Nitro-Oleic Acid Inhibits Angiotensin II–Induced Hypertension

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Rationale: Nitro-oleic acid (OA-NO2) is a bioactive, nitric-oxide derived fatty acid with physiologically relevant vasculoprotective properties in vivo. OA-NO2 exerts cell signaling actions as a result of its strong electrophilic nature and mediates pleiotropic cell responses in the vasculature.

Objective: The present study sought to investigate the protective role of OA-NO2 in angiotensin (Ang) II–induced hypertension.

Methods and Results: We show that systemic administration of OA-NO2 results in a sustained reduction of Ang II–induced hypertension in mice and exerts a significant blood pressure lowering effect on preexisting hypertension established by Ang II infusion. OA-NO2 significantly inhibits Ang II contractile response as compared to oleic acid (OA) in mesenteric vessels. The improved vasoconstriction is specific for the Ang II type 1 receptor (AT1R)-mediated signaling because vascular contraction by other G-protein–coupled receptors is not altered in response to OA-NO2 treatment. From the mechanistic viewpoint, OA-NO2 lowers Ang II–induced hypertension independently of peroxisome proliferation-activated receptor (PPAR)γ activation. Rather, OA-NO2, but not OA, specifically binds to the AT1R, reduces heterotrimeric G-protein coupling, and inhibits IP3 (inositol-1,4,5-triphosphate) and calcium mobilization, without inhibiting Ang II binding to the receptor.

Conclusions: These results demonstrate that OA-NO2 diminishes the pressor response to Ang II and inhibits AT1R-dependent vasoconstriction, revealing OA-NO2 as a novel antagonist of Ang II–induced hypertension. 

Key Words: nitroalkenes • hypertension • angiotensin II • angiotensin II type 1 receptor • peroxisome proliferation-activated receptor-γ

The nitrate-nitrite (NO2−–nitric oxide (NO) pathway1 is emerging as an important mediator of cell signaling and bioenergetics events, with therapeutic implications in blood flow regulation and tissue responses to hypoxia.2,3 Nitroalkene derivatives of unsaturated fatty acids represent the convergence of such NO pathway with lipids4 because they are formed by NO and NO2−–dependent redox reactions.1 Thus, they constitute a subset of molecules in the emerging physiological pool of the nitric oxide derived metabolome5,6 that control the physiological homeostasis by coordinating adaptive responses.7,8 At present, the mechanisms underlying the production of nitrated lipids under biological conditions, specific structural isomer distributions, nitrated fatty acid-susceptible proteome, and detailed biological signaling actions are incompletely characterized.6 It is agreed that under conditions in which O2 tension is low (eg, ischemia and anoxia), the balance between peroxyl radical formation and coupling with NO shifts toward nitration reactions.9,10 Thus, nitroalkenes are significantly increased in the heart after reperfusion in an ischemia/reperfusion injury model,11 and these species are abundantly generated in the mitochondria of hearts exposed to ischemic preconditioning.12,13

Nitroalkenes transduce signaling reactions by covalently modifying nucleophilic protein targets and altering their structure and function.6 For instance, nitro derivatives of linoleic and oleic (OA-NO2) acids are activators of peroxisome proliferator-activated receptor (PPAR)γ,14,15 form covalent adducts with the p65 subunit of nuclear factor κB.16

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and activate the Keap1/Nrf2 antioxidant response, reactions that promote adaptive responses in vascular cells. These derivatives of NO-mediated redox reactions represent an inflammatory-resolving adaptive response. In the context of the vasculature, OA-NO₂ inhibits neointimal hyperplasia and exerts vasorelaxation of preconstricted aortic rings. For these reasons, and because of the well-established vasorelaxant properties of pharmacological doses of nitrite, it is plausible to suggest that nitroalkenes will have a significant impact in the regulation of blood pressure and vascular tone. Herein, we determined whether a nitroalkene derivative ameliorate angiotensin (Ang) II–induced hypertension. As the major component of the renin–angiotensin–aldosterone system, deregulation of Ang II production plays a critical role in the pathogenesis of hypertension. Thus, inhibition of the catalytic activity of the angiotensin converting enzyme (ACE) or competitive inhibition of Ang II type 1 receptor (AT₁R) ligand binding by angiotensin receptor blockers (ARBs) are 2 prevalent treatment options for hypertension. Also, molecular targets of nitroalkenes, such as PPARγ and the AT₁R reported herein, reflect common signaling targets that influence the progression of hypertension and other cardiovascular and metabolic diseases.

**Methods**

**Ang II–Induced Hypertension Model**

Blood pressure (BP) in mice was measured by radiotelemetry. Mice were subjected to subcutaneous implantation of osmotic minipumps for delivery of OA-NO₂ or oleic acid (OA) at a concentration supporting an infusion rate of 5 mg/kg per day, and Ang II was administered at an infusion rate of 500 ng/kg per minute.

**In Vivo Infusion for Blood Pressure Tracings and In Vitro Vessel Contraction Analysis**

OA or OA-NO₂ and the PPARγ inhibitor GW9662 were delivered via the jugular vein. Both BP recordings were monitored before and after Ang II infusion (10 μg/mL, infusion rate: 1 μL/min) using a 1.4F microtip catheter sensor inserted into the right carotid artery. Second-order mesenteric arteries from SD rats were mounted onto a myograph. Vessels were pretreated with OA-NO₂ or OA at 2.5 μmol/L and 5 μmol/L for 10 minutes and then contracted with Ang II, phenylephrine (PE), or endothelin-1 (ET-1), respectively.

**Analysis of OA-NO₂ Binding to the AT₁R**

The transalkylation reaction of AT₁R-bound OA-NO₂ was performed according to the methodology described previously.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**OA-NO₂ Reduces Ang II–Induced Hypertension**

To determine whether OA-NO₂ plays a role on Ang II–mediated hypertension, we delivered OA-NO₂ (5 mg/kg per day), as well as its nonnitrated counterpart, OA, systemically via osmotic minipumps in C57/B6L mice. Systemic delivery of OA and OA-NO₂ did not affect baseline systolic or diastolic BP recordings measured by radiotelemetry during the first week of the analysis as compared with polyethylene glycol–treated group that served as vehicle control (Figure 1A and 1C). Subsequently, mice were further treated with Ang II, supporting an infusion rate of 500 ng/kg per minute for 2 additional weeks. Under these conditions, OA-NO₂ but not OA significantly reduced Ang II–dependent increases in systolic (≈15 mm Hg reduction) and diastolic BP (≈12 mm Hg reduction) compared to control OA- or vehicle-treated animals (Figure 1B and 1D), without any apparent changes in the heart rate (Online Figure I). These results strongly indicate that OA-NO₂ treatment has a significant impact on Ang II–induced hypertension through a prolonged and sustained reduction of BP.

