Testosterone Induces Vascular Smooth Muscle Cell Migration by NADPH Oxidase and c-Src–Dependent Pathways


Abstract—Testosterone has been implicated in vascular remodeling associated with hypertension. Molecular mechanisms underlying this are elusive, but oxidative stress may be important. We hypothesized that testosterone stimulates generation of reactive oxygen species (ROS) and migration of vascular smooth muscle cells (VSMCs), with enhanced effects in cells from spontaneously hypertensive rats (SHRs). The mechanisms (genomic and nongenomic) whereby testosterone induces ROS generation and the role of c-Src, a regulator of redox-sensitive migration, were determined. VSMCs from male Wistar-Kyoto rats and SHRs were stimulated with testosterone (10⁻⁷ mol/L, 0–120 minutes). Testosterone increased ROS generation, assessed by dihydroethidium fluorescence and lucigenin-enhanced chemiluminescence (30 minutes [SHR] and 60 minutes [both strains]). Flutamide (androgen receptor antagonist) and activin A (gene transcription inhibitor) diminished ROS production (60 minutes). Testosterone increased Nox1 and Nox4 mRNA levels and p47phox protein expression, determined by real-time PCR and immunoblotting, respectively. Flutamide, activin A, and cycloheximide (protein synthesis inhibitor) diminished testosterone effects on p47phox. c-Src phosphorylation was observed at 30 minutes (SHR) and 120 minutes (Wistar-Kyoto rat). Testosterone-induced ROS generation was repressed by 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (c-Src inhibitor) in SHRs and reduced by apocynin (antioxidant/NADPH oxidase inhibitor) in both strains. Testosterone stimulated VSMCs migration, assessed by the wound healing technique, with greater effects in SHRs. Flutamide, apocynin, and 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine blocked testosterone-induced VSMCs migration in both strains. Our study demonstrates that testosterone induces VSMCs migration via NADPH oxidase–derived ROS and c-Src–dependent pathways by genomic and nongenomic mechanisms, which are differentially regulated in VSMCs from Wistar-Kyoto rats and SHRs. (Hypertension. 2012;59:1263-1271.)

Key Words: testosterone ■ hypertension ■ c-Src ■ NADPH oxidase ■ vascular smooth muscle

Testosterone has been associated with a higher prevalence of arterial hypertension in several experimental animal models.1–5 This steroid hormone contributes not only to the development of arterial hypertension but also to hypertension-associated vascular and renal abnormalities.6 In humans, it is still controversial whether testosterone contributes to or protects from cardiovascular disease. Although many reports indicate that low testosterone levels are associated with high risk of cardiovascular disease and that testosterone replacement improves cardiovascular and metabolic functions,7–9 other studies indicate that older men with higher testosterone levels are more likely to have cardiovascular disease.10,11

Testosterone effects on the vasculature are contradictory and include modulation of vascular tone,12 proliferation of vascular smooth muscle cells (VSMCs),13 and regulation of apoptosis in endothelial cells.14 These processes can be mediated by genomic (classic) or nongenomic (nonclassic) mechanisms. The classic effects of testosterone depends on its binding to the androgen receptor (AR), which acts as a transcription factor that, on association with the androgen
response element, modulates transcription. The nonclassic actions of testosterone do not require the association of AR with DNA and rely on the regulation of signaling pathways.  

The first studies addressing the nonclassic actions of testosterone suggested the existence of a new receptor located at the cell membrane, with an androgen-binding site being considered the mediator of nongenomic actions of androgens. However, increasing evidences demonstrate that there is a transient translocation of AR to the cell membrane. In support, Hamzeh and Robaire have described recently that reduction of AR activity (by hydroxyflutamide, a classic AR antagonist) represses fast (1 minute) dihydrotestosterone-induced extracellular-regulated kinase 1/2 phosphorylation in epididymal cells, suggesting that this effect occurs by direct regulation of cell signaling pathways by AR instead of binding of its cognate nuclear receptor to DNA.

