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Enhanced Vascular PI3K/Akt-NOX Signaling Underlies the Peripheral NMDAR-Mediated Pressor Response in Conscious Rats

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Abstract

The molecular mechanisms for peripheral N-Methyl-D-Aspartate receptor (NMDAR)-mediated vascular oxidative stress and pressor response are not known. We conducted integrative (in vivo) and ex vivo biochemical studies to test the hypothesis that ROS-dependent calcium influx, triggered by activation of vascular kinases, underlies the NMDAR-mediated pressor response. Pharmacological inhibition of PI3K/Akt (Wortmannin; 15 µg/kg), PKC (Chelerythrine; 5 mg/kg, i.v.), Ca²⁺ influx (nifedipine; 0.35 or 0.75 mg/kg) or NOX (apocynin; 5 mg/kg) attenuated the peripheral NMDAR-mediated pressor response in conscious male Sprague-Dawley rats. NMDAR activation enhanced the phosphorylation of Akt, ERK1, JNK and p38 (Western blot) and NADPH oxidase (NOX) activity in vascular tissues collected during the pressor response caused by NMDA infusion (180 µg/kg/min, 30 min). Further, ex vivo studies showed that wortmannin, chelerythrine or apocynin abrogated the NMDAR-mediated vascular NO and ROS generation and NOX activation in the vasculature. These findings implicate vascular PI3K/Akt-PKC signaling in the peripheral NMDAR-mediated increases in vascular NO and NOX activation (ROS), which ultimately lead to calcium influx and pressor response in conscious rats.

Keywords

NMDA receptor; vasculature; blood pressure; PI3K; NO; ROS

Introduction

Recent findings including ours implicated the peripheral N-Methyl-D-Aspartate receptor (NMDAR) in NO-dependent oxidative stress in systemic and retinal blood vessels, which resulted in pressor response and retinal damage, respectively^{1, 2}. However, the underlying mechanisms for the NMDAR-mediated NO and ROS generation, which lead to these physiopathological consequences, are not fully known. This knowledge gap could be attributed, at least partly, to a scant number of publications that reported conflicting blood pressure responses caused by peripheral NMDAR activation^{3, 4}. Notably, peripheral

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NMDAR activation (up to 1 mg/kg) produced modest dose-unrelated depressor response in anesthetized rats³ in marked contrast to dose-related pressor responses in conscious rats in our previous study¹. Furthermore, previous studies, conducted in anesthetized rats, demonstrated pressor responses following central or peripheral NMDA administration^{5, 6}. However, in the reported studies, systemic NMDA was administered in much higher doses (2.5-12 mg/kg), and the molecular mechanisms of the peripherally mediated pressor response were not investigated. Therefore, there is an unmet need to elucidate the molecular mechanisms of the NMDAR-mediated pressor response, particularly in the absence of confounding and deleterious cardiovascular effects of anesthesia^{7, 8}.

NMDAR activation in different tissues triggers phosphoinositide 3-kinase (PI3K)-dependent events^{9,10}, which include calcium influx, NO generation¹¹ and NADPH oxidase (NOX) activation¹². Notably, PI3K/Akt activation within cardiovascular tissues causes ROS-dependent vasoconstriction, at least partly, via enhancement of MAPK kinases (ERK1/2, JNK 3, p38) phosphorylation¹³⁻¹⁵. Further, PI3K-dependent calcium influx serves an important role in NOS-derived NO¹⁶ and NOX-derived ROS¹⁷ generation via protein kinase C (PKC) activation. Importantly, NMDAR-mediated increases in NOX2 activity and NO contribute to ROS generation in neuronal tissue¹⁸, and increased NOX2 expression within the vasculature is associated with pressor responses¹⁹. Despite this knowledge, there are no mechanistic studies on the potential role of the PI3K/Akt-NOX pathway in vascular NMDAR-mediated oxidative stress and pressor response.

In the present study, we tested the hypothesis that enhanced vascular PI3K/Akt signaling underlies the peripheral NMDAR-mediated increases in vascular NO and NOX activation, which ultimately lead to the calcium influx-dependent pressor response. Realizing the importance of MAPK phosphorylation and protein kinase C (PKC) in the PI3K-dependent activation of NOS (NO) and NOX (ROS), we investigated the effect of pharmacological inhibition of PI3K/Akt pathway (Wortmannin), PI3K downstream effector, protein kinase C (Chelerythrine), Ca²⁺ influx (nifedipine) or NOX (apocynin) on NMDAR-mediated pressor response. We also investigated the effect of peripheral NMDAR activation on the phosphorylation state of Akt (PI3K product) and its downstream kinases, ERK1/2, JNK1/3 and p38 in vascular tissues. To facilitate data interpretation, the ex vivo measurements of vascular NOX activity, NO (nitrite/nitrate) and ROS levels were conducted in aortas collected during the NMDA-evoked pressor response and compared with control values obtained from vehicle-treated rats. In addition to elucidating the molecular mechanisms for the vascular oxidative stress and pressor responses caused by vascular NMDAR activation, the findings will yield new insight into potential therapeutics against NMDAR-mediated vascular anomalies.

Methods

Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 275–325 grams (11–12 weeks old) were used in this study. Rats were housed individually in separate cages and allowed free access to Purina chow and water. The temperature was maintained at 22 ± 1°C and a 12–12-hour light-dark cycle with the lights automatically turned off at 7:00 p.m. Surgical procedures and animal experiments were conducted in accordance with the

institutional animal use and care guidelines and the Guide for the Care and Use of Laboratory Animals as indicated by the U.S. National Institutes of Health.

Intravascular Catheterization

Femoral artery and vein catheterization was performed as previously done in our laboratory²⁰. Animals received buprenorphine for analgesia (0.03 mg/kg) 30 min prior to surgery and were anesthetized with i.p. ketamine (9 mg/100g) and xylazine (1 mg/100g). Catheters consisting of 5-cm PE-10 tubing bonded to 15-cm PE-50 tubing were placed into the abdominal aorta and vena cava via the left femoral vessels for measurement of arterial pressure and intravenous injections, respectively. Two venous catheters were inserted into a femoral vein to permit i.v. bolus administration and/or infusion of drugs. Catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. Vascular catheters were flushed with heparinized saline and plugged by stainless steel pins. Incisions were closed with surgical clips and swabbed with povidine-iodine solution. Postoperative care included buprenorphine (0.03 mg/kg) and penicillin G procaine (100,000 U/kg; Durapen, Vedco Inc., Overland Park, KS). The animals were allowed 2 days following surgery before conducting experiments.

