# Differential upregulation of Nox homologues of NADPH oxidase by tumor necrosis factor-α in human aortic smooth muscle and embryonic kidney cells

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## Abstract

NADPH oxidases are important sources of vascular superoxide, which has been linked to the pathogenesis of atherosclerosis. Previously we demonstrated that the Nox4 subunit of NADPH oxidase is a critical catalytic component for superoxide production in quiescent vascular smooth muscle cells. In this study we sought to determine the role of Nox4 in superoxide production in human aortic smooth muscle cells (AoSMC) and embryonic kidney (HEK293) cells under proinflammatory conditions. Incubation with tumor necrosis factor-α (TNF-α, 10 ng/ml) for 12h increased superoxide production in both cell types, whereas angiotensin II, platelet-derived growth factor or interleukin-1β had little effects. Superoxide production was completely abolished by the NADPH oxidase inhibitors diphenyline iodonium and apocynin, but not by inhibitors of xanthine oxidase, nitric oxide synthase or mitochondrial electron transport. TNF-α upregulated the expression of Nox4 in AoSMC at both message and protein levels, while Nox1 and Nox2 were unchanged. In contrast, upregulation of Nox2 appeared to mediate the enhanced superoxide production by TNF-α in HEK293 cells. We suggest that Nox4 may be involved in increased superoxide generation in vascular smooth muscle cells under proinflammatory conditions.

**Keywords**: NADPH oxidase ▪ Nox2 ▪ Nox4 ▪ HEK293 ▪ aortic smooth muscle cell ▪ TNF-α

## Introduction

Oxidative stress induced by superoxide ($O_2^-\cdot$) and other reactive oxygen species (ROS) has critical roles in the pathogenesis of cardiovascular disease [1]. Potential enzymatic sources of ROS in vasculature include the mitochondrial respiration chain, lipoxygenase and cyclooxygenase, xanthine oxidase, NADPH oxidase, nitric oxide (NO) synthase and cytochrome P450 enzymes. Although our understanding of the mechanisms for superoxide production in vascular cells is incomplete, increasing evidence suggests that NADPH oxidase is a

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major source [1, 2]. This enzyme was originally reported as a multi-component complex consisting of cytosolic p47phox and p67phox subunits, a small G protein Rac, membrane-bound Nox (non-phagocytic NADPH oxidase) and p22phox subunits and later reported that cytosolic subunits and G protein Rac were not required for its activity [3]. This enzyme generates superoxide by one-electron reduction of oxygen *via* its Nox subunit using reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor. The prototype of the Nox subunit, Nox2 (formerly called gp91phox), was originally identified in phagocytic leukocytes, where it is responsible for the generation of bactericidal respiratory bursts in these cells. Nox2 is a flavo-hemoprotein containing one flavin adenine dinucleotide (FAD), two hemes and a C-terminal NADPH binding site, thus is thought to be essential in mediating the electron transfer from NADPH to oxygen [4]. In addition to Nox2, four other isoforms have been identified in both human and animal tissues and based on homologies with each other and their apparent evolution from an ancestral Nox, these are named Nox1, Nox3, Nox4 and Nox5 [5–7]. In mature vasculature, all of Nox1, Nox2, Nox4 and Nox5 have been found to be expressed in both vascular endothelial and smooth muscle cells [8–12].

NADPH oxidase and subsequent ROS generation has been described to modulate intracellular signaling, leading to aberrant proliferation or apoptosis, cellular hypertrophy, expression of inflammatory molecules, remodeling of extracellular matrix, or impairment of NO release [2, 13]. In phagocytes, the role of NADPH oxidase in 'respiratory burst' has been defined. The Nox4 isoform of NADPH, also known as Renox, was found to be abundantly expressed in the kidney cortex [14] with a proposed role in superoxide production, oxygen sensing and regulation of cell growth in HEK293 cells [15]. The physiological or pathophysiological functions of NADPH oxidase expressed in vascular cells are unclear. In vascular smooth muscle cells, Nox4 was demonstrated to be a catalytic component, pivotal for superoxide production wherein downregulation of Nox4 expression by antisense oligonucleotides attenuated superoxide production by 41% [10].The importance of Nox4 in superoxide generation has also been demonstrated in human endothelial cells [9]. However, there is little information about the role of Nox4 in cellular oxidative stress under pathophysiological conditions, for example in the presence of proinflammatory cytokines.