Although several studies have addressed the effects of androgens on vascular tone, as well as its actions on endothelial cells, there are few studies focusing on the generation and regulation of reactive oxygen species (ROS) by testosterone in the vasculature. In the cardiovascular system, ROS are mainly produced by vascular NADPH oxidase and are key players in the maintenance and development of hypertension-associated end-organ injury.

Seven members of the NADPH oxidase family have been described (Nox1–7), of which Nox1, Nox2, and Nox4 have been identified in rodent vascular tissue. The NADPH oxidase complex is formed by regulatory and cytosolic subunits (NOXO1, NOXA1, p47phox, p67phox, p40, and Rac). The oxidase complex can be activated by oxidases, a role for c-Src in testosterone-induced activation of extracellular-regulated kinase 1/2 phosphorylation in epididymal cells, suggesting that this effect occurs by direct regulation of cell signaling pathways by AR instead of binding of its cognate nuclear receptor to DNA.

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(Carlsbad, CA). Dihydroethidium was purchased from Invitrogen (Carlsbad, CA). 3-(4-Chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2; c-Src inhibitor) was bought from Tocris (Ellisville, MO). Antibodies were as follows: antiphospho Src and anti-Src were obtained from Cell Signaling (Danvers, MA); anti47phox was obtained from Santa Cruz Biotechnology (St Cruz, CA), and antirat smooth muscle actin was from Sigma.

Animals
Housing conditions and experimental protocols were in accordance with the ethical animal committees from the University of São Paulo and University of Ottawa. Animals were housed under standard laboratory conditions with free access to food and water. Male WKY rats and SHRs were euthanized by decapitation. VSMCs from many), with exposure intensity adjusted relatively to baseline. Imaged by fluorescence microscopy (Axioskop Zeiss, Berlin, Germany), with exposure intensity adjusted relatively to baseline.

Lucigenin-Enhanced Chemiluminescence
VSMCs were stimulated with testosterone 10^-7 mol/L (5–120 minutes). In some experiments, cells were pre-exposed for 30 minutes to PP2 (10^-5 mol/L), actinomycin D (10^-5 mol/L), or flutamide (10^-5 mol/L). After stimulation, cells were washed and harvested in lysis buffer (20 mmol/L of KH2PO4, 1 mmol/L of EGTA, 1 μmol/L of aprotinin, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, and 1 mmol/L of PMSF). Fifty microliters of the sample were added to a suspension containing 175 μL of assay buffer (50 mmol/L of KH2PO4, 1 mmol/L of EGTA, and 150 mmol/L of sucrose) and lucigenin (5 μmol/L). NADPH (10^-4 mol/L) was added to the suspension (300 μL) containing lucigenin. Luminescence was measured every 18 seconds for 3 minutes by a luminometer (AutoLumat LB 953, Berthold) before and after stimulation with NADPH. A buffer blank was subtracted from each reading. The results are expressed as counts per milligram of protein (percentage of control).

Real-Time PCR
Quiescent VSMCs were stimulated with testosterone 10^-7 mol/L for 2 to 24 hours. The analysis of mRNA expression was made by the real-time PCR technique, which was performed as described previ-
Primers used are listed in the Table. The reaction conditions consisted of 2 steps at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 3 steps, 15-second denaturation at 95°C, and 15 seconds at 72°C. The relative expression (target gene/housekeeping gene) of mRNAs were calculated and reported as arbitrary units. Calculated and reported as arbitrary units.

**Figure 3.** Testosterone positively modulates Nox1 and Nox4 mRNA in Wistar-Kyoto (WKY, □) rat vascular smooth muscle cells (VSMCs). Real-time analysis of testosterone-stimulated (10^{-7} mol/L; 2–24 hours) mRNA expression of Nox1 (A), Nox4 (B), and p22phox (C) in WKY rat and spontaneously hypertensive rat (SHR, ■) VSMCs. Data represent the mean±SEM of n=5 experiments; *P<0.05 vs control (vehicle).