On the day of the experiment, the arterial catheter was connected to a pressure transducer for measurement of blood pressure in conscious freely moving rats. At least 30 min were allowed for stabilization of blood pressure and heart rate at the beginning of an experiment. Blood pressure (BP) was recorded by ML870 (PowerLab 8/30) and analyzed by LabChart (v.6) pro software (AD Instruments, Colorado Springs, CO). Heart rate was extracted from BP recording by the LabChart (v.6) blood pressure analysis module, and both variables were continuously recorded and stored for offline analysis. At the end of each experiment, animals were euthanized with i.p. sodium pentobarbital (>100 mg/kg) and tissues were collected and stored for ex vivo studies.

Quantification of aortic reactive oxygen species—The 2', 7'-dichlorofluorescein (DCF) biochemical assay was utilized for quantification of ROS as reported²¹ with the following modifications, which was employed in our recent study¹). Homogenization was performed using Radnoti tissue grinders (Radnoti Glass Technology, Monrovia, CA) to increase protein yield and kinetic readings were taken at 5 min intervals for 30 min at 37°C. ROS levels were calculated by DCF fluorescence per µg protein.

Measurement of nitrite/nitrate (NOx) level—Vascular NOx (nitrite/nitrate) content was measured using a colorimetric assay kit according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI) and as in reported studies including ours^{22, 23}.

Quantification of NADPH oxidase (NOX) activity—Fresh unfixed aorta sections (10 µm) were incubated with 10 µM dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO₂ in a moist chamber for 30 min. Positive and negative controls were used to validate the assay (see experimental groups and protocols). Images were visualized with a Zeiss LSM 510 microscope. Three-five images were acquired

from 3–4 animals for each experimental condition. Quantification was conducted using Image J software (NIH) and changes in total fluorescence intensity, normalized to control, were calculated.

Western Blotting—Following euthanasia, aortas were collected from systemic NMDA- or saline-infused rats and flash frozen in 2-methylbutane (Sigma Aldrich, St. Louis, MO) in dry ice and stored at -80°C for subsequent measurements of the level of phosphorylated Akt, ERK1/2, JNK1/3 and p38 in relation to the corresponding total protein as reported in our previous studies^{23, 24}. Tissues were homogenized, on ice, in lysis buffer containing 20 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM b-glycerolphosphate, 1mM activated sodium orthovanadate, 1 $\mu\text{g}/\text{ml}$ leupeptin) with protease inhibitor cocktail (Roche, Indianapolis, IN). Following centrifugation (12,000 g for 20 min), protein in the supernatant was quantified using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA). Protein extracts for p-Akt, Akt, p-ERK1/2, ERK, p-p38, p38, p-JNK1/3 and JNK 1/3 from aorta (50 $\mu\text{g}/\text{lane}$) were run on a NUPAGE 4–12% Bis-Tris polyacrylamide gel by electrophoresis at 100 volts (V) (Invitrogen, Grand Island, NY). Gels were electroblotted to nitrocellulose membranes via a semi-dry transfer utilizing the Bio-Rad TransBlot SD transfer cell (Bio-Rad Laboratories, Hercules, and CA) at 25 V for 1 hr. The nitrocellulose membranes were then incubated for two hr. in Odyssey Blocking Buffer (Licor Biosciences, Lincoln, NE). Following blocking, membranes were incubated overnight at 4°C with mouse antibodies to p-Akt (1:200, BD Biosciences, San Jose, CA), p-ERK1/2, p-p38, p-JNK1/3 (1:200, Cell Signaling, Danvers, MA) or rabbit antibodies to Akt, ERK1/2, p38 and JNK 1/3 (1:200, Cell Signaling, Danvers, MA) diluted in Odyssey blocking buffer and 0.1% Tween. The blots were washed with Phosphate Buffered Saline-Tween 0.1% buffer four times, incubated for one hr. at room temperature with infrared secondary antibodies: anti-mouse for p-Akt, p-ERK1/2, ERK1/2, p-p38, p-JNK1/3 or anti-rabbit for Akt, ERK1/2, p38 and JNK1/3 (1:15,000, Licor Biosciences, Lincoln, NE) in Odyssey Blocking Buffer. Following four additional washes, the nitrocellulose membrane was placed in PBS and imaging acquisition was performed via the Odyssey Infrared Imaging system (Licor Biosciences, Lincoln, NE). Bands of p-Akt, p-ERK1/2, p-p38 and pJNK1/3 were quantified by measuring the integrated density using Odyssey software (version 3.0, Licor Biosciences, Lincoln, NE) and normalized to total Akt, ERK1/2, p38 or JNK1/3 and expressed as a percentage of the control (saline).

Experimental Groups and Protocols

Experiment 1: Effect of pharmacological inhibition of PI3K-Akt signaling on peripheral NMDAR-mediated pressor response—We investigated the effect of PI3k/Akt inhibition by wortmannin on peripheral NMDAR-mediated pressor response in 4 groups of conscious male rats ($n=4-6$). In 2 groups, we investigated the blood pressure and heart rate responses elicited by NMDA (125, 250, 500 or 1000 $\mu\text{g}/\text{kg}$; i.v.) 15 min after wortmannin (15 $\mu\text{g}/\text{kg}$, i.v.) or its vehicle (saline). NMDA was administered at 15 min intervals to allow full recovery from the preceding dose except for the final (1000 $\mu\text{g}/\text{kg}$) dose where the animal was sacrificed immediately after the pressor response peak was achieved. The remaining two groups received i.v. wortmannin or saline alone. The dose of wortmannin was based on our previous study²³.

Experiment 2: Effect of pharmacological inhibition of PKC on peripheral

NMDAR-mediated pressor response—Four groups of rats (n=5–6 each) were used in this experiment. The dose of the PKC inhibitor, chelerythrine (5 mg/kg, i.v.), was based on a reported study ²⁵. Chelerythrine or an equal volume of DMSO (vehicle for chelerythrine) was administered 15 min prior to i.v. NMDA (125, 250, 500 or 1000 µg/kg). In the remaining 2 groups, chelerythrine or an equal volume of DMSO was administered.