We then monitored BP in isoflurane-anesthetized mice by in vivo tracing recordings on short-term infusion of Ang II, which results in a rapid increase in systemic BP. OA and OA-NO₂ (1 mmol/L, infusion rate: 1 μL/min) administered before Ang II delivery (10 μg/mL, infusion rate: 1 μL/min) did not alter basal BP recordings (Figure 2A and 2B). Rather, OA-NO₂, but not similar concentrations of the native OA, significantly prevented BP elevation after short-term infusion of Ang II (Figure 2C). To determine whether OA-NO₂ actually reduces the pressor response to Ang II, we then administered OA-NO₂ to mice 3 days after Ang II osmotic minipump implantation (Figure 2D and 2E). Under these experimental conditions, OA-NO₂ but not OA results in a dose-dependent reduction of BP to the preexisting Ang II–induced hypertension (Figure 2F). Taken together, these results demonstrate that OA-NO₂ diminishes the pressor response to Ang II in vivo, regardless of whether the nitroalkene is administered before or after Ang II challenge.

**OA-NO₂ Specifically Inhibits Ang II–Induced Vasoconstriction**

To determine the specificity of OA-NO₂ effects on Ang II–induced hypertension, we evaluated whether OA-NO₂ may result in changes of vascular contractility. In isolated second-order rat mesenteric vessels, the presence of OA-NO₂ (2.5 μmol/L) resulted in ~50% reduction of Ang II–mediated vasoconstriction at 10⁻⁷ mol/L (corresponding to ~70% of the contractile response induced by 50 mmol/L KCl), whereas 5 μmol/L OA-NO₂ but not OA almost completely abolishes

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**Non-standard Abbreviations and Acronyms**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<td>ARB</td>
<td>angiotensin receptor blocker</td>
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<td>AT₁R</td>
<td>angiotensin type 1 receptor</td>
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<td>BME</td>
<td>β-mercaptoethanol</td>
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<td>BP</td>
<td>blood pressure</td>
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<td>ET-1</td>
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<td>GPCR</td>
<td>G-protein–coupled receptor</td>
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<td>IP₃</td>
<td>inositol-1,4,5-trisphosphate</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor-γ</td>
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contraction induced by Ang II (Figure 3A and Online Figure IV). Thus, OA-NO2 treatment dose-dependently reduced Ang II–dependent vasoconstriction. Interestingly, vasoconstriction elicited by signaling through other members of the G-protein–coupled receptor (GPCR) family, such as by treatment with PE and ET-1, was not affected on OA-NO2 treatment at any concentration given (Figure 3B and 3C; Online Figure IV). These results are clearly indicative that OA-NO2 reduces vasoconstriction by specifically interfering with Ang II signaling in the vasculature.

OA-NO2 Reduction of Ang II–Induced Hypertension Is a PPARγ-Independent Phenomenon

Because (1) OA-NO2 activates PPARγ,14,25 (2) PPARγ activators downregulate AT1R in vascular cells,26,27 and (3) certain ARBs inhibit AT1R through PPARγ activity,28–30 we assessed whether PPARγ activation by OA-NO2 contributes to the observed reduction of Ang II–induced hypertension in vivo.29 To this aim, we first monitored BP by tracing recordings on stimulation with Ang II in response to the treatment with the PPARγ antagonist GW9662 (Figure 4). Efficient inhibition of PPARγ by GW9662 was directly demonstrated monitoring PPARγ transcripational activity by means of EGFP expression in the aorta of 3XPPRE-driven EGFP transgenic reporter mice (Online Figure V). Inhibition of PPARγ alone does not result in basal BP reduction in vivo (Figure 4A and 4B). Moreover, GW9662 administration (10 mg/kg) compared to vehicle treated mice at the same infusion rate did not attenuate BP increases after Ang II challenge (Figure 4 and Online Figure II). Of importance, pretreatment with OA-NO2 but not equimolar concentrations of OA persistently reduced approximately 10 mm Hg of Ang II–mediated BP elevation in the presence or absence of GW9662 (Figure 4C). Further experiments were performed to determine whether PPARγ activation by OA-NO2 affects already established hypertension. To this aim, we compared the BP lowering effect of OA-NO2 versus rosiglitazone after preincubation with GW9662 in Ang II–induced hypertensive mice as described above. In response to rosiglitazone treatment, BP reduction (~7 mmHg) was efficiently antagonized by GW9662 (Online Figure II), whereas OA-NO2 delivery in the presence of GW9662 results in a dose-dependent BP reduction comparable to the lack of the PPARγ antagonist (Online Figure III). Next, we determine the contractile response of mesenteric rings in response to partial inhibition of Ang II–mediated vasoconstriction by OA-NO2 (2.5 µmol/L) after treatment with GW9662 (10 µmol/L). As shown in Figure 4D, OA-NO2 similarly diminished Ang II–dependent vasoconstriction with or without PPARγ antagonism by GW9662 treatment. This series of experiments indicate that PPARγ activation by OA-NO2 is not the major mechanism involved in OA-NO2 antagonism of both Ang II–mediated hypertension and vasoconstriction.