**Figure 4.** Testosterone upregulates p47phox expression by genomic mechanisms. Representative immunoblots and corresponding bar graphs of p47phox expression in Wistar-Kyoto (WKY, □, rat and spontaneously hypertensive rat (SHR, ■) vascular smooth muscle cells (VSMCs) stimulated with 10^{-7} mol/L of testosterone (Test) for 120 minutes in the presence or absence of cycloheximide (Cyc) and actinomycin D (Act D). Data represent the mean±SEM of n=6. *P<0.05 vs control (vehicle/2 hours); ≠P<0.05 versus Test.

**Immunoblotting**

Quiescent VSMCs were stimulated with testosterone 10^{-7} mol/L for 1 to 120 minutes. Whenever necessary, cells were preincubated for 30 minutes with actinomycin D (10^{-5} mol/L), cycloheximide (10^{-5} mol/L), flutamide (10^{-5} mol/L), or apocynin (3×10^{-5} mol/L). Cells were harvested in lysis buffer (in mmol/L, sodium pyrophosphate 50, NaF 50, NaCl 5, EDTA 5, EGTA 5, HEPES 10, Na3VO4 2, PMSF 50, Triton 100 0.5%, and leupeptin/aproptin/pepsatin 1 mg/mL). Proteins were extracted, separated by electrophoresis on a 10% polyacrylamide gel (20–30 μg), and transferred to a nitrocellulose membrane, as described previously.27 Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with 1% of Tween for 1 hour at room temperature. Membranes were then incubated with specific antibodies (antiphospho-Src, anti-Src, anti-p47phox, and anti-α-actin) overnight at 4°C. Membranes were washed 3 times with Tris-buffered saline solution with 1% of Tween and incubated with specific secondary antibodies for 1 hour at room temperature. Signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results were normalized by the total protein and are expressed relatively to vehicle (100%) in the experimental protocols.

**Cell Migration**

For the monolayer-wounding cell migration assay, VSMCs that had been grown to confluence in 24-well culture plates were subjected to wounding, as described previously.28 Briefly, cells were maintained overnight in DMEM supplemented with 0.4% of bovine albumin. Cell layers were wounded with a sterile 10-μL pipette tip. After washing away suspended cells, DMEM supplemented with 0.4% of bovine albumin was reintroduced in the culture plates. Migration was determined by wound length and photographed immediately or after testosterone stimuli (0–24 hours). According to the protocols, cells were incubated previously for 30 minutes with flutamide (10^{-5} mol/L), apocynin (3×10^{-5} mol/L), or PP2 (10^{-5} mol/L). Five different fields were monitored, and 3 measurements were performed...
Testosterone, ROS, VSMCs, and Hypertension

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Results

Testosterone Induces ROS Generation in VSMCs From WKY Rats and SHRs With a Differential Time Course

To elucidate whether testosterone increases ROS generation and whether there are differences between WKY and SHR VSMCs, we determined ROS production in VSMCs from WKY rats and SHRs (Figure 1A) by lucigenin-enhanced chemiluminescence and by dihydroethidium staining (Figure 1B and 1C; VSMCs from WKY rats and SHRs, respectively). The concentration- and time-dependent effects of testosterone on ROS production are illustrated in the Figure S1 and S2 (available in the online-only Data Supplement). Testosterone induced ROS generation in a strain- and time-dependent manner. ROS production by testosterone occurred earlier in SHR VSMCs (30 minutes) than in WKY rat VSMCs (60 minutes; P<0.05). In addition, ROS formation by testosterone was higher in SHR VSMCs (2.0-fold) than in WKY rat VSMCs (0.5-fold). Moreover, testosterone-induced ROS production increased constantly with time in SHR VSMCs (0–2 hours), whereas a plateau was observed in WKY rat VSMCs (60–120 minutes; *P<0.05 versus vehicle). Testosterone induced ROS production by direct mechanisms and not via conversion to 17β-estradiol, because the effects of testosterone were not altered by anastrazole, an aromatase inhibitor (10–3 mol/L, 30 minutes of preincubation; Figure S3).