The vascular NADPH oxidases are activated and regulated by a variety of hormonal and mechanical factors known to be important players in vascular disease, including angiotensin II, various cytokines and growth factors, thrombin, and changes in laminar shear stress (for review see [16]). The proinflammatory cytokine tumor necrosis factor-α (TNF-α) activates NADPH oxidase in neutrophils [17–19], through phosphorylation of p47phox and subsequent translocation to the plasma membrane and association with the membrane-bound subunits [20]. Moreover, it has been shown to modulate NADPH oxidase through upregulation of Nox2 in HL-60 cells [21]. However the effects of TNF-α on other NADPH oxidase subunits, especially the Nox1 and Nox4 isoforms, are unclear. Since TNF-α-induced oxidative stress has been implicated in many pathophysiological conditions such as atherosclerosis [22], heart failure [23] and insulin resistance [24], the present study was carried out to investigate whether TNF-α modulates Nox1, Nox2 and Nox4 expression and function in human aortic smooth muscle cells (AoSMC) and HEK293 human embryonic kidney cells.

# Materials and methods

## Materials

Angiotensin II and platelet-derived growth factor were purchased from Calbiochem (EMD Biosciences). Human recombinant interleukin-1β and tumor necrosis factor-α were purchased from Merck. Sodium diethyldithiocarbamate trihydrate (DETCA), β-nicotinamide adenine dinucleotide phosphate sodium (NADPH), allopurinol, acetovanillone (apocynin), diphenyleneiodonium chloride (DPI), N-omega-nitro-L-arginine methyl ester hydrochloride (L-NAME), superoxide dismutase from bovine erythrocytes (SOD), 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), and rotenone were purchased from Sigma-Aldrich. Lucigenin (bis-N-methylacridinium nitrate) was purchased from Molecular Probes. Pre-designed Assay-on-Demand® TaqMan® probes and primer pairs for Nox2 (NM_000397), Nox1 (NM_007052) and Nox4 (NM_016931) were obtained from Applied Biosystems (Foster City, CA, USA). Anti-human Nox2, Nox1, Nox4 and GAPDH polyclonal antibodies were purchased from Santa Cruz (CA).

## Cells and treatment protocols

HEK293 cells were maintained in DMEM with 10% heat-inactivated fetal bovine serum (FBS). Human AoSMC were obtained from Cambrex (Clonetics) and maintained in SmGM-2 (reconstituted according to manufacturer's instruction). Both cells were used between passage 3 to 5 and at 85–90% confluence. During experiments, HEK293 cells ($5 \times 10^6$/well) or AoSMC ($5 \times 10^5$ /well) in 6-well plates were starved with low serum (1% FBS) media, treated with angiotensin II ($10^{-5}$ M), platelet-derived growth factor (20 ng/ml), interleukin-1β (10 ng/ml) and TNF-α (10 ng/ml) for different time periods.

## Measurement of superoxide anion

Superoxide production was determined by lucigenin-enhanced chemiluminescence assay, as described previously by our group [25]. Briefly, enzyme assays were carried out in a final volume of 1 ml of Krebs-Hepes buffer containing 98.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 11.1 mM D-glucose, and 20 mM Hepes-Na. Cells were pretreated with 3 mM DETCA for 1h to inactivate endogenous $Cu^{2+}/Zn^{2+}$ SOD activity. Chemiluminescence was detected by dark-adapted lucigenin (5 µM) with Wallac TriLux MicroBeta scintillation counter (PerkinElmer) at different time intervals [26], and expressed as luminescence counts per second (LCPS).

To characterize the chemiluminescence signal, cells were treated with the NADPH oxidase inhibitor DPI ($10^{-5}$ M) and apocynin (1 mM), the xanthine oxidase inhibitor allopurinol (1 mM), superoxide dismutase (SOD) (60 units/ml), the SOD mimetic tiron (10 mM), the inhibitor of mitochondrial respiration rotenone (1 µM) or the NO synthase inhibitor L-NAME (100 µM). NADPH (100 µM) substrate was used to provide substrate for NADPH oxidase. Lucigenin (5 µM) alone was used as background control for superoxide measurement.