Figure 1. OA-NO2 inhibits Ang II–induced hypertension. Mice implanted with osmotic pumps adjusted to deliver 5 mg/kg per day OA-NO2 (red) or OA (blue) and vehicle control (black) were infused with Ang II at a pressor rate of 500 ng/kg per minute. Systolic (A) and diastolic (C) blood pressure was measured by radiotelemetry. Data are shown as means ± SEM (n=6 in each group). *P<0.05. OA-NO2–treated mice showed significantly lower systolic (~15 mm Hg reduction) (B) and diastolic pressure (~12 mm Hg reduction) (D) after Ang II challenge than did OA and vehicle-treated mice as determined both at the night and day time periods. Data in B and D are shown as means ± SEM of measurements collected before (3 days) and after Ang II (12 days) (n=6 in each group). *P<0.05.
OA-NO₂ Forms Covalent Adducts With AT₁R

Nitroalkenes are highly electrophilic and react with cellular nucleophiles via a reversible Michael addition reaction. Thus, we hypothesized that OA-NO₂ could directly modify nucleophilic residues of AT₁R and impact the propagation of downstream signaling events. A covalent interaction between OA-NO₂ and AT₁R was revealed by treatment of HEK293 cells overexpressing AT₁R with OA-NO₂ or OA. Following immunoprecipitation of AT₁R from cell lysates, a transalkylation reaction of AT₁R-bound OA-NO₂ permitted the detection and quantification of OA-NO₂ adduction by HPLC-MS/MS (Online Methods). HEK293 cells treated with increasing concentrations of OA-NO₂ revealed significantly increased levels of AT₁R-adducted OA-NO₂ compared with control cells (Figure 5A). OA-NO₂ bound to the AT₁R, quantified as a β-mercaptoethanol (BME) derivative after nucleophilic exchange of OA-NO₂ from the AT₁R to BME, revealed a ≈20-fold increase of adducted OA-NO₂ in AT₁R-expressing cells when compared to controls (Figure 5C). The MS/MS-induced fragmentation of recovered BME-OA-NO₂ adducts gave product ions and ion intensities similar to a synthetic BME-OA-NO₂ standard and BME-[¹³C₁₈]-OA-NO₂ used as an internal control (Figure 5B). Thus, the presence of BME-exchangeable OA-NO₂ demonstrates the

Figure 2. OA-NO₂ inhibits the pressor response to Ang II. BP was monitored on treatment with OA (A) and OA-NO₂ (B) (10 mg/kg) before Ang II infusion (10 μg/mL, infusion rate: 1 μL/min). All drugs were delivered via jugular vein administration starting at the time indicated by the arrows. BP tracings were recorded by insertion of a microtip catheter sensor into the right carotid artery. C, Systolic BP was averaged for a 10 minutes period before and after Ang II infusion (10 μg/mL, infusion rate: 1 μL/min). Data are shown as means±SEM (n=6 in each group). OA-NO₂ significantly reduced the pressor response to Ang II infusion compared to OA, P<0.05 (OA-NO₂ vs OA after Ang II infusion). Hypertension was developed in mice in 3 days after implantation of osmotic pumps to infuse Ang II at a pressor rate of 500 ng/kg per minute. OA (D) or OA-NO₂ (E) were then delivered to mice via the jugular vein at increasing concentrations as indicated by the arrows. BP tracings were recorded as described above. F, Data in D and E are summarized as systolic BP reduction (in mm Hg) after either OA or OA-NO₂ treatment as compared to maximal systolic pressure in Ang II hypertensive mice. OA-NO₂ treatment but not OA dose-dependently reduced established hypertension on Ang II delivery. Data are shown as means±SEM (n=4 in each group). *P<0.05.

Figure 3. OA-NO₂ reduces AT₁R-dependent vessel contraction. Second-grade mesenteric artery rings were mounted into a myograph as described in the Online Data Supplement. The mesenteric artery rings were preincubated for 10 minutes with 2.5 or 5 μmol/L either OA or OA-NO₂ as indicated. Vascular contraction was induced by serial addition of increasing concentrations of Ang II (A), PE (B), or ET-1 (C) to the myograph. Contractile response was quantified and shown in the figure as percentage of the vessel contraction force afforded by 50 mmol/L KCl. OA-NO₂ dose-dependently reduced Ang II-induced vasoconstriction but not that of PE or ET-1. Data are shown as means±SEM, n=6 in each group, *P<0.05 and **P<0.01 (OA-NO₂ vs OA or vehicle (0.1% ethanol-treated arteries).
Muscle Cells Contractile Responses in Vascular Smooth NO2 adduction of the AT1R does not affect Ang II binding 

G AT1R in ligand-receptor binding competition analyses using OA-NO2 nor OA affected binding of radio-labeled Ang II to II binding, we next examined whether OA-NO2 affects AT1R and represents a novel manner of AT1R antagonism that is treated HEK293 cells overexpressing HA-tagged AT1R, the figure as percentage of the vessel contraction force afforded by 50 mmol/L KCl. OA-NO2 reduced Ang II–induced vasoconstriction by serial addition of increasing concentrations of Ang II to the bath in the myograph. Contractile response was quantified and shown in independent of the PPARq11-EGFP, Gq11, and HA-tagged Gq11 with OA-NO2 or OA and determined Gq11 coupled to the AT1R following immunoadducted G-protein (Gq11). To this aim, show that AT1R is a relevant cellular target of OA-NO2 alkylation.

OA-NO2 Does Not Interfere With Ang II Binding to the Receptor

Next, we sought to determine whether the adduction of the AT1R by OA-NO2 interferes with Ang II binding. Unlike the ARB losartan, that inhibits Ang II binding to AT1R, neither OA-NO2 nor OA affected binding of radio-labeled Ang II to its receptor (Figure 5E). Furthermore, neither OA-NO2 nor OA significantly reduced AT1R coupled to the immunoprecipitated Gq11 after 1 minute stimuli with Ang II (100 nmol/L) (Figure 6A and 6B). Taken together, adduction of OA-NO2 with the AT1R does not affect Ang II binding but reduces G-protein coupled to the AT1R. Finally, the impact of OA-NO2 on the contractile response of vascular smooth muscle cells (VSMCs) downstream of AT1R reaction was evaluated, where Ang II–triggered second messenger mobilization was measured. Competitive radioreceptor analysis showed that OA-NO2, but not OA, reduced Ang II–induced intracellular inositol-1,4,5-trisphosphate (IP3) mobilization (Figure 6C). Accordingly, we show a dose-dependent inhibition of Ang II–mediated increase in intracellular calcium mobilization ([Ca2+]i) on OA-NO2 treatment, as determined by fluorescence imaging of fura-2 (Figure 6D). Pretreatment of VSMC with 2.5 μmol/L OA-NO2 significantly reduced the ratio of fura-2 fluorescent emission induced by Ang II (75%), whereas 1 μmol/L losartan completely inhibited Ang II–mediated increases in [Ca2+]i. By comparison, OA had no impact in Ang II–induced [Ca2+]i increase (Figure 6D, Online Figure VI, and Online Movies I through III).

