCSF-treated ABD and SO rats. CGS21680 increased pERK1/2 level in the RVLM of ABD and SO rats, compared with their respective (aCSF) control (Fig. 3); such increase was greater in ABD rats and paralleled the greater hypotensive response in the same rats (Fig. 2).

Central Adenosine A2A R Blockade Abrogates Clonidine-Evoked Enhancement of pERK1/2 Generation in the RVLM of ABD Rats. In our recent study (Nassar and Abdel-Rahman, 2006a), we showed that central adenosine A2A R blockade virtually abolished clonidine-evoked hypotension in conscious ABD rats. The brains of these rats were used in the present study to investigate the effect of i.c. clonidine on neuronal pERK1/2 in the RVLM in the absence or presence of central A2A receptor blockade. Likewise, as shown in Fig. 4, clonidine-evoked hypotension was substantially attenuated following central NOS inhibition (i.e. l-NAME) as detailed below. The experimental protocol used for i.c. clonidine administration in the present and in our previous study (Nassar and Abdel-Rahman, 2006a) was similar to the corresponding values in the present study (Table 1). Similar to CGS21680, i.c. clonidine caused a significant (P < 0.05) increase in pERK1/2 level in the RVLM of ABD rats (Fig. 5), along with a hypotensive response (−26 ± 2.5 mm Hg; Fig. 5). Central adenosine A2A R blockade (i.c. SCH58261) abrogated (P < 0.05; ANOVA) clonidine-evoked hypotension and the associated increase in pERK1/2 production in the RVLM; pERK1/2 in the RVLM of the ABD rats that received i.c. clonidine following SCH58261 was not significantly (P > 0.05; ANOVA) different from control (aCSF) level (Fig. 5).

Central NOS Inhibition Attenuates the Hypotension but Not the Enhancement of pERK1/2 Generation in the RVLM Elicited by Clonidine in ABD Rats. This experiment was conducted to determine whether pERK1/2 generation in the RVLM mediates the hypotensive action of clonidine, at least partly, via an enhancement of downstream NOS-NO signaling. L-NAME (4 μg i.c.) had no effect on baseline MAP and HR (Table 1), which agrees with reported findings (Moreira et al., 2004). Pretreatment with L-NAME, compared with aCSF, virtually abolished clonidine-evoked hypotension (Figs. 4 and 5). However, in the same rats, clonidine-evoked pERK1/2 generation in the RVLM was not affected by L-NAME pretreatment. As shown in Fig. 5, i.c. clonidine elicited similar increases in pERK1/2 levels in the RVLM of ABD rats pretreated with L-NAME or aCSF; the level of pERK1/2 in the RVLM of either treatment group was significantly (P < 0.05; ANOVA) higher than control level (Fig. 5).

Discussion

In the present study, we investigated the role of the RVLM pERK1/2-NOS signaling in the central adenosine A2A R-dependent hypotensive action of clonidine. The following are the most important findings of the study: 1) the A2A R, along with the molecular targets for clonidine, a2A R, and I1 recep-
tors, is up-regulated in the RVLM of ABD, compared with SO, rats; 2) activation of the central adenosine $A_2A$R elicits greater hypotension in ABD rats, supporting a functional relevance of the anatomical up-regulation of the receptor in the RVLM; 3) pERK1/2 level in the RVLM is significantly increased in association with the hypotensive response elicited by i.c. clonidine or CGS21680; 4) blockade of the central $A_2A$R abrogates the RVLM pERK1/2 generation and the hypotension elicited by clonidine in ABD rats; 5) inhibition of central NOS (L-NAME) virtually abolished the hypotension, but not the generation of RVLM pERK1/2, elicited by clonidine in ABD rats. Together, the findings yield insight into a key role for brainstem pERK1/2-NOS signaling in the A2AR-dependent hypotension elicited by clonidine in conscious ABD rats.

Recently, we have shown that the enhanced clonidine-evoked hypotension in ABD rats, the same model used in the present study, is dependent on central $A_2A$R signaling (Nassar and Abdel-Rahman, 2006a). Therefore, we investigated the possibility that the $A_2A$R is anatomically and/or functionally up-regulated in ABD rats compared with SO rats. Furthermore, we investigated whether the up-regulation of the $A_2A$R parallels an up-regulation in the molecular targets for clonidine, $I_1$, and/or $A_2A$R receptors, in the same neuronal pool. Results of the present investigation demonstrate that the $A_2A$R is up-regulated along with the $A_2A$R and $I_1$ receptors in the RVLM of ABD rats. The latter agrees with and extends earlier findings, which demonstrated the up-regulation of $A_2A$ and $I_1$ receptors in the RVLM of the same animal model (el-Mas and Abdel-Rahman, 1995, 1997). However, the use of specific antibodies in the present study addressed a major limitation of the previous studies, the use of nonselective autoradiographic ligands, which made it difficult to ascertain the specificity/subtype of the receptor under investigation. It might be argued that the adenosine $A_2A$R up-regulation is a nonspecific response to barodenervation because it was paralleled with similar increases in $A_2A$R and $I_1$ receptor in the RVLM. To the contrary, such parallel up-regulation might be physiologically/pharmacologically relevant because 1) for the first time, we demonstrate up-regulation of the $A_2A$R, along with the molecular targets for clonidine, $A_2A$R, and the $I_1$ receptors, in the RVLM of ABD rats; 2) all three receptors mediate hypotension (el-Mas and Abdel-Rahman, 1995; Szabo et al., 1999; Szabo, 2002; Scislo and O’Leary, 2005); 3) physiological interaction between these receptors is highly likely given their shared signaling pathways (Zhang et al., 2001; Schulte and Fredholm, 2003; Zhang and Abdel-Rahman, 2005); 4) contrary to the adenosine $A_2A$R, the $A_2A$R is down-regulated in the RVLM of ABD, compared with SO, rats (Nassar and Abdel-Rahman, 2006b). Together, these findings argue against the notion that aortic barodenervation causes nonspecific up-regulation of the investigated receptors. Furthermore, the findings lend anatomical support to the key role of the adenosine $A_2A$R in clonidine (mixed $A_2A$R/$I_1$ receptor agonist)-evoked hypotension in conscious ABD rats, which was first demonstrated in our recent study (Nassar and Abdel-Rahman, 2006a).