## RNA isolation and Real-time PCR (RT-PCR) analysis

Total RNA was extracted from HEK293 and AoSMC cells after incubation with TNF-α (10 ng/ml) for different times with Trizol® reagent (Invitrogen) following the manufacturer's protocol. Total RNA was quantified using Turner® spectrophotometer (Barnstead International) and contaminating genomic DNA was removed with DNA-free™ reagent (Ambion) before reverse transcription. First stand synthesis was performed using the SuperScriptII™ first-strand synthesis system for RT-PCR (Invitrogen). Two micro liters of first strand reaction was used for each 50 µl PCR reaction using TaqMan® Universal PCR Master Mix together with 2.5 µl of Assay-on-Demand primers and probes. Real-time quantitative PCR analysis was conducted using ABI PRISM® 7000 Sequence Detection System. After an initial incubation step for 2 min at 50°C and denaturation for 10 min at 95°C, PCR was performed using 40 cycles (95°C for 15s, 60°C for 60s). Equal amounts of input RNA were used for all RT-PCR reactions, reactions were performed in duplicate, and TaqMan® 18S ribosomal RNA was used as an internal control. Differential gene expression analysis was calculated using the Comparative ($\Delta\Delta C_T$) method as described [27].

## Western blotting

Total protein was extracted from HEK293 and AoSMC cells after incubation with TNF-α using M-PER™ Mammalian Protein Extraction Reagent (Pierce) and mixed with Halt™ Protease Inhibitor Cocktail Kit (Pierce) following manufacturer's instruction. Western blotting was performed using anti-human gp91phox, Nox4 and Nox1 polyclonal antibodies. After incubation with HRP-conjugated secondary antibody, proteins were detected by SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Each membrane was re-probed with anti-human GAPDH after removing with Restore™ Western Blot Stripping Buffer (Pierce). The signals were analyzed using Molecular Analyst software (BioRad) and imaging densitometer (Model GS.670 BioRad).

## Data analysis

Lucigenin assay data and densitometric ratios were normalized to the respective control values. Data are expressed as means ± SEM. Statistical analysis of data was performed using Student *t* test or Mann-Whitney test, as appropriate. $P < 0.05$ was considered statistically significant [28].
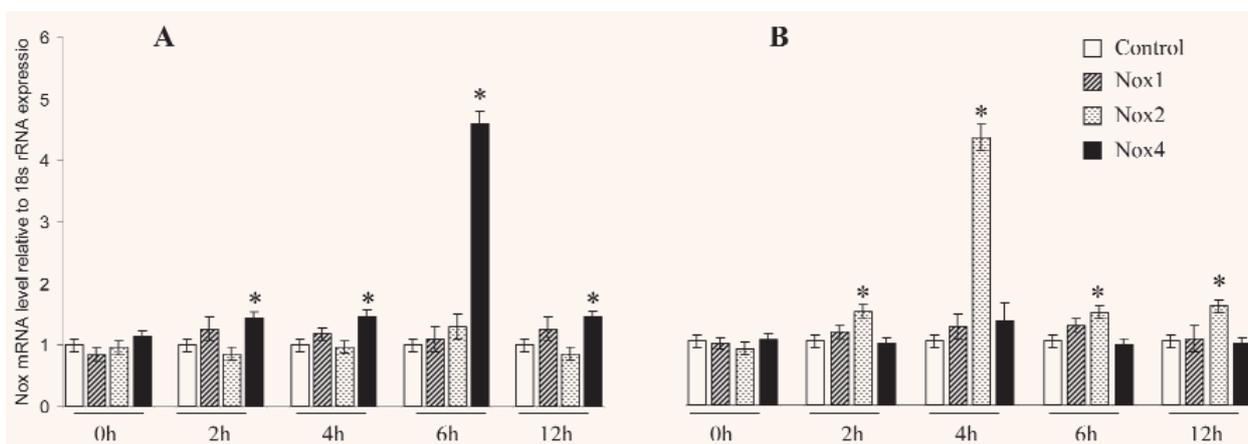
**Fig. 1** Effects of TNF-α (10 ng/ml) on Nox1, Nox2 and Nox4 expression in AoSMC (A) and HEK293 cells (B). Real-time RT-PCR analysis showed upregulation of Nox4 in AoSMC at 2, 4, 6, and 12h with a peak at 6h and upregulation of Nox2 in HEK293 at 2, 4, 6, and 12h with a peak at 4h. (*$p < 0.05$, n = 4, Mann-Whitney test).