Discussion

Early studies deciphering the physiological and/or pharmacological roles of nitrated fatty acids indicate that nitro derivatives of fatty acids exert relevant biological effects in the vasculature (also reviewed). More recent data, using cardiovascular models of disease, strongly support the notion that nitroalkenes serve as novel therapeutic tools associated with vascular and cardiac tissue damage.11,16,19

Figure 4. OA-NO2 reduces Ang II–induced hypertension independently of PPARγ activation. BP in mice was monitored with a microtip catheter sensor inserted into the right carotid artery by arteriotomy on treatment with OA (A) and OA-NO2, (B) (10 mg/kg) delivered via jugular vein administration after infusion of the PPARγ inhibitor GW9662 (10 mg/kg). A and B show representative BP recordings of n=6 in each treatment before and after Ang II infusion (10 μg/mL), delivered at an infusion rate of 1 μL/min. C, OA-NO2 delivery results in a significant reduction of Ang II–induced hypertension (=10 mm Hg) independently of PPARγ inhibition by GW9662. Data are depicted as systolic BP increase (in mm Hg) after Ang II infusion as compared to baseline systolic pressure and are shown as means±SEM (n=6 in each group). P<0.01 (OA-NO2 vs OA in either GW9662-treated or DMSO vehicle control-treated group after Ang II infusion). D. The mesenteric artery rings were preincubated for 10 minutes with 10 μmol/L GW9662 and then with 2.5 μmol/L, either OA or OA-NO2 as indicated. Vascular contraction was induced by serial addition of increasing concentrations of Ang II to the bath in the myograph. Contractile response was quantified and shown in the figure as percentage of the vessel contraction force afforded by 50 mmol/L KCl. OA-NO2 reduced Ang II–induced vasoconstriction independent of the PPARγ antagonist GW9662. Data are shown as means±SEM (n=6 in each group). *P<0.05.
In the present study, we provide compelling evidence supporting that OA-NO2 reduces Ang II–mediated hypertension in a model of Ang II infusion in mice. Systemic delivery of pharmacological doses of OA-NO2 but not OA resulted in a prolonged and sustained reduction of BP throughout the 14 days of Ang II infusion. The differences in BP in the OA-NO2 group as compared to OA or vehicle group were more pronounced during the first week of Ang II infusion, when BP raised and remained highest, and persisted significantly lower during the second week of Ang II infusion, when physiological compensatory mechanisms might occur to reduce BP in response to a long-term hypertensive challenge.36 Thus, our long-term radiotelemetry analysis confirmed that OA-NO2 diminished the pressor response to Ang II rather than simply delay Ang II–dependent hypertension. Furthermore, comparative analysis with the inclusion of the OA-treated group, as the nonnitrated counterpart for OA-NO2 also indicates that OA alone at the dosage used in the present study does not reduce Ang II-mediated hypertension in contrast to previous studies indicating that OA or related species might be hypotensive.37 Thus, at an infusion rate of 5 mg/kg per day, reduction of Ang II–mediated hypertension is afforded by the nitrated but not free form of OA.

Under our experimental conditions, Ang II infusion did not result in significant changes of free nitroalkenes in plasma (data not shown), indicating that chronic infusion of Ang II from AT,R to BME reveals a significant increase of added OA-NO2 to the AT,R (270 vs 13 pmol/L BME-OA-NO2, respectively, after 5 μmol/L OA-NO2 treatment). E, HEK293 cells were similarly transfected with the pcDNA3.1-AT,R plasmid and treated with biotin-labeled OA-NO2 or OA for 3 hours at the indicated concentrations. Biotin-labeled adducts were visualized by Western blot after immunoprecipitation of the Flag-AT,R. OA-NO2 dose-dependently increased the covalent adduction to the immunoprecipitated AT,R. The data shown are a representative set of 3 independent experimental repetitions. F, Radioactivity was measured using a γ-counter, and values are expressed as means ± SEM (n=4). The experiments were performed 3 times with similar results.

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Under our experimental conditions, Ang II infusion did not result in significant changes of free nitroalkenes in plasma (data not shown), indicating that chronic infusion of Ang II might not generate sufficient systemic reactions to be detected at the serum level of these hypertensive mice. It has previously shown that proinflammatory stimuli increases fatty acid nitration in macrophages.38 In vivo, nitroalkene production is abundantly generated in the mitochondria of the heart after ischemia/reperfusion injury.12,13 Therefore, it seems critical to determine the local production of nitroalkenes in response to certain types of injury and on specific subcellular compartments locally in tissues. From the present study, it cannot be excluded that local generation of
The transnitroalkylation reaction of OA-NO2 from AT1R adduction to BME revealed a dose-dependent increase of bound OA-NO2 to the AT1R. These results were further confirmed by immunoprecipitation analysis of AT1R and detection of biotinylated OA-NO2. However, addition of the AT1R by OA-NO2 did not interfere with Ang II binding to its receptor, suggesting that the molecular interactions between OA-NO2 and AT1R are unlikely similar to typical competitive inhibitors of AT1R.44,45

Crystal structures for some proteins of the seven transmembrane family, to which the AT1R belongs, show that monomers associate through a lipophilic interface with limited protein–protein contacts.46 It is becoming increasingly evident that lipid/membrane structures interact with GPCRs, exerting posttranslational modification within the GPCR.47 It is likely that these protein/lipid interactions48,49 may serve as regulatory mechanisms with diverse impact on downstream signaling actions.47 Thus, AT1R adduction by OA-NO2 may result in an allosteric inhibition by adding to the hydrophobic interface in a fashion reminiscent of the A2A adenosine receptor antagonist.50 This could promote an inactive state of the receptor, thus uncoupling Ang II binding from downstream activation.51 Further studies will be aimed at dissecting the molecular mechanisms by which electrophilic fatty acid derivatives reduce Ang II–mediated hypertension, including biochemical and crystallographic approaches, thus aiding in the design of a new generation of AT1R antagonists with increased selectivity.

In summary, this is the first demonstration that OA-NO2 has a long-lasting impact on hypertension induced by Ang II infusion and exerts a significant BP-lowering effect on
preexisting hypertension. OA-NO2 antagonizes Ang II signaling through formation of stable adducts with the AT1R, leading to a reduced vasoconstriction via uncoupling G-protein signaling and subsequently reducing second messenger mobilization.