Experiment 3: Effect of pharmacological inhibition of calcium influx on

peripheral NMDAR-mediated pressor response—The objective of this experiment was to test the hypothesis that enhanced Ca²⁺ influx plays a pivotal role in vascular NMDAR-mediated pressor response in our model system. In this experiment, we exploited the findings of a preliminary study that showed that the NMDAR-mediated response is reproducible over 3 days. Therefore, we utilized a crossover design to investigate the effect of the Ca²⁺ entry blocker, nifedipine on the NMDAR-mediated pressor responses. On experimental day 1, NMDA dose (125, 250, 500 and 1000 µg/kg) pressor response curves were constructed in 6 rats. Following recovery of BP to baseline after the last dose of NMDA, 3 rats received nifedipine in divided doses (0.05, 0.1, 0.2 mg/kg) at 5 min intervals to achieve a cumulative dose of 0.35 mg/kg, while the other 3 rats received equal volume of saline. The NMDA dose-pressor response curve was reconstructed in all 6 rats. On day 2, the same protocol was repeated in the 5 remaining rats except that nifedipine was administered to the 3 rats that received saline on day 1, and the other 2 rats received saline instead of nifedipine. On day 3, the NMDA dose-pressor response curve was constructed before and after nifedipine, administered in divided doses (0.05, 0.1, 0.2 and 0.4 mg/kg) at 5 min intervals to achieve a cumulative dose of 0.75 mg/kg, in the 4 rats that completed this experiment. Nifedipine was administered in divided doses to achieve calcium channel blockade without causing drastic reduction in BP in accordance with reported protocol ²⁶. Notably, we modified the reported regimen so that we administered half of the reported nifedipine dose in the first part, and the full nifedipine dose in the second part, of this experiment. This modified protocol permitted investigation of the dose-related effects of nifedipine on the NMDAR-mediated pressor response.

Experiment 4: Effect of pharmacological inhibition of NADPH Oxidase (NOX) on peripheral NMDAR-mediated pressor response

—Under this experiment, we elucidated the functional role of NOX-derived oxidative stress in the peripheral NMDAR-mediated pressor and biochemical response. Four groups of rats (n=5–6) were used; two groups received apocynin (5 mg/kg, i.v.) 30 min before NMDA (180 µg/kg/min) or saline infusion at a rate of 9 µl/min (Harvard compact pump, Model 975) for 15 min. The other two groups received saline (vehicle) followed by NMDA or saline infusion. The apocynin dose was based on a reported study ²⁷.

Experiment 5: Effect of NMDA infusion on vascular Akt, ERK1/2, JNK1/2/3 and p38 phosphorylation

—Based on the findings of experiment 1, and the important role of phosphorylated Akt in activating other MAP kinases ²⁸, the objective of this experiment was to determine the impact of peripheral NMDAR activation on the phosphorylation state of vascular kinases known to mediate pressor response and oxidative stress ²⁸. To achieve this

goal, aortic tissues were collected during the sustained pressor response caused by NMDA infusion (180 µg/kg/min) using a regimen employed in our previous study¹. Control aortic tissues were obtained from saline infused rats. The levels of the investigated phosphorylated kinases normalized to the corresponding total protein were measured in NMDA and saline infused rats (n=4–5 each) by Western blot analysis as detailed above.

Ex vivo biochemical studies were conducted in vascular (aortic) tissues collected from treatment and control animals employed in the integrative (in vivo) cardiovascular studies during the NMDA-evoked pressor response or the corresponding time in controls. In these studies, we investigated the effect of the pharmacological interventions used in the integrative studies on peripheral NMDAR-mediated increases in vascular nitrite/nitrate (NO_x), ROS and NOX activity.

Drugs

N-Methyl-D-Aspartic Acid, 2'-7'-Dichlorofluorescein, 2'-7'-Dichlorofluorescein diacetate and Apocynin were purchased from Sigma Aldrich (St. Louis, MO). Wortmannin and Chelerythrine were purchased from Tocris Biosciences (Ellisville, MO). Sterile Saline was purchased from B. Braun Medical (Irvine, CA). Dihydroethidium (DHE) was purchased from Life Sciences (Grand Island, NY).

Data Analysis & Statistics

Values were expressed as means ± SEM. Mean Arterial Pressure (MAP) was computed as 1/3 (Systolic-Diastolic) + Diastolic. Statistical analyses were conducted using a one way ANOVA with Bonferroni post-hoc test. Graphpad Prism 5.0 software was used to perform statistical analyses. P<0.05 was considered significant.

Results

Baseline blood pressure and heart values prior to vehicle or pharmacological intervention were similar (P>0.05 ANOVA; Table 1).

PI3K or PKC inhibition attenuates NMDA evoked pressor response

Bolus injections of NMDA (125, 250, 500 or 1000 µg/kg, i.v.) dose dependently increased blood pressure (Fig. 1A, 2A), but caused dose-unrelated tachycardic responses; the tachycardic responses did not achieve statistical significance, when compared with corresponding control (saline) values, because of their variability (Fig. 1B and 1C). PI3K (Wortmannin) or PKC (chelerythrine) inhibition significantly (P<0.05) attenuated the peripheral NMDAR-mediated pressor response and shifted the NMDA dose-pressor response curve downwards (Figs. 1A, 2A). Further, Wortmannin abrogated the NMDAR-mediated tachycardia and its variability (Fig. 1B). However, in the presence of chelerythrine or its vehicle (DMSO), NMDA had no effect on heart rate (Fig. 2B). When administered alone, wortmannin or chelerythrine produced little to no change in blood pressure (Figs. 1C, 2C) or heart rate (Figs. 1D, 2D).

Calcium influx contributes to peripheral NMDAR-mediated pressor response

The NMDA dose-pressor response curves constructed prior to nifedipine or saline were similar (Figs. 3A–C). Nifedipine (0.35 or 0.75 mg/kg), administered in divided doses to circumvent substantial drop in blood pressure²⁶, resulted in slowly developing hypotensive response amounting to -9.1 ± 2.1 , -10.9 ± 2.1 , -15.3 ± 1.9 and -21.1 ± 2.8 mm Hg following the 0.05, 0.15, 0.35 and 0.75 mg/kg cumulative nifedipine doses, respectively. Further, nifedipine caused dose-related and significant ($P < 0.05$) attenuation of the dose-dependent pressor response elicited by peripheral NMDAR activation (Fig. 3B and 3C). Heart rate responses of NMDA treated rats in presence or absence of nifedipine were non consistent and not significantly different (data not shown).