## Results

### Modulation of Nox2, Nox4 and Nox1 mRNA expression in AoSMC and HEK293 cells

Real-time RT-PCR analysis was performed to investigate the differential expression of Nox1, Nox2 and Nox4 in both cells after treatment with TNF-α for 0, 2, 4, 6 and 12h. In AoSMC, TNF-α significantly increased Nox4 mRNA expression in a time dependent manner, peaking at 6 h with a 4.6-fold increase over the baseline level (Fig. 1A). However, Nox1 and Nox2 expression was not changed by TNF-α in AoSMC. In HEK293 cells, TNF-α increased Nox2 mRNA expression over the observation period with a peak (4.2-fold increase) at 4h , whereas Nox1 and Nox4 mRNA showed no significant change (Fig. 1B).

### Modulation of Nox proteins in AoSMC and HEK293 cells by TNF-α

We also confirmed the differential expression of Nox2, Nox4 and Nox1 in these cells by western blot analysis. All three proteins were detected in both cell lines. In line with the mRNA expression profile, the Nox4 protein level in AoSMC was significantly upregulated by TNF-α at 12h (Fig. 2), while the protein levels of Nox1 and Nox2 were not changed during the period. In

contrast, in HEK293 cells, expression of Nox2 protein was significantly increased by TNF-α at 6h and 12h after incubation (Fig. 3). Again, like mRNA expression, Nox1 and Nox4 protein levels did not change after TNF-α treatment in HEK293 cells.

### Modulation of NADPH oxidase function by TNF-α

To evaluate the effects of TNF-α treatment on NADPH oxidase function in AoSMC and HEK293 cells, these cells were treated with TNF-α and superoxide production assayed with lucigenin-enhanced chemiluminescence. The chemiluminescence signal in either AoSMC or HEK293 cells was very low under basal conditions, indicating that $O_2^-\cdot$ production in the untreated cells is low. Treatment with TNF-α (10 ng/ml) similarly increased $O2^-\cdot$ production in both AoSMC (Fig. 4A) and HEK293 cells (Fig. 4B) after 12h of incubation. We found that this effect of TNF-α is specific because treatment with angiotensin II, platelet-derived growth factor or interleukin-1β had little effects.

The TNF-α-induced chemiluminescence signal was reduced by SOD (60 units/ml) (53% in AoSMC and 65% in HEK293) and abolished by the cell permeable superoxide scavenger tiron (10 mM) (85% in AoSMC and 90% in HEK293).

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**Fig. 2** Effect of TNF-α on Nox4 protein level in AoSMC. (**A**) Cells were treated with TNF-α (10 ng/ml) at different times and western blot was performed. C: control; T: TNF-α; (**B**) Densitometric analysis showed the significant increase at 12h. (*$p < 0.05$, n = 4, Mann-Whitney test).
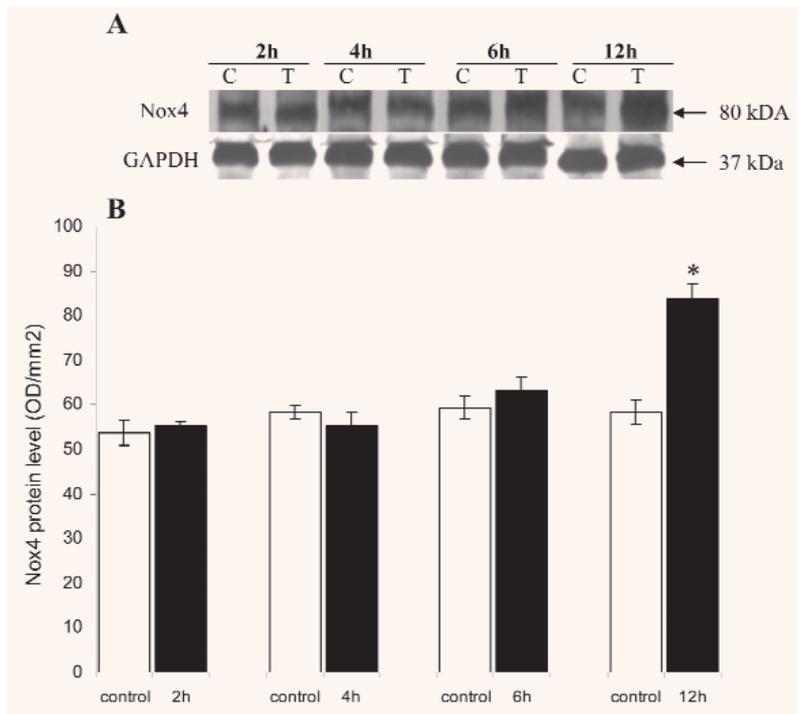
Moreover, TNF-α-induced $O_2^-$· production in AoSMC and HEK293 cells was completely blocked by DPI (10 µM) and apocynin (1 mM), two inhibitors of NADPH oxidase. In contrast, the xanthine oxidase inhibitor allopurinol (1 mM), the mitochondrial respiration inhibitor rotenone (1 µM) or the nitric oxide synthase inhibitor L-NAME (100 µM) had no effect on TNF-α-stimulated superoxide production in either cell type (Fig. 5).