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**Disclosures**

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**References**


Inhibition of AT1R by OA-NO2 uncouples downstream G-protein signaling and calcium mobilization.

OA-NO2 and rosiglitazone reduce established hypertension through elevation of cAMP. AO-NO2 reduces blood pressure and vasoconstriction in response to Ang II binding to the receptor unlike typical competitive angiotensin receptor blockers, thus defining a novel mechanism for antagonism.

Nitroalkenes are nitric oxide derivatives of unsaturated fatty acids with pleiotropic biological activities of relevance for metabolic and cardiovascular diseases.

Nitro-oleic acid (OA-NO2) protects against vascular lesion, atherosclerosis, cardiac ischemia/reperfusion injury, and diabetes in experimental animal models.

What New Information Does This Article Contribute?

- OA-NO2 reduces blood pressure and vasoconstriction in response to angiotensin (Ang) II.
- OA-NO2 binds Ang II receptor (AT1R), but it does not interfere with Ang II binding to the receptor unlike typical competitive angiotensin receptor blockers, thus defining a novel mechanism for antagonism.
- OA-NO2 and rosiglitazone reduce established hypertension through different pathways.
- Inhibition of AT1R by OA-NO2 uncouples downstream G-protein signaling and calcium mobilization.

We demonstrate for the first time that OA-NO2 has a long-lasting impact on hypertension induced by Ang II infusion in an animal model where systemic delivery of pharmacological doses of OA-NO2, but not its parental fatty acid, oleic acid, results in a prolonged and sustained reduction of blood pressure. We show that OA-NO2 lowers blood pressure on preexisting hypertension and inhibits vascular contractility in an AT1R-dependent fashion. OA-NO2 shows specificity for the AT1R, because OA-NO2 dose-dependently inhibits Ang II-mediated vessel contraction but does not reduce vascular contractility induced by alternative vasoconstrictors, such as endothelin-1 and phenylephrine, signaling through other G-protein coupled receptors. We describe that OA-NO2 lowers Ang II–induced hypertension independent of peroxisome proliferation-activated receptor-γ activation. Rather, OA-NO2 binds directly to the AT1R without interfering with Ang II binding, suggesting that the molecular interactions between OA-NO2 and AT1R are likely to differ from angiotensin receptor blockers competitive inhibition. OA-NO2 reduces heterotrimeric G-protein coupling to the AT1R and inhibits downstream signaling. This novel regulatory mechanism of the AT1R by OA-NO2 could inform the design of a new generation of AT1R antagonists with increased selectivity.
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SUPPLEMENTAL MATERIAL

Nitro-oleic acid inhibits angiotensin II-induced hypertension

Zhang: Nitro-oleic acid and blood pressure.

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DETAILED METHODS

Animals

Male C57BL/6J mice, 8-10 weeks old, were housed in a temperature-controlled animal facility with a 12h:12h light-dark cycle and free access to water and rodent chow. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals.

Blood Pressure Measurements

Blood pressure (BP) was measured by radiotelemetry as previously described in detail. Briefly, mice were anesthetized with isoflurane and placed on a controlled warming pad (37°C). The catheter is attached to a combination pressure transducer, transmitter, and battery, all encapsulated in an implantable microminiaturized electronic monitor (PA-C10, Data Sciences International, St. Paul, MN). After preparation of the left common carotid artery, arteriotomy was performed using the bent tip of a 25g needle allowing the 0.4 mm catheter to pass into the vessel toward the heart. The transducer was secured within the abdominal cavity allowing stable long-term pressure recordings. Telemetry measurements of pulsatile arterial BP were a continuous 24-h/day, 7-days/week readouts of systolic, diastolic and mean arterial pressure and heart rate. Baseline BP measurements were initiated after 1 week of postsurgery recovery and subsequent OA, OA-NO₂ and PEG-vehicle control delivery (see below).

Angiotensin II (Ang II)-induced hypertension model and nitroalkene delivery in vivo

After baseline BP recordings were performed (1 week), mice were again anesthetized and subjected to subcutaneous implantation of osmotic mini-pumps (model 2004, Alzet) for delivery of nitro-oleic acid (OA-NO₂) or oleic acid (OA). We adjusted the stock concentration of synthetic OA-NO₂ in the pump to provide an infusion rate of 5mg/kg/day. During the infusion regime, blood analyses
revealed a plasma concentration within the nanomolar range as recently demonstrated\(^2\). This concentration must be considered in the context that the infused OA-NO\(_2\) is also metabolized to other derivatives not specifically determined here but extensively studied in\(^3\), \(^4\). After an additional week, a second osmotic pump (model 2002, Alzet), containing Ang II (Sigma) at a concentration supporting an infusion rate of 500 ng/kg/min, was implanted subcutaneously on a side of the back of each mouse. Depicted in Figure 1 are the mean values (n=6) of individual systolic and diastolic BP recordings every 4 h. Heart rate determinations are also shown in Online Figure I.

**Short-term nitroalkene delivery *in vivo* before and after Ang II-induced hypertension and BP recordings.**

For short-term delivery of OA, OA-NO\(_2\), Ang II and the PPAR\(\gamma\) antagonist GW9662, BP measurements were determined as previously described\(^5\). Briefly, after preparation of the right carotid artery, a 1.4F micro-tip catheter sensor (model SPR-671, Millar Instruments Inc, Houston, TX) was inserted. BP was measured for a period of 10 min after steady-state levels were reached using the data-acquisition system Powerlab 8/30 and chart software (ADInstruments). The drugs were administrated *via* jugular vein by a GENIE Plus Infusion Syringe Pump (Kent Scientific, Torrington, CT, USA). OA or OA-NO\(_2\) (1 \(\mu\)mol/L) were delivered at an infusion rate of 1 \(\mu\)L/min (equivalent to a final concentration of 10 mg/kg) and the PPAR\(\gamma\) inhibitor, GW9662 (0.1mmol/L) at an infusion rate of 1 \(\mu\)L/min (equivalent to a final concentration of 10 mg/kg), all delivered before Ang II infusion (10 \(\mu\)g/mL, infusion rate: 1 \(\mu\)L/min) as indicated by the arrows in Figures 2A and 2B and Figure 4A and 4B).