Short-Term Testosterone-Induced ROS Generation in SHR VSMCs Is Not a Genomic Effect

To investigate the possible mechanisms (genomic and non-genomic) whereby testosterone induces ROS production at 30 minutes (short term) and 2 hours (long term), we measured ROS generation by lucigenin-enhanced chemiluminescence in the presence of flutamide and actinomycin D. VSMCs from WKY rats and SHRs were incubated with one of the above-mentioned drugs for 30 minutes before being stimulated with testosterone 10–7 mol/L for 30 and 120 minutes. Testosterone increased ROS generation at 30 minutes only in SHR VSMCs, an effect not inhibited by flutamide or actinomycin D, indicating a nongenomic effect (Figure 2A). Neither flutamide nor actinomycin D, per se, altered ROS production (Figure 2B and 2D). In addition, testosterone augmented ROS formation after 2 hours in both WKY rat and SHR VSMCs, an effect blocked by both flutamide and actinomycin D, thus by a genomic pathway (Figure 2C; *P<0.05 versus control; #P<0.05 versus testosterone).

Testosterone Increases the Expression of NADPH Oxidase Subunits

To analyze whether NADPH oxidase plays a role in ROS production by testosterone, we determined the effects of testosterone on the mRNA expression of Nox1, Nox4, (physiologically more abundant in SHRs; data not shown), p22phox, and the protein expression of p47phox in VSMCs from WKY rats and SHRs. Testosterone augmented mRNA expression of Nox1 after 8 hours (Figure 3A) and Nox4 after 4 hours (Figure 3B) only in WKY VSMCs. p22phox (Figure 3C) expression was not altered by testosterone. Testosterone augmented the expression of p47phox, a key regulator of

in each field. Accordingly, 15 measurements were done at each time point per well. Images were captured with an inverted microscope equipped with a digital camera (Axio Observer equipped with AxioCam MRc, Carl Zeiss, Berlin, Germany) and analyzed with AxioVision software. Results are expressed as percentage of control (100%; time 0 hours).

Statistical Analysis

Data are presented as mean±SEM. Groups were compared using 1-way ANOVA or Student t test, as appropriated. Newman-Keuls posttest was used to compensate for multiple testing procedures. P<0.05 was considered statistically significant.
Figure 6.
NOX1 activity, in both WKY rats and SHRs after 2 hours (Figure 4). To determine whether the regulation of NADPH oxidase subunit expression by testosterone is a genomic effect, we determined the protein expression of p47phox in the presence of actinomycin D and cycloheximide. Both inhibitors abolished the augmentation of p47phox expression by testosterone in WKY rat and SHR VSMCs (Figure 4; P<0.05).

**Testosterone Induces c-Src Activation in VSMCs**

To determine whether testosterone activates c-Src, a previously described NADPH oxidase regulator in VSMCs, we assessed phosphorylation of c-Src by immunoblotting in WKY rat and SHR VSMCs treated with testosterone for 0 to 120 minutes. As shown in Figure 5A, testosterone augmented the activity of c-Src in both WKY rat and SHR VSMCs in a time-dependent and different manner (30 minutes only in SHR VSMCs and at 120 minutes both in WKY rat and SHR VSMCs; P<0.05).

**c-Src Mediates Testosterone-Induced NADPH-Driven ROS Production in SHR VSMCs**

To further elucidate a role for c-Src in testosterone-induced activation of NADPH oxidase, we determined NADPH oxidase–driven ROS generation in the presence of PP2, a c-Src inhibitor. Inhibition of c-Src had no effect on testosterone-induced ROS production in WKY rat VSMCs but abolished testosterone-induced ROS generation in SHR VSMCs (Figure 5B; P<0.05).

**Testosterone-Induced ROS Leads to VSMC Migration via c-Src–Mediated Mechanisms**

Because VSMC migration plays an important role in vascular remodeling and in hypertension-associated vascular changes, we determined whether testosterone induces VSMC migration and whether ROS play a role in this effect. As illustrated in Figure 6A and 6B, testosterone increases VSMC migration after 8 hours in WKY rat VSMCs and after 6 hours in SHR VSMCs and with greater effects in SHR VSMCs. These effects were inhibited by flutamide (Figure 6A and 6B), apocynin (Figure 6C and 6D), and PP2 (Figure 6E and Figure 6F; P<0.05). Representative pictures are shown in Figure 6G and 6H.