NOX inhibition attenuates the sustained NMDA evoked pressor response

NMDA infusion significantly ($P < 0.05$) increased blood pressure with a maximal response (~ 35 mmHg) at 10 min. Despite its gradual decline, the pressor response was still evident at the end of the 15 min NMDA infusion period (Fig. 4A) when the tissues were collected for the ex vivo studies. Apocynin, which had no significant effect on BP or heart rate (Fig. 4) significantly ($P < 0.05$) attenuated the NMDA-evoked pressor (Fig. 4A), but had little effect on the associated bradycardic response (Fig. 4B).

Effect of NMDA infusion on vascular Akt, ERK1/2, JNK and p38 phosphorylation

Aortic tissues collected during the NMDA-evoked sustained pressor response exhibited significantly ($P < 0.05$) greater levels of phosphorylated Akt, ERK1, JNK1 and p38 compared with the values obtained from control (saline treated) rats (Fig. 5).

PI3K, PKC and NOX contribute to vascular NMDAR-mediated ROS generation and NOX activation

NMDA injection (Fig. 6A, B) or infusion (Fig. 6C) significantly ($P < 0.05$) increased vascular NO (nitrate/nitrite; NOx). Prior PI3K/Akt (Wortmannin), PKC (chelerythrine) or NOX (apocynin) inhibition abrogated ($P < 0.05$) the NMDAR-mediated increases in vascular nitrate/nitrite (NOx) (Fig. 6A–C). ROS generation was measured by DCFH-DA in aortas obtained from rats treated with: saline (vehicle), NMDA, Wortmannin or Wortmannin + NMDA (D), DMSO (vehicle), NMDA, Chelerythrine, Chelerythrine + NMDA (E) or saline (vehicle), NMDA, apocynin or apocynin + NMDA (F). Aorta homogenates were incubated at 37°C with DCF for 30 min ($n = 4-6$). Kinetic readings were recorded at 5 min intervals; relative fluorescence units (RFU) were normalized to protein and were expressed as RFU/ μ g protein/min in the inset in each panel. ROS was significantly ($P < 0.05$) higher in the vasculature of NMDA-, compared with saline-, treated animals. Wortmannin, chelerythrine or apocynin abrogated ($P < 0.05$) the peripheral NMDAR-evoked increases in vascular ROS (Fig. 6D–F) and NADPH oxidase (NOX) activity (Figs. 7, 8). NOX activity was measured in fresh unfixed aorta sections (10 μ m) incubated with 10 μ M dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO₂ in a moist chamber for 30 min. Positive and negative controls were used to validate the assay (see experimental groups and protocols). Images were visualized with a Zeiss LSM 510 microscope. Three-five images were acquired from 3–4 animals for each experimental

condition. Quantification was conducted using Image J software (NIH) and changes in total fluorescence intensity, normalized to control, were calculated. These biochemical findings paralleled the blood pressure responses described above.

Discussion

The present study is the first to demonstrate, in conscious unrestrained rats, the following important findings: (i) pharmacological inhibition of PI3K/Akt signaling (Wortmannin), PKC (Chelerythrine) or calcium influx (nifedipine) significantly attenuated the dose-related pressor responses elicited by peripheral NMDAR activation; (ii) peripheral NMDAR activation enhanced the phosphorylation of vascular Akt and its downstream kinases (ERK1, JNK1, p38); (iii) NOX inhibition (apocynin) attenuated the NMDA-evoked pressor response; (iv) Wortmannin or apocynin abrogated the peripheral NMDAR-mediated increases in vascular NOX activity, and vascular NO and ROS levels. Further, it is possible that under non-pathological conditions, ROS might mask the vasodilating effect of NO, and result in a pressor response²⁹. Collectively, the present integrative (in vivo) and ex vivo molecular/biochemical findings implicate vascular PI3K/Akt and its downstream kinases in the vascular NMDAR-mediated oxidative stress and calcium-dependent pressor response.

Our in vivo pharmacologic and ex vivo molecular studies yielded functional evidence that implicates vascular Akt phosphorylation in the increases in vascular NO, ROS and NOX caused by peripheral NMDAR activation. We focused on the PI3K/Akt pathway because it activates multiple interrelated downstream targets such as MAPK, PKC¹⁶, and NOX³⁰, which ultimately lead to Ca²⁺ influx and vasoconstriction. We show for the first time that PI3K/Akt inhibition (Wortmannin) attenuated the peripheral NMDAR-mediated pressor response (Fig. 1). This finding supports the involvement of the PI3K/Akt signaling pathway in vascular contraction¹¹. It is important to note that baroreceptor involvement³¹ or direct effect of NMDA on the heart might explain the tachycardia and bradycardia observed following bolus (Fig. 1B) and infused (Fig. 4B) NMDA, respectively. Further, if the bradycardic response, observed during NMDA infusion, reflects myocardial dysfunction, it is possible that such response contributes to the decline in the pressor response, despite the continued infusion of NMDA (Fig. 4A). Future studies are needed to elucidate the mechanisms of the peripheral cardiac effects of peripheral NMDAR activation. It is also imperative to note that Wortmannin abrogation of the NMDAR-mediated increase in vascular NO (Fig. 6A) suggests a pivotal role for vascular PI3K/Akt signaling in vascular NOS-derived NO generation caused by vascular NMDAR activation. The present findings with Wortmannin and our previous findings, which demonstrated the ability of nonselective NOS (L-NAME) or selective nNOS (NPLA) inhibition to attenuate the peripheral NMDAR-pressor response and vascular NO generation¹, suggest an important role for vascular PI3K/Akt in downstream activation of nNOS following vascular NMDAR activation. This notion is further supported by reported findings, including ours, which implicated PI3K in downstream NOS signaling, at least partly, via Akt phosphorylation^{23, 32}. Nonetheless, the latter study identified PKC as an important intermediate signaling step in the PI3K activation of NOX in vitro. Therefore, we determined whether PKC is similarly involved in the PI3K-dependent NOX activation in vivo in our model system.