For short-term delivery of OA, OA-NO\(_2\), the PPAR\(\gamma\) agonist rosiglitazone and antagonist GW9662, in the preexisting hypertension model, mice were first implanted with Ang II minipumps
(model 2002, Alzet) supporting an infusion time of 14 days and a rate of 500 ng/kg/min, as described above. 3 days after Ang II delivery, systolic BP in mice stably rose to 150mmHg (Figure 2D and 3E). Increasing concentrations of OA or OA-NO₂ were then administered to anesthetized mice via jugular vein delivery supporting a final dosages of 1.25, 2.5, 5, 10 and 20 mg/kg (Figure 2D to 2F). Under similar experimental conditions, increasing doses of OA-NO₂ or rosiglitazone (final concentrations of 1.25, 2.5, 5, 7.5, 12.5 and 20 mg/kg) were delivered in the presence or absence of the PPARγ antagonist, GW9662 (10 mg/kg) (Online Figure II and Online Figure III). As described in other experimental models of hypertension⁶,⁷, rosiglitazone reduced preexisting hypertension, which herein was afforded by Ang II infusion (Online Figure IIA) In response to rosiglitazone treatment, maximal reduction of BP reached ~7 mmHg and GW9662 efficiently antagonized this effect (Online Figure II). In a similar approach, when OA-NO₂ is delivered into hypertensive mice reduction of BP is dose-dependent (Online Figure IIIA) and pretreatment with GW9662 cannot antagonize this reduction (Online Figure IIIB). Thus, OA-NO₂ equally inhibits Ang-II-induced hypertension in the presence or absence of GW9662 (Online Figure IIIC).

Mesenteric artery contraction and relaxation assays

Sprague-Dawley rats (200-250 g) were sacrificed through i.p. injection of urethane (1 g/kg) and the mesenteric artery isolated and dissected from the animal and placed on ice in a physiological saline solution (PSS) consisting of 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.18 mmol/L Mg₂SO₄, 1.18 mmol/L KH₂PO₄, 24.9 mmol/L NaHCO₃, 10 mmol/L glucose, and 0.03 mmol/L EDTA before being mounted in the myograph. After the connective tissue was dissected away from the vessels, second-order mesenteric arteries (2 mm long) were prepared and mounted onto a computer-controlled, myograph (Multiwire Myograph System. Model 610M, DMT) that was connected to a force transducer
to measure the wall tension developed by the vessel. A computer-assisted normalization protocol was then performed to set the pretension units to 100 mmHg for each vessel. After the normalization procedure was complete, the second-order mesenteric rings were balanced for 1h, and then challenged three times with a high-potassium solution (KPSS, PSS with 50 mmol/L KCl) to determine whether they were viable. Only segments that demonstrated a contraction in response to KPSS were used for further studies. After a washout period, vessels were pretreated with OA-NO₂ or OA at 5 µmol/L and 2.5 µmol/L and then 10 min contracted with increasing concentrations of Ang II, endothelin-1 (ET-1) and phenylephrine (PE) as shown in Figure 3. Data acquisition was performed using the DMT Normalization Module (AD Instruments). Data are shown as mean ± SD, n=6 in each group, *P < 0.05 and **P < 0.01 (OA-NO₂ vs. OA or vehicle (0.1% ethanol)-treated arteries). Trace analysis of the contractile curve was determined using Chart v5 (AD Instruments) and representative contractile curves in response to Ang II, PE and ET-1 are shown in Online Figure IV.

**Generation of PPRE-EGFP transgenic mice and determination of PPARγ transcriptional activity in the aorta.**

The PPRE-EGFP construct contains three tandem consensus PPRE sites, consisting in the following sequence: AGGACAAAGGTCA, followed by a mini promoter of the thymidine kinase (TK) gene coupled to the gene encoding enhanced green fluorescent protein (EGFP). The PPRE-EGFP transgene was also flanked by two pieces of chicken HS4 insulator at each side to avoid positional effect of the transgene expression (Online Figure VA). The construct was tested in HEK293 and COS-1 cells before generation of transgenic mice to verify the integrity of the PPRE-EGFP construct. Our preliminary studies demonstrated that co-transfection with any PPAR isoform expression vector plus
treatment of corresponding ligand increased the EGFP expression in the cell culture system (data not shown).

Generation of transgenic founder lines was subsequently accomplished using standard techniques of microinjection, which was performed at the Transgenic Animal Model Core of the University of Michigan. Briefly, PPRE-EGFP construct was microinjected into (C57BL/6 X SJL)F2 mouse eggs and surgically transferred to recipients. We obtained a total of 81 offspring (F0) from the microinjection. 5 out of 81 are transgene positive as screened by genomic PCR. The F1 generation was subsequently obtained by cross breeding with C57 mice. All 5 lines showed germline transmission for PPRE-EGFP transgene. Line #62 was used for the current experiments and positive transgene expression was further confirmed by genotyping with the F2 generation (Online Figure VB) using the primers, EGFP-F2: 5’-CGCCCAGCGTCTTGTCATTG-3’ and EGFP-R2: 5’-ACGCCGTAGGTAGTTGTC-3’

To determine PPAR transcriptional activity in the vasculature in vivo, male PPRE-EGFP transgenic mice were administered via oral gavage with rosiglitazone (5 mg/kg) with or without the PPARγ antagonist GW9662 (10 mg/kg). After 20 h, the aortas were harvested, homogenized and subjected to Western blot analysis with an anti-GFP antibody (Living Colors, Clontech). EGFP expression as a measure of PPAR transcriptional activity was robustly increased in the aortic tissue in response to rosiglitazone treatment. Coadministration of GW9662 significantly attenuated rosiglitazone-dependent PPAR activity (Online Figure VC).