**Fig. 3** Effect of TNF-α on Nox2 protein level in HEK293. (**A**) Cells were treated with TNF-α (10 ng/ml) at different times and western blot was performed. C: control; T: TNF-α; (**B**) Densitometric analysis showed the significant increase at 6 and 12h. (*$p < 0.05$, n = 4, Mann-Whitney test).
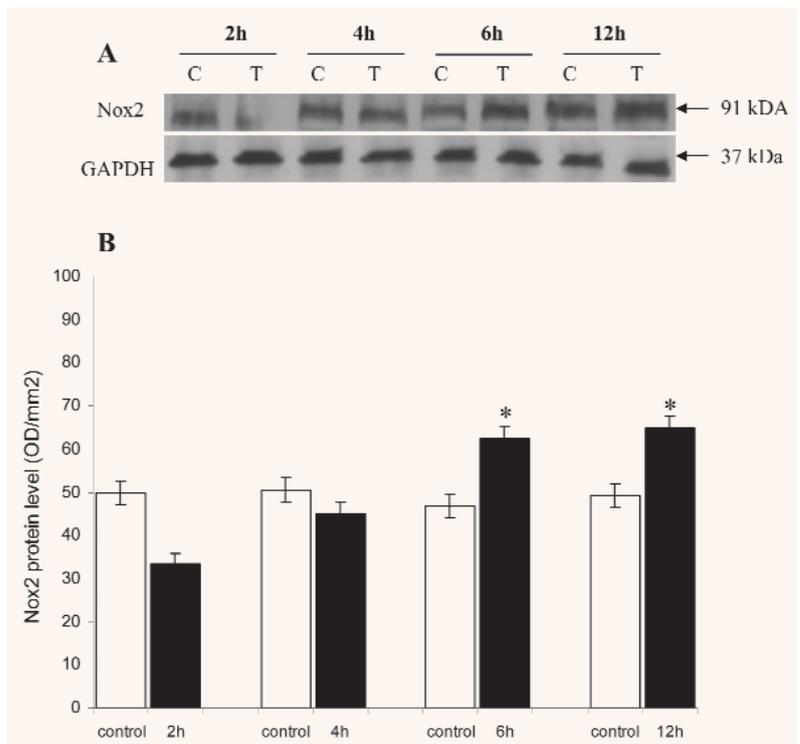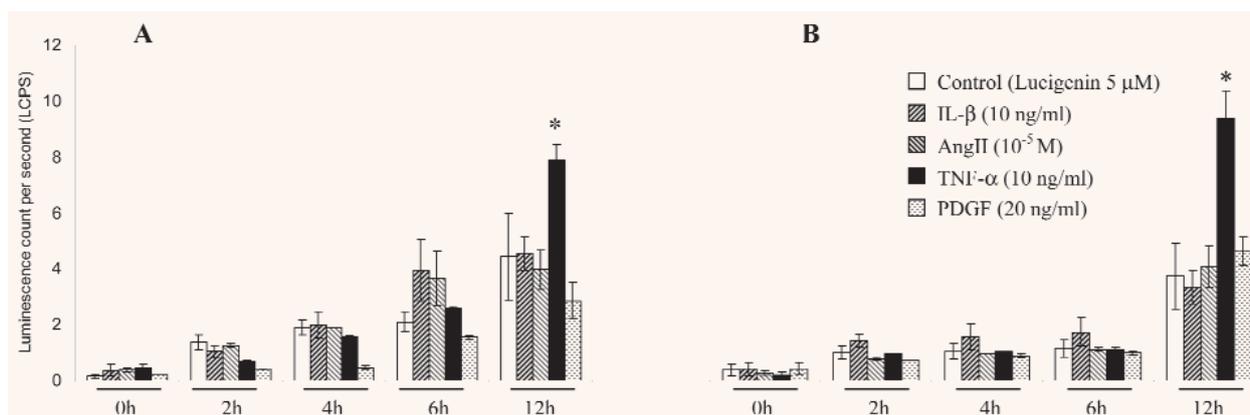
**Fig. 4** $O_2^-\cdot$ production in AoSMC (**A**) and HEK293 (**B**). Cells were treated with interleukin-1β (10 ng/ml), angiotensin II ($10^{-5}$ M), TNF-α (10 ng/ml) and platelet derived growth factor (20 ng/ml) in the presence of DETCA and chemiluminescence change was measured with lucigenin (5 µM) at 0, 2, 4, 6 and 12h. (*$p < 0.05$, n = 4, Mann-Whitney test).