Results showed that when administered in a commonly used dose in reported cardiovascular studies^{25, 33}, the PKC inhibitor chelerythrine attenuated NMDA-evoked pressor response (Fig. 2A). This finding agrees with chelerythrine ability to attenuate another peripherally (angiotensin II)-mediated pressor response³⁴. Given the dual PKC activation of NOS¹⁶ and NOX³⁵, and the involvement of nNOS-derived NO in the vascular NMDAR-mediated pressor response in the our model system¹, it was important to determine if PKC was implicated in the NMDAR-mediated NO generation in the vasculature. First, we showed that chelerythrine abrogated the NMDAR-mediated NOx (index of NO) accumulation in the vasculature (Fig. 6B). This finding, which agrees with a similar finding in cerebellar granule cells³⁶, supports the view that PKC contributes to PI3K-mediated NOS activation¹⁶, and identify the PI3K-PKC signaling network as a molecular trigger for the NMDAR-mediated vascular nNOS activation in our model system. A second objective of this experiment was the elucidation of the role of PKC in NMDAR-mediated ROS generation in the vasculature. We show that PKC inhibition (chelerythrine) abrogated the NMDAR-mediated ROS generation (Fig. 6E), which agrees with chelerythrine inhibition of superoxide levels in human arteries and veins³⁷, at least partly, via abrogation of the NMDAR-mediated NOX activation (Fig. 7). The latter finding is consistent with PKC involvement in NOX-derived ROS generation³⁸. Collectively, these findings implicate the PI3K-PKC signaling network in the NOS-derived NO and NOX-derived ROS generation, which play pivotal roles in the peripheral NMDAR-mediated pressor response¹. It is also imperative to note that PI3K and PKC signaling contributes to calcium influx, which is a well-known mediator of vascular contractility. Our finding that nifedipine produced dose-dependent attenuation of the peripheral NMDAR-mediated pressor response (Fig. 3) suggests the involvement of calcium entry in the peripheral NMDAR-mediated pressor response. The adopted nifedipine dose regimen confirmed the contribution of calcium influx, through L-type of voltage-dependent calcium channels (L-VDCC), to pressor responses caused by exogenous vasopressor agents²⁶.

To further elucidate the molecular mechanisms for vascular NO-dependent oxidative stress (ROS) in peripheral NMDAR-mediated pressor response, we investigated the role of vascular MAP kinases, which are activated by Akt, and play a key role in NO and ROS generation. In addition to enhancing Akt phosphorylation, vascular NMDAR activation enhanced the phosphorylation of ERK1, JNK 1 and p38 (Fig. 5). The enhanced Akt phosphorylation in the vasculature of NMDA-treated rats (Fig. 5A) is consistent with in vitro findings in neurons and VSMCs incubated with NMDA^{39, 40}. However, our findings highlight a functional role for this vascular kinase because Akt phosphorylation was evident during the NMDA-evoked pressor response and the later was attenuated following Akt inhibition with Wortmannin. Further, Akt phosphorylation might account for downstream phosphorylation of ERK1 (Fig. 5B) because the latter could be mediated via a PI3K/Akt-dependent mechanism²⁸ or directly⁴¹ following NMDAR activation in striatal neurons. While the functional relevance of the enhanced Akt and ERK1 phosphorylation will be discussed further below, it is highly likely that the enhancement of the phosphorylation of the other investigated kinases JNK1 (Fig. 5C) and p38 (Fig. 5D) contributes to the NMDAR-mediated pressor response for the following reasons. First, although we report the first evidence for NMDAR-mediated p38 phosphorylation in vasculature, our finding is

consistent with similar findings in neuronal cultures⁴². Second, enhanced phosphorylation of JNK and p38 increases vascular tone, at least partly, via NOX activation and ROS generation¹³. Collectively, the present study demonstrates peripheral NMDAR-mediated activation of these vascular kinases and infer a role for these kinases in the downstream signaling that lead to NO and ROS generation, and ultimately the pressor response.

Another major role for PI3K/Akt signaling in vascular biology is its mediation of ROS generation, at least partly, via NOX activation⁴³. The present findings support the involvement of this signaling cascade in NMDA-evoked increase in vascular ROS levels and pressor response because: (i) Wortmannin abrogated the NMDAR-mediated ROS elevation (Fig. 6D); (ii) peripheral NMDAR activation significantly increased vascular dihydroethidium (DHE) fluorescence (Fig. 7B), which reflects increased NOX catalytic activity⁴⁴; (iii) Wortmannin abrogated the NMDAR-mediated increase in vascular NOX activity (Fig. 7D), which is supported by Wortmannin ability to abrogate NOX activation in other model systems³⁵. These findings implicate the PI3K/Akt signaling in the NOS (NO) and NOX (ROS) generation and Ca²⁺ influx, which play pivotal roles in the peripheral NMDAR-mediated pressor response.

It is important to comment on our pharmacological and biochemical findings with the NOX inhibitor apocynin. When used in a dose that caused no behavioral effects in normotensive rats⁴⁵, apocynin abrogated the peripheral NMDA-evoked pressor response (Fig. 4). This finding fully agrees with apocynin abrogation of pressor responses elicited by other vasoactive agents such as angiotensin II or endothelin⁴⁶. Equally important, the ex vivo findings that demonstrated apocynin abrogation of the NMDAR-mediated vascular NOX activation (Fig. 8) and ROS generation (Fig. 6F) lend credence to our in vivo findings. Interestingly, apocynin also abrogated the NMDAR-mediated elevation in vascular NO. However, this latter finding contradicts a reported increase in NO following apocynin^{47, 48}. It is possible that the relatively short time allowed after acute apocynin administration in our study accounts for such discrepancy because NO decreases and increases following acute (minutes) and extended (hours/days) exposure to apocynin⁴⁹.

Together, these findings suggest that vascular NOX (ROS) and NOS (NO) signaling, via their common upstream activator PI3K/Akt, play important roles in the NMDAR-mediated pressor response in conscious rats.