**Discussion**

Findings from our study demonstrate that testosterone induces migration of VSMCs via NADPH oxidase–driven ROS and c-Src–dependent pathways by different mechanisms in WKY rat and SHR VSMCs. These effects might be related to important features in cardiovascular pathologies, such as arterial calcification, increased levels of C-reactive protein and endothelin 1, and myocardial inflammation.

Testosterone induces long-term, genomic ROS production in both strains in a time- and concentration-dependent manner and by direct mechanisms. However, it stimulates short-term, nongenomic ROS production by unique mechanisms only in SHR VSMCs. c-Src is a key factor for ROS production by testosterone exclusively in SHRs, and it is a crucial player in VSMC migration by testosterone in both strains.

These conclusions are based on several observations. First, testosterone augmented ROS production in VSMCs from WKY rats (60 and 120 minutes) and SHRs (30, 60, and 120 minutes). Second, flutamide and actinomycin D inhibited only long-term (120 minutes) ROS generation in WKY rat and SHR VSMCs, having no effect on short-term (30 minutes) ROS production in SHR VSMCs. Third, testosterone enhanced the expression of NADPH oxidase subunits (Nox1 and Nox4 in WKY rat VSMCs; p47phox in WKY rat and SHR VSMCs). Fourth, testosterone induced phosphorylation of c-Src in a time-dependent manner and with a similar time course observed for ROS formation in SHRs (30 minutes) and after augmentation of ROS generation in WKY VSMCs (120 minutes). Fifth, PP2 decreased ROS production only in SHR VSMCs. Sixth, flutamide, apocynin, and PP2 inhibited both WKY rat and SHR VSMCs migration. Thus, our data strongly indicate that testosterone has an important role in hypertension-associated processes, such as vascular oxidative stress, activation of redox-sensitive pathways, and VSMC migration and that c-Src has a fundamental role in these effects.

Because ROS play a key role in hypertension, we hypothesized that testosterone regulates its production and modulates redox-sensitive processes by different mechanisms in cells isolated from normotensive (WKY rats) and hypertensive animals (SHRs) and in a marked manner in SHR VSMCs. Herein, we have described for the first time a direct effect of testosterone on NADPH oxidase in VSMCs. We have also demonstrated by different methodologies that testosterone augments ROS generation in VSMCs from WKY rats and SHRs in a time- and strain-dependent manner. Although there were no differences in long-term (genomic) ROS formation between cells from WKY rats and SHRs, VSMCs from SHRs were more sensitive to testosterone stimulus, because the increase in ROS production occurred earlier and was significantly higher in SHR VSMCs. More importantly, we provide novel data characterizing a nongenomic pathway whereby testosterone induces short-term ROS production exclusively in SHR VSMCs. In addition, we show a key role of c-Src in both genomic and nongenomic effects of testosterone in VSMCs. Our data corroborate previous studies showing that testosterone induces oxidative stress.
stress in rabbit and rat prostate, and is related to augmentation of blood pressure levels in different animal models of hypertension.

c-Src is considered a key element in hypertension-associated activation of NADPH oxidase, playing a role both upstream and downstream of the enzyme. Although the Src kinase family acts as a cofactor in the activation of AR in prostatic cells, and inhibition of c-Src results in increased dehydroepiandrosterone production by human adrenal tumor cells, there are no data indicating modulation of c-Src by testosterone in VSMCs.

Here we show that testosterone is an important regulator of c-Src–mediated redox signaling. Our data provide complementary evidence that, in SHR VSMCs, c-Src is an upstream modulator of NADPH oxidase, and inhibition of c-Src results in increased ROS production only in SHR VSMCs, in a time-independent manner, suggesting that c-Src mediates regulation of NADPH oxidase by testosterone exclusively in SHRs by not fully elucidated mechanisms. Our data support the concept that c-Src is involved in redox-sensitive signaling pathways in hypertension.