## Discussion

In the present study, we investigated TNF-α induced-oxidative stress mediated by NADPH oxidase in AoSMC and HEK293 cells, and we examined the differential regulation of NADPH oxidase subunits Nox1, Nox2 and Nox4 in these cells. TNF-α stimulated superoxide production and this was derived from NADPH oxidase. Using real-time PCR and western blot analysis, we found that TNF-α upregulated Nox2, but not Nox4, in HEK293 cells; but in AoSMC, the opposite was true. Nox1 remained unchanged in both cells throughout the observation period of 12h.

Inflammatory cytokines including TNF-α, interferon-γ, interleukin-1 and interleukin-6 induce superoxide production from vascular smooth muscle cells, endothelial cells [29–31] and HEK293 cells [32, 33]. TNF-α-induced-oxidative stress in the heart may be mediated by NADPH oxidase, NO synthase and xanthine oxidase enzyme systems, and it contributes to myocardial contractile failure [34]. Our study showed that in both AoSMC and HEK293, TNF-α was able to induce $O_2^-\cdot$ production which was not inhibited by xanthine oxidase inhibitor allopurinol and nitric oxide synthase inhibitor L-NAME, but completely abolished by NADPH oxidase inhibitors (DPI and apocynin). This suggests that NADPH oxidase is the predominant source of ROS production in both cell types, in agreement with other reports [1, 2, 35].

However we did not exclude that the effect might be stimulus-specific.

NADPH oxidase activation has been proposed as causative in TNF-α induced endothelial dysfunction, which is implicated in pathophysiological conditions such as adult respiratory distress syndrome [36]. TNF-α induces NADPH oxidase-dependent superoxide production through several mechanisms, including increase in p22phox expression, and translocation of p47phox [37]. Our results have further revealed that TNF-α may activate the NADPH oxidase system by modulating the expression of Nox subunits. Our study showed that TNF-α was able to specifically induce Nox2 in HEK293 and Nox4 in AoSMC, at both mRNA and protein levels. We did not detect any change in Nox1 expression levels in these cells, and therefore what roles Nox1 might play in vascular cell dysfunction is uncertain. It is noted that Nox1 has been reported to be upregulated during TNF-α-induced oxidative stress in coronary arteries of hyperhomocysteinemic rats [28].

We and others have shown that all of Nox1, Nox2 and Nox4 isoforms are present in vascular smooth muscle cells, and that Nox1 and Nox4 might be the vascular specific NADPH oxidase subunits involved in the vascular remodeling process which occurs in hypertension and atherosclerosis [8, 13, 38]. Recently, Nox1 and Nox4 were found to be compartmentalized differentially in specific signaling domains in the

**Fig. 5** NADPH oxidase mediates TNF-$\alpha$-induced $O_2^-\cdot$ production in AoSMC and HEK293. Both cells were treated with TNF-$\alpha$ (10 ng/ml) in the presence of DETCA and various enzyme inhibitors and $O_2^-$ scavenger, and chemiluminescence change was measured with lucigenin (5 $\mu$M) at 12h. (*$p$ < 0.05, n = 4, Mann-Whitney test).