Conclusion

The integrative pharmacologic and ex vivo molecular findings provide the first evidence that enhancement of vascular PI3K/Akt/PKC signaling triggers oxidative stress and the Ca²⁺-dependent pressor response caused by vascular NMDAR activation. The findings implicate phosphorylation of vascular MAP kinases (Akt, ERK1, JNK1 and p38) in the NMDAR-mediated pressor response. Specifically, the pharmacological evidence, complemented with the ex vivo biochemical and fluorescence findings, strongly support the involvement of the PI3K/Akt-PKC-NOX pathway in the NMDAR-mediated oxidative stress and pressor response. The current findings yield new insight into the understanding of the vascular biology of NMDAR signaling and set the stage for detailed molecular studies on the role of

vascular NMDAR in blood pressure control in health and in pathophysiological states; e.g. hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. McGee MA, Abdel-Rahman AA. Enhanced vascular neuronal nitric-oxide synthase-derived nitric-oxide production underlies the pressor response caused by peripheral n-methyl-d-aspartate receptor activation in conscious rats. *The Journal of pharmacology and experimental therapeutics*. 2012; 342:461–471. [PubMed: 22580349]
2. Leonelli M, Martins DO, Britto LR. Retinal cell death induced by trpv1 activation involves nmda signaling and upregulation of nitric oxide synthases. *Cellular and molecular neurobiology*. 2013; 33:379–392. [PubMed: 23324998]
3. Sitniewska EM, Wi niewska RJ, Wi niewski K. The role of ionotropic receptors of glutaminic acid in cardiovascular system. *Amino Acids*. 2003; 24:397–403. [PubMed: 12768502]
4. Kaczy ska K, Szereda-Przestaszewska Mg. Cardiorespiratory effects of intravenous n-methyl-d-aspartate challenge in anaesthetized rats. *Clinical and Experimental Pharmacology and Physiology*. 2004; 31:101–106. [PubMed: 14756692]
5. Dong XD, Mann MK, Kumar U, Svensson P, Arendt-Nielsen L, Hu JW, Sessle BJ, Cairns BE. Sex-related differences in nmda-evoked rat masseter muscle afferent discharge result from estrogen-mediated modulation of peripheral nmda receptor activity. *Neuroscience*. 2007; 146:822–832. [PubMed: 17382479]
6. Santini CO, Fassini A, Scopinho AA, Busnardo C, Corrêa FMA, Resstel LBM. The ventral hippocampus nmda receptor/nitric oxide/guanylate cyclase pathway modulates cardiovascular responses in rats. *Autonomic Neuroscience*. 2013; 177:244–252. [PubMed: 23735844]
7. Reinke LA, Kotake Y, Moore DR, Nanji AA. Free radical formation during ketamine anesthesia in rats: A cautionary note. *Free Radical Biology and Medicine*. 1998; 24:1002–1006. [PubMed: 9607611]
8. Kevin LG, Novalija E, Stowe DF. Reactive oxygen species as mediators of cardiac injury and protection: The relevance to anesthesia practice. *Anesth Analg*. 2005; 101:1275–1287. [PubMed: 16243980]
9. Brennan-Minnella A, Shen Y, El-Benna J, Swanson R. Phosphoinositide 3-kinase couples nmda receptors to superoxide release in excitotoxic neuronal death. *Cell Death and Disease*. 2013; 4:e580. [PubMed: 23559014]
10. Zhu D, Wu X, Strauss KI, Lipsky RH, Qureshi Z, Terhakopian A, Novelli A, Banaudha K, Marini AM. N-methyl-d-aspartate and trkb receptors protect neurons against glutamate excitotoxicity through an extracellular signal-regulated kinase pathway. *Journal of Neuroscience Research*. 2005; 80:104–113. [PubMed: 15744743]
11. Morello F, Perino A, Hirsch E. Phosphoinositide 3-kinase signalling in the vascular system. *Cardiovasc Res*. 2009; 82:261–271. [PubMed: 19038971]
12. Chen C, Druhan L, Varadharaj S, Chen Y, Zweier J. Phosphorylation of endothelial nitric-oxide synthase regulates superoxide generation from the enzyme. *J Biol Chem*. 2008; 283:27038–27047. [PubMed: 18622039]
13. Bao W, Behm DJ, Nerurkar SS, Ao Z, Bentley R, Mirabile RC, Johns DG, Woods TN, Doe CP, Coatney RW, Ohlstein JF, Douglas SA, Willette RN, Yue T-L. Effects of p38 mapk inhibitor on

- angiotensin ii-dependent hypertension, organ damage, and superoxide anion production. *J Cardiovasc Pharmacol.* 2007; 49:362–368. [PubMed: 17577100]
14. Yoshizumi M, Tsuchiya K, Tamaki T. Signal transduction of reactive oxygen species and mitogen-activated protein kinases in cardiovascular disease. *J Med Invest.* 2001; 48:11–24. [PubMed: 11286012]
 15. Pandya N, Santani D, Jain S. Role of mitogen-activated protein (map) kinases in cardiovascular diseases. *Cardiovasc Drug Rev.* 2005; 23:247–254. [PubMed: 16252017]
 16. Hammer B, Davis Parker W, Bennett JP. Nmda receptors increase oh radicals in vivo by nitric oxide synthase and protein kinase c. *Neuroreport.* 1993; 5:72–74. [PubMed: 7506590]
 17. Frey R, Gao X, Javaid K, Siddiqui S, Rahman A, Malik A. Phosphatidylinositol 3-kinase gamma signaling through protein kinase c zeta induces nadph oxidase-mediated oxidant generation and nf-kappab activation in endothelial cells. *J Biol Chem.* 2006; 281:16128–16138. [PubMed: 16527821]
 18. Girouard H, Wang G, Gallo EF, Anrather J, Zhou P, Pickel VM, Iadecola C. Nmda receptor activation increases free radical production through nitric oxide and nox2. *J Neurosci.* 2009; 29:2545–2552. [PubMed: 19244529]
 19. Winkler K, Hermans JJR, Schiffers P, Moens AL, Paul M, Schmidt H. Nox1, 2, 4, 5: Counting out oxidative stress. *British Journal of Pharmacology.* 2011; 164:866–883. [PubMed: 21323893]
 20. Nassar NN, Li G, Strat AL, Abdel-Rahman AA. Enhanced hemeoxygenase activity in the rostral ventrolateral medulla mediates exaggerated hemin-evoked hypotension in the spontaneously hypertensive rat. *Journal of Pharmacology and Experimental Therapeutics.* 2011; 339:267–274. [PubMed: 21768222]
 21. Zou Y, Jung K, Kim J, Yu B, Chung H. Alteration of soluble adhesion molecules during aging and their modulation by calorie restriction. *FASEB J.* 2003; 18:320–322. [PubMed: 14688195]
 22. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. *Analytical Biochemistry.* 1993; 214:11–16. [PubMed: 7504409]
 23. El-Mas MM, Fan M, Abdel-Rahman AA. Facilitation of myocardial pi3k/akt/nnos signaling contributes to ethanol-evoked hypotension in female rats. *Alcoholism: Clinical and Experimental Research.* 2009; 33:1158–1168.
 24. Ibrahim BM, Abdel-Rahman AA. Differential modulation of brainstem phosphatidylinositol 3-kinase/akt and extracellular signal-regulated kinase 1/2 signaling underlies win5,212-2 centrally mediated pressor response in conscious rats. *Journal of Pharmacology and Experimental Therapeutics.* 2012; 340:11–18. [PubMed: 21946192]
 25. Yamanaka K, Takahashi N, Ooie T, Kaneda K, Yoshimatsu H, Saikawa T. Role of protein kinase c in geranylgeranylacetone-induced expression of heat-shock protein 72 and cardioprotection in the rat heart. *Journal of Molecular and Cellular Cardiology.* 2003; 35:785–794. [PubMed: 12818569]
 26. Pinterova M, Karen P, Kunes J, Zicha J. Role of nifedipine-sensitive sympathetic vasoconstriction in maintenance of high blood pressure in spontaneously hypertensive rats: Effect of gi-protein inactivation by pertussis toxin. *J Hypertens.* 2010; 28:969–978. [PubMed: 20224433]
 27. Ahmad A, Mondello S, Di Paola R, Mazzon E, Esposito E, Catania MA, Italiano D, Mondello P, Aloisi C, Cuzzocrea S. Protective effect of apocynin, a nadph-oxidase inhibitor, against contrast-induced nephropathy in the diabetic rats: A comparison with n-acetylcysteine. *European Journal of Pharmacology.* 2011; 674:397–406. [PubMed: 22094062]
 28. Perkinson MS, Ip J, Wood GL, Crossthwaite AJ, Williams RJ. Phosphatidylinositol 3-kinase is a central mediator of nmda receptor signalling to map kinase (erk1/2), akt/pkb and creb in striatal neurones. *Journal of Neurochemistry.* 2002; 80:239–254. [PubMed: 11902114]
 29. Burgoyne JR, Mongue-Din H, Eaton P, Shah AM. Redox signaling in cardiac physiology and pathology. *Circulation Research.* 2012; 111:1091–1106. [PubMed: 23023511]
 30. Li J, Li W, Liu W, Altura BT, Altura BM. Mechanisms of hydroxyl radical-induced contraction of rat aorta. *European Journal of Pharmacology.* 2004; 499:171–178. [PubMed: 15363964]
 31. Su D, Miao C. Arterial baroreflex function in conscious rats. *Acta Pharmacol Sin.* 2002; 23:673–679. [PubMed: 12147187]