Like testosterone, c-Src is associated with cell migration, an important player in many physiological processes, including embryonic development, inflammatory responses, response to injury, and vascular remodeling, a major structural change in hypertension. Basal levels of VSMC migration were different between the 2 strains (data not shown), in accordance with previous reports. Previous studies have associated increased ROS levels, and activation of c-Src with VSMC migration. However, there are no data showing that testosterone induces VSMC migration. Although c-Src mediates ROS production only in SHR VSMCs, our data indicate that it has a crucial role in VSMC migration in both WKY rat and SHR VSMCs. Our original findings demonstrate that testosterone induces VSMCs migration in a classic manner, by a genomic mechanism mediated by the AR in a ROS-dependent and c-Src–sensitive manner in WKY rat and SHR VSMCs, indicating a role for testosterone in vascular processes that occur in hypertension.

Testosterone activates c-Src in both strains, a key mechanism on testosterone-induced VSMC migration. Activation of c-Src is accompanied by increased ROS production, suggesting a direct link between c-Src and NADPH oxidase. Inhibition of c-Src blocks ROS production by testosterone only in SHR VSMCs, suggesting that c-Src is upstream NADPH oxidase in SHR VSMCs. On the other hand, ROS production by testosterone is not altered by c-Src inhibition in WKY rat VSMCs. Because the effects of testosterone on VSMC migration depend on both c-Src and ROS, it is possible that c-Src might be downstream NADPH oxidase in WKY rat VSMCs. The mechanisms whereby c-Src modulates the vascular effects of testosterone are still under investigation, and further experiments are needed to clarify this process. Limitations of the present study include the fact that testosterone effects on ROS production were observed with nonphysiological levels of the androgen, which may be associated with the in vitro experimental conditions, and a molecular approach (eg, silencing of the AR) would reinforce pharmacologically obtained data.

In conclusion, testosterone increases ROS production by genomic (WKY rat and SHR VSMCs) and nongenomic (SHR VSMCs) pathways. This effect is markedly increased in SHR VSMCs, in which c-Src is a crucial player. c-Src is not required for ROS production by testosterone in WKY rat VSMCs, but it seems to be modulated by redox-sensitive processes. Our data define an ROS-sensitive pathway whereby testosterone regulates signaling cascades and processes associated with vascular remodeling in hypertension.

**Perspectives**

Testosterone upregulates ROS production in VSMCs and may cause oxidative stress and vascular dysfunction, especially in the context of hypertension. Because ROS have been implicated in cell contraction, growth, migration, and apoptosis, testosterone may have a wide range of effects in the vascular system and can be particularly important in males, especially in patients with augmented testosterone levels, cardiovascular diseases, and arterial hypertension.

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**Disclosures**

None.

**References**


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ONLINE SUPPLEMENT

TESTOSTERONE INDUCES VASCULAR SMOOTH MUSCLE CELL MIGRATION BY NADPH OXIDASE AND c-SRC-DEPENDENT PATHWAYS.
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Figure S1. Testosterone induces ROS production in a concentration-dependent manner. VSMCs were stimulated with testosterone $10^{-9}$, $10^{-8}$, $10^{-7}$ or $10^{-6}$ mol/L for 2 hours. ROS production was assessed by DHE fluorescence microscopy. Representative images for ROS production, assessed by DHE microscopy fluorescence, in WKY-VSMCs ($n=4$).
<table>
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<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
<th>12.0</th>
<th>24.0</th>
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Figure S2. Testosterone induces ROS production in a time-dependent manner. VSMCs were stimulated with testosterone $10^{-7}$mol/L for 0.5 up to 24 hours. ROS production was assessed by DHE fluorescence microscopy. Representative images for ROS production, assessed by DHE microscopy fluorescence, in WKY-VSMCs (n=4).
Figure S3. Direct effects of testosterone on ROS production. Testosterone-induced ROS production is not altered by inhibition of aromatase. VSMCs were incubated with anastrazol® $10^{-5}$ mol/L for 30 minutes and then stimulated with testosterone $10^{-7}$ mol/L for 5-120 minutes. ROS production was assessed by lucigenin-enhanced chemiluminescence. Data represent the mean ± SEM of n= 4-6 experiments, * p<0.05 vs. control (Vehicle).