Detection and quantification of AT1R modification by OA-NO2

HEK293 cells were either transfected with control plasmid (pcDNA3.1) or plasmid encoding FLAG-tagged-AT1R using lipofectamine 2000 (Invitrogen). 24 h after transfection, cells were starved in DMEM containing 0.1% fatty acid-free bovine serum albumin (BSA) for 24 h, and then incubated with
either 2 μmol/L or 5 μM OA-NO₂ for 2 h. Cells were then harvested and lysed using immunoprecipitation buffer (1% Triton X-100, 10% glycerol, 150 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA) supplemented with Protease inhibitor cocktail (Roche) and phosphatase inhibitors. The cell lysates were briefly sonicated and clarified by centrifugation at 14000 g for 10 min and 3.5 mg of total lysates were subjected to immunoprecipitation with anti-FLAG M2 monoclonal antibody-conjugated agarose (Sigma) overnight. After immunoprecipitation, the beads were washed with washing buffer (50 mmol/L Tris HCl, 150 mmol/L NaCl, pH 7.4) three times and then eluted by competition with 3 X FLAG peptide (Sigma). The trans-nitroalkylation reaction was carried out in 50 mM phosphate buffer pH 7.4 in the presence of [13C18]-OA-NO₂ internal standard (final 0.5 ng/ml) and β-mercaptoethanol (BME) (500 mmol/L final) for 2 h at 37°C. The reaction was stopped by addition of 1% formic acid. The trans-nitroalkylated BME-OA-NO₂ (transfer of OA-NO₂ from protein to BME) was then further detected and quantified using tandem mass spectrometry³ (see below).

Quantification of BME-OA-NO₂ adducts

Quantitative determination of the different BME-adducted molecules was performed through analysis in the multiple reaction monitoring (MRM) scan mode using a 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA) as previously reported³. Briefly, BME-adducts were detected in the negative ion mode by monitoring ions that undergo the M⁻/[M - BME]⁻ transition. Thus, the transitions used were as follows: BME-OA-NO₂, m/z 404.4/326.3; BME-[13C18]-OA-NO₂, m/z 422.4/344.3 (Figure 5B). The declustering potential was -50 V and the collision energy was set at -17 for BME adducts. Zero grade air was used as source gas, and nitrogen was used in the collision chamber. Data were acquired and analyzed using Analyst 1.4.2 software (Applied Biosystems, Framingham, MA). Quantification (Figure 5C) was achieved by comparing peak area ratios between
analytes and their corresponding internal standards and calculating analytes concentration using an internal standard curve.

**AT1R immunoprecipitation and detection of biotin-labeled OA-NO2.**

The synthesis of biotinylated OA and OA-NO2 was performed as previously described\(^8\), \(^9\). HEK293 cells were transiently transfected with the Flag-AT1R plasmid (0.5 μg/well). After 24 h of serum deprivation cells overexpressing AT1R were treated with OA-NO2 and OA in a dose-dependent manner for 3 h as described in the figure legends (Figure 5D). Cells were then washed with cold PBS and lysed using Nonidet P-40 buffer. Cell lysates were then precleared using 50 μl of a 50% slurry of protein G-Sepharose 4 Fast Flow (Sigma) and incubated at 4°C for 1 h with gentle shaking. The precleared lysates (5 μg) were incubated with an anti-biotin antibody at 4°C overnight and then further incubated with protein G-Sepharose 4 Fast Flow for 1 h. The precipitates were subjected to SDS-PAGE and electrotransfered onto nitrocellulose membranes. To detect the direct binding of biotin-labeled OA or OA-NO2 to AT1R, immunoblotting was done using an anti-biotin HRP-linked antibody (Cell Signaling).

**Gα\(_{q11}\) immunoprecipitation and detection of HA-tagged-AT1R.**

For identification of coupled Gα\(_{q11}\) to the AT1R in response to Ang II treatment, HEK293 cells were transiently transfected with the following plasmids: HA-tagged-AT1R (0.3 μg/well) was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org); Gα\(_{q11}\)-EGFP (0.3 μg/well), Gβ\(_1\) (0.1 μg/well) and HA-tagged-Gγ\(_2\) (0.1 μg/well) were a generous gift of Dr. Catherine H. Berlot (Weis Center for Research, Danville, PA)\(^10\). EGFP was tagged to the Gα\(_{q11}\) avoiding modification at the N- or C-termini of Gα\(_{q11}\), important for interaction of the G-protein subunits and the receptor as described
before. Cells were then treated with OA-NO2 and OA (2.5 μmol/L) 10 min before the addition of Ang II (100 nmol/L). After 1 min, cells were washed with cold PBS, lysed and precleared as described above. The precleared lysates were incubated with an anti-GFP antibody (Living Colors, Clontech) at 4°C overnight and then further incubated with protein G-Sepharose 4 Fast Flow for 1h. The precipitates were subjected to a deglycosylation step using the Protein Deglycosylation Mix (New England Biolabs) following the manufacturer’s instructions before SDS-PAGE. Detection of associated HA-tagged-AT1R to the Goq11 was obtained by immunoblotting using an anti-HA antibody (Cell Signaling) (Figure 6A). Western blots were quantified using the ImageJ software (NIH, Bethesda, MD) after densitometric scanning of the films and expressed as relative ratio of AT1R vs. Goq11 (Figure 6B).

**Angiotensin II type 1 receptor binding assays**

The AT1R binding assay was performed as described previously. Briefly, confluent primary cultured rat aortic smooth muscle cells (RASMC) in 24-well plates were cultured in DMEM supplemented with 0.1% FBS for 2 h. The cells pretreated with different dose of OA or OA-NO2 for 10 min as indicated in Figure 5F, and then incubated for 30 min at 37°C with 0.1 nmol/L of [125I]-[Sar^1,Ile^8]-Ang II (Perkin Elmer). Cells were then washed three times with ice-cold PBS containing 0.1% BSA and lysed in 0.5 N NaOH. The radioactivity count of the lysate was measured by γ-counter. For AT1R competition binding studies, the cells were pretreated with 2.5 μmol/L of OA or OA-NO2 for 10 min and then incubated for 30 min with 0.3 nmol/L [125I]-[Sar^1,Ile^8]-Ang II and serial concentrations of the cold Ang II as indicated in Figure 5E. Cells were harvested and counted with a γ counter.

**Inositol-1,4,5-trisphosphate (IP3) radioreceptor assays**
RASMC were cultured in DMEM-F/12 (Invitrogen) with 10% FBS in 6-well plate until reaching 95% confluence. Cells were washed once with PBS and then incubated with DMEM-F/12, 1% FBS containing 10 mmol/L of LiCl for 30 min. Cells were then pre-treated with 2.5 μmol/L OA-NO2 or OA for 5 min. 100 nmol/L of Ang II was added to the cells and incubated for 45 min. IP3 was measured by radioreceptor assay (PerkinElmer) following the manufacturer’s protocol (Figure 6). The experiments were performed in triplicates and repeated 3 independent times.