32. Yoshitomi H, Xu Q, Gao M, Yamori Y. Phosphorylated endothelial nos ser1177 via the pi3k/akt pathway is depressed in the brain of stroke-prone spontaneously hypertensive rat. *J Stroke Cerebrovasc Dis.* 2011; 20:406–412. [PubMed: 20813549]
33. Das A, Ockaili R, Salloum F, Kukreja RC. Protein kinase c plays an essential role in sildenafil-induced cardioprotection in rabbits. *American Journal of Physiology - Heart and Circulatory Physiology.* 2004; 286:H1455–H1460. [PubMed: 15020304]
34. Mollnau H, Wendt M, Szocs K, Lassegue B, Schulz E, Oelze M, Li H, Bodenschatz M, August M, Kleschyov AL, Tsilimingas N, Walter U, Forstermann U, Meinertz T, Griendling K, Munzel T. Effects of angiotensin ii infusion on the expression and function of nad(p)h oxidase and components of nitric oxide/cgmp signaling. *Circ Res.* 2002; 90:e58–e65. [PubMed: 11884382]
35. Yamamori T, Inanami O, Nagahata H, Kuwabara M. Phosphoinositide 3-kinase regulates the phosphorylation of nadph oxidase component p47phox by controlling cpkc/pkc delta but not akt. *Biochemical and Biophysical Research Communications.* 2004; 316:720–730. [PubMed: 15033459]
36. Gunasekar PG, Kanthasamy AG, Borowitz JL, Isom GE. Nmda receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: Implications for cell death. 1995; 65:2016–2021.
37. Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, Korbut R, Channon KM. Systemic regulation of vascular nad(p)h oxidase activity and nox isoform expression in human arteries and veins. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2004; 24:1614–1620.
38. Ungvari Z, Csiszar A, Kaminski PM, Wolin MS, Koller A. Chronic high pressure-induced arterial oxidative stress: Involvement of protein kinase c-dependent nad(p)h oxidase and local renin-angiotensin system. *The American Journal of Pathology.* 2004; 165:219–226. [PubMed: 15215177]
39. Doronzo G, Russo I, Del Mese P, Viretto M, Mattiello L, Trovati M, Anfossi G. Role of nmda receptor in homocysteine-induced activation of mitogen-activated protein kinase and phosphatidyl inositol 3-kinase pathways in cultured human vascular smooth muscle cells. *Thrombosis Research.* 2010; 125:e23–e32. [PubMed: 19766294]
40. Mao L-M, Tang Q-S, Wang JQ. Regulation of extracellular signal-regulated kinase phosphorylation in cultured rat striatal neurons. *Brain Research Bulletin.* 2009; 78:328–334. [PubMed: 19056470]
41. Wang JQ, Fibuch EE, Mao L. Regulation of mitogen-activated protein kinases by glutamate receptors. *Journal of Neurochemistry.* 2007; 100:1–11. [PubMed: 17018022]
42. Waxman EA, Lynch DR. N-methyl-d-aspartate receptor subtype mediated bidirectional control of p38 mitogen-activated protein kinase. *Journal of Biological Chemistry.* 2005; 280:29322–29333. [PubMed: 15967799]
43. Clemmus RE, Griendling KK. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovascular Research.* 2006; 71:216–225. [PubMed: 16616906]
44. Zhang C, Yi F, Xia M, Boini K, Zhu Q, Laperle L, Abais J, Brimson C, Li P-L. Nmda receptor-mediated activation of nadph oxidase and glomerulosclerosis in hyperhomocysteinemic rats. *Antioxid Redox Signal.* 2010; 13:975–986. [PubMed: 20406136]
45. Viridis A, Colucci R, Fornai M, Duranti E, Giannarelli C, Bernardini N, Segnani C, Ippolito C, Antonioli L, Blandizzi C, Taddei S, Salvetti A, Del Tacca M. Cyclooxygenase-1 is involved in endothelial dysfunction of mesenteric small arteries from angiotensin ii-infused mice. *Hypertension.* 2007; 49:679–686. [PubMed: 17145980]
46. Baumer AT, Kruger CA, Falkenberg J, Ten Freyhaus H, Rosen R, Fink K, Rosenkranz S. The nad(p)h oxidase inhibitor apocynin improves endothelial no/superoxide balance and lowers effectively blood pressure in spontaneously hypertensive rats: Comparison to calcium channel blockade. *Clinical and Experimental Hypertension.* 2007; 29:287–299. [PubMed: 17653964]
47. Hamilton CA, Brosnan MJ, Al-Benna S, Berg G, Dominiczak AF. Nad(p)h oxidase inhibition improves endothelial function in rat and human blood vessels. *Hypertension.* 2002; 40:755–762. [PubMed: 12411473]
48. Williams HC, Griendling KK. Nadph oxidase inhibitors: New antihypertensive agents? *J Cardiovasc Pharmacol.* 2007; 50:9–16. [PubMed: 17666910]

49. Zhang R, Ran H, Ma J, Bai Y, Lin L. Nad(p)h oxidase inhibiting with apocynin improved vascular reactivity in tail-suspended hindlimb unweighting rat. *J Physiol Biochem.* 2012; 68:99–105. [PubMed: 22015782]

Figure 1.

Effect of the PI3K-Akt inhibitor wortmannin (15 μ g/kg, i.v.) or saline (vehicle) on i.v. NMDA-evoked changes in (A) mean arterial pressure (Δ MAP) and (B) heart rate (Δ HR) in conscious male Sprague Dawley rats. Right panels show the effects of Wortmannin or saline, given alone, on MAP (C) and HR (D). Values are means $\pm$ SEM of 4-6 observations. * P <0.05 vs. NMDA alone (one-way ANOVA with Bonferroni post-hoc test).

Figure 2.

Effect of the PKC inhibitor chelerythrine (5 mg/kg, i.v.) or DMSO (vehicle) on i.v. NMDA-evoked changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) in conscious male Sprague Dawley rats. Right panels show the effects of chelerythrine or DMSO, given alone, on MAP (C) and HR (D). Values are means $\pm$ SEM of 4–6 observations. *P<0.05 vs. NMDA alone (one-way ANOVA with Bonferroni post-hoc test).

Figure 3.

Dose-pressor responses constructed by systemic NMDA before (open symbols) and after (closed symbols) saline (A), 0.35 mg/kg nifedipine, (B) or 0.75 mg/kg nifedipine (C) in conscious male Sprague Dawley rats. Values are means $\pm$ SEM of 4–6 observations. * $P < 0.05$ vs. corresponding pretreatment values (one-way ANOVA with Bonferroni post-hoc test). Note that all 3 NMDA dose-pressor response curves constructed before saline (A) or nifedipine (B, C) were similar ($P > 0.05$).

Figure 4.

Effect of the NADPH oxidase inhibitor apocynin (5 mg/kg, i.v.), compared with saline (vehicle), on systemic NMDA-evoked changes in mean arterial pressure (MAP; **A**) and heart rate (HR; **B**) in conscious male Sprague Dawley rats. Also shown in the figure, the lack of changes in MAP or HR in rats treated only with apocynin, compared with saline. Values are means $\pm$ SEM of 4–6 observations. * $P < 0.05$ vs. saline; # $P < 0.05$ vs. NMDA alone (one-way ANOVA with Bonferroni post-hoc test).

Figure 5.

Effect of NMDA infusion (180 $\mu\text{g/kg/min}$) on phosphorylated Akt (A), ERK1/2 (B), JNK (C), and p38 (D) as a ratio of the corresponding total protein. Western blot analyses were conducted in the vasculature of male Sprague Dawley rats during sustained pressor response caused by 30 min i.v NMDA infusion; vasculature from saline-infused rats served as control. Values are means $\pm$ SEM of 3 to 5 observations. * $P < 0.05$ vs. control (saline); data analyzed by one-way ANOVA with Bonferroni post-hoc test.

Figure 6.

Effect of NMDA on aortic nitrite/nitrate (NOx) content in animals pretreated with the PI3K-Akt inhibitor Wortmannin (15 μ g/kg, i.v., **A**), the PKC inhibitor chelerythrine (5 mg/kg, i.v., **B**) or the NADPH oxidase inhibitor apocynin (5 mg/kg, i.v., **C**) compared with the corresponding vehicle control values. ROS generation, measured by DCFH-DA, in aortas obtained from rats treated with: Saline (vehicle), NMDA, Wortmannin or Wortmannin + NMDA (**D**), DMSO (vehicle), NMDA, chelerythrine or chelerythrine + NMDA (**E**) or Saline (vehicle), NMDA, apocynin or apocynin + NMDA (**F**). Values are means $\pm$ SEM of 4–6 observations and data analyzed by one-way ANOVA with Bonferroni post-hoc test.

Figure 7.

NOX activity measured by dihydroethidium (DHE) fluorescence in aortic sections from treated and control rats (3–4 images/rat were acquired from 3–4 animals for each experimental condition). (A) saline (control), (B) NMDA, (C) the PI3K inhibitor wortmannin, (D) wortmannin + NMDA, (E) the PKC inhibitor chelerythrine (CH), and (F) chelerythrine (CH) + NMDA. Sections were incubated at 37°C with DHE (10 μM) for 30 min. (G) Bar graphs showing DHE fluorescence intensity; data normalized to vehicle. * $P < 0.05$ vs. vehicle (saline for NMDA or wortmannin and DMSO for chelerythrine) and # $P < 0.05$ vs. NMDA alone (one-way ANOVA with Bonferroni post-hoc test).

Figure 8.

NOX activity in aortic sections measured by dihydroethidium (DHE) fluorescence in aortic sections from treated and control rats (3–4 images/rat were acquired from 3–4 animals for each experimental condition): (A) saline (control), (B) NMDA infusion, (C) NOX inhibitor apocynin, (D) apocynin + NMDA infusion. Sections were incubated at 37°C with DHE (10 μ M) for 30 min. (E) Bar graphs showing DHE fluorescence intensity; data normalized to vehicle. * $P < 0.05$ vs. saline (vehicle) and # $P < 0.05$ vs. NMDA alone (one-way ANOVA with Bonferroni post-hoc test).