We demonstrate an approximately 2-fold increase in the number of the $A_2A$R in the RVLM of ABD compared with SO
A main objective of the present study was to delineate the cellular mechanisms that underlie central A2AR-dependent hypotensive action of clonidine. We present the first evidence that central A2AR activation (i.c. CGS21680) or i.c. clonidine increased pERK1/2 level in the RVLM along with the hypotensive response in ABD rats. Interestingly, the magnitude of the hypotensive response seems to be dependent, at least partly, on the level of pERK1/2 expression in the RVLM. In support of this notion are the findings that the greater hypotensive response elicited by the highest dose of CGS21680 in ABD compared with SO rats (Fig. 2) was associated with greater expression of pERK1/2 expression in the RVLM (Fig. 3). Conversely, it may be argued that the increase in RVLM pERK1/2 is a result, rather than a cause, of the hypotensive response because increased RVLM pERK1/2 has been shown to follow hydralazine-evoked hypotension (Springell et al., 2005). It is noteworthy that in the latter study, the rats were anesthetized, the hypotension was peripherally mediated, and it was at least 2-fold greater than the centrally mediated hypotensive response elicited by CGS21680 or clonidine in our studies. It is also imperative to note that a role of pERK1/2 as a mediator of centrally evoked hypotension is supported by the following findings: 1) enhanced pERK1/2 production in the RVLM is associated with centrally mediated hypotension elicited by rilmenidine but not by α-methylnorepinephrine (Zhang and Abdel-Rahman, 2005); and 2) the ERK1/2 inhibitor PD98059 significantly attenuated rilmenidine-evoked hypotension (Zhang and Abdel-Rahman, 2005). Furthermore, as discussed below, clonidine enhancement of RVLM pERK1/2 production persisted when clonidine-evoked hypotension was abrogated by central NOS inhibition (Fig. 5). Together, the findings suggest pERK1/2 generation in the RVLM is implicated, at least partly, in the hypotension elicited by clonidine in conscious ABD rats.

We have shown that central adenosine A2AR blockade abrogated the increase in RVLM pERK1/2 caused by i.c. clonidine in the present study (Fig. 5) and that it virtually abolished clonidine-evoked hypotension in the same rats in our previous study (Nassar and Abdel-Rahman, 2006a). These intriguing findings present the first evidence for a cellular mechanism that might explain the dependence of clonidine-evoked hypotension on central adenosine A2AR. The present and our previous findings with rilmenidine (Zhang and Abdel-Rahman, 2005) bolster the conclusion that pERK1/2 generated in the RVLM plays a causal role in the hypotension caused by clonidine-like drugs. However, these findings did not elucidate the cellular events downstream of pERK1/2. We reasoned that pERK1/2 would activate downstream NOS-NO signaling based on an established signaling pathway in cultured cells (Wyatt et al., 2002; Vázquez et al., 2004), and the following in vivo findings: 1) NOS-derived NO in the RVLM mediates sympathoinhibition and hypotension in rats (Chan et al., 2001), and 2) central NOS inhibition (1-NAME) attenuated moxonidine-evoked hypotension (Moreira et al., 2004). Our finding that central NOS inhibi-
tion (i.e. L-NAME) attenuated clonidine-evoked hypotension fully agrees with the latter study (Moreira et al., 2004). More importantly, we demonstrated the preservation of clonidine-evoked enhancement of RVLM pERK1/2 generation in L-NAME-pretreated rats in spite of the abolition of the hypertensive response (Figs. 4 and 5). These findings strongly suggest that the A2AR-dependent hypotension elicited by clonidine in conscious ABD is mediated, at least partly, as a result of the pERK1/2 generated in the RVLM, which subsequently triggers the activation of downstream NOS-NO signaling.

In summary, the present cellular and cardiovascular findings support the hypothesis that central adenosine A2AR plays a pivotal role in clonidine enhancement of pERK1/2 generation in the RVLM, which constitutes a critical cellular event in the mediation of the hypotensive response. Furthermore, the findings that following central NOS inhibition, clonidine-evoked hypotension was aborted, whereas the enhanced RVLM pERK1/2 production was preserved, suggest the latter initiates centrally mediated hypotension via the activation of downstream NOS-NO signaling in the brainstem of ABD rats.

Acknowledgments
We thank Dr. Kevin O’Brien for valuable assistance with the statistical analyses. We also thank Dr. Jian Zhang for help with the photomicrographs and Kui Sun for technical assistance.

References

Address correspondence to: Dr. Abdel A. Abdel-Rahman, Department of Pharmacology and Toxicology, School of Medicine, East Carolina University, Greenville, NC 27834. E-mail: abdelrahmana@ecu.edu