**Intracellular Calcium Concentration Measurement**

Ratiometric fluorescence imaging was performed using the fluorescent indicator dye fura-2 to obtain the intracellular calcium concentration ([Ca^{2+}]_i) from the ratio of emissions at 510 nm with 340 nm and 380 nm excitation\(^\text{13}\). Cells were incubated for 30min at 37°C in culture medium containing 5 μmol/L fura-2 (Invitrogen, Carlsbad, CA), the membrane permeant acetoxymethyl ester form of fura-2 and 0.05% v/v of 10% w/v Pluronic F-127 (Invitrogen, Carlsbad, CA) in water. Excess dye was removed by washing the cells three times with culture medium. The dish was then placed on a 37°C heating stage of an inverted microscope (Nikon Eclipse Ti-U, Melville, NY). To perform real-time imaging, a monochromator (DeltaRAM X™, PTI, Birmingham, NJ) with a 5 nm bandpass was used to filter light from a 75W xenon lamp alternately between the wavelengths 340 nm and 380 nm, each with excitation exposure time of 656 ms. The excitation light was directed through a 20x Super Fluor objective with a numerical aperture of 0.75 (Nikon, Melville, NY), and the subsequently emitted light from the cells due to fura-2 fluorescence was passed through an appropriate dichroic filter. The resulting series of grayscale photomicrographs were acquired with a cooled CCD camera (Photometrics QuantEM, Tucson, AZ) at a 512x512 resolution (Online Figure VI and Online Movies I to III).
Image analysis was performed using software package Easy Ratio Pro (PTI, Birmingham, NJ). The background-corrected fluorescence intensity was obtained by subtracting the fluorescence intensity from an area free of cells. The ratio was calculated as $R = \frac{F_{340}}{F_{380}}$, where $F_{340}$ and $F_{380}$ are the emission fluorescent intensity with 340 nm and 380 nm excitation respectively. Change in ratio of $F_{340}$ and $F_{380}$ is related to the change in the $[Ca^{2+}]_{i}^{14}$. 

Statistical analysis

Mean ± SEM values were analyzed using Prism (version 4). Statistical comparisons between two groups were performed by Student’s $t$ test, and among three groups were performed by one-way ANOVA. Groups were considered significantly different if $P$ values were <0.05.
Supplemental References


Online Figure I. Heart rate measurements by telemetry. Mice implanted with osmotic pumps adjusted to deliver 5 mg/kg/day OA-NO₂ (red) or OA (blue) and vehicle control (black) were infused with Ang II at a pressor rate of 500 ng/kg/min. Heart rate was measured by radiotelemetry. Data are expressed in heart beats per min and shown as mean ± SEM, n=6 in each group.
**Online Figure II. Rosiglitazone reduces Ang II-induced hypertension.** Hypertension was developed in mice in 3 days after implantation of osmotic pumps to infuse Ang II at a pressor rate of 500 ng/kg/min. (A) Rosiglitazone was then administered to the mice via the jugular vein at increasing concentrations as indicated by the arrows. BP tracings were recorded as described above. (B) GW9662 (10 mg/kg) was delivered as a single dose at the time indicated by the arrow before treatment with rosiglitazone as described in A. (C) Data in A and B are summarized as systolic BP reduction (in mmHg) after either rosiglitazone or rosiglitazone + GW9662 treatment as compared to maximal systolic pressure in Ang II hypertensive mice and are shown as mean ± SEM, n=4 in each group, P < 0.05 (rosiglitazone vs. rosiglitazone + GW9662).
Online Figure III. OA-NO₂ reduces Ang II-induced hypertension independently of PPARγ.

Hypertension was developed in mice in 3 days after implantation of osmotic pumps to infuse Ang II at a pressor rate of 500 ng/kg/min. (A) OA-NO₂ was then delivered to mice via the jugular vein at increasing concentrations as indicated by the arrows. BP tracings were recorded as described above. (B) GW9662 (10 mg/kg) was delivered as a single dose at the time indicated by the arrow before treatment with OA-NO₂ as described in A. (C) Data in A and B are summarized as systolic BP reduction (in mmHg) after either OA-NO₂ or OA-NO₂ + GW9662 treatment as compared to maximal systolic pressure in Ang II hypertensive mice and are shown as mean ± SEM, n=4 in each group, P < 0.05 (rosiglitazone vs. rosiglitazone + GW9662).
Online Figure IV. Contraction curves after OA-NO₂ treatment in response to Ang II, PE or ET-1.

The mesenteric artery rings were preincubated for 10 min with 2.5 μmol/L or 5 μmol/L of either OA-NO₂, OA or vehicle as indicated. Vascular contraction was induced by serial addition of increasing concentrations of Ang II (A), PE (B) and ET-1 (C) to the myograph at the time indicated by the arrows.
Online Figure V. Generation of PPRE-EGFP transgenic mice model. (A). Schematic diagram of PPRE-EGFP transgenic mouse structure. A 3x tandem repeat of the consensus PPRE sequence was inserted upstream of the mini-TK promoter. EGFP coding sequence and a BGH polyadenylation signal was cloned downstream of mini-TK promoter. The arrows show the genotyping primer sites. (B) Genomic PCR to genotype the PPRE-EGFP transgenic mice. P: positive control (transgenic construct); N: negative control (water); Wt: wild type mouse; Tg: transgenic mouse. (C) EGFP expression in PPRE-EGFP transgenic mice detected by Western blot. Protein was extracted from aortic tissue of PPRE-EGFP transgenic mice after 20h of treatment via oral gavage with 5mg/kg rosiglitazone or the combination of rosiglitazone + GW9662 (10 mg/kg).
Online Figure VI. OA-NO₂ inhibits Ang II-mediated intracellular calcium efflux in VSMC. Three cells (left panel) were randomly selected at the 380 nm channel for monitoring calcium efflux induced by 0.1 µmol/L Ang II (A). Ang II-induced calcium mobilization was dramatically inhibited by 2.5 µmol/L OA-NO₂ (B), but not by 2.5 µmol/L OA (C). Right panels show the normalized ratio-time traces from three typical cells (each of them depicted in different colors) in the field of view for the randomly selected cells. Calcium mobilization can be visualized for each treatment with Ang II alone or in the presence of OA-NO₂ or OA in Online Movies I to III, respectively. Movies were acquired at a 512x512 resolution with a cooled CCD camera as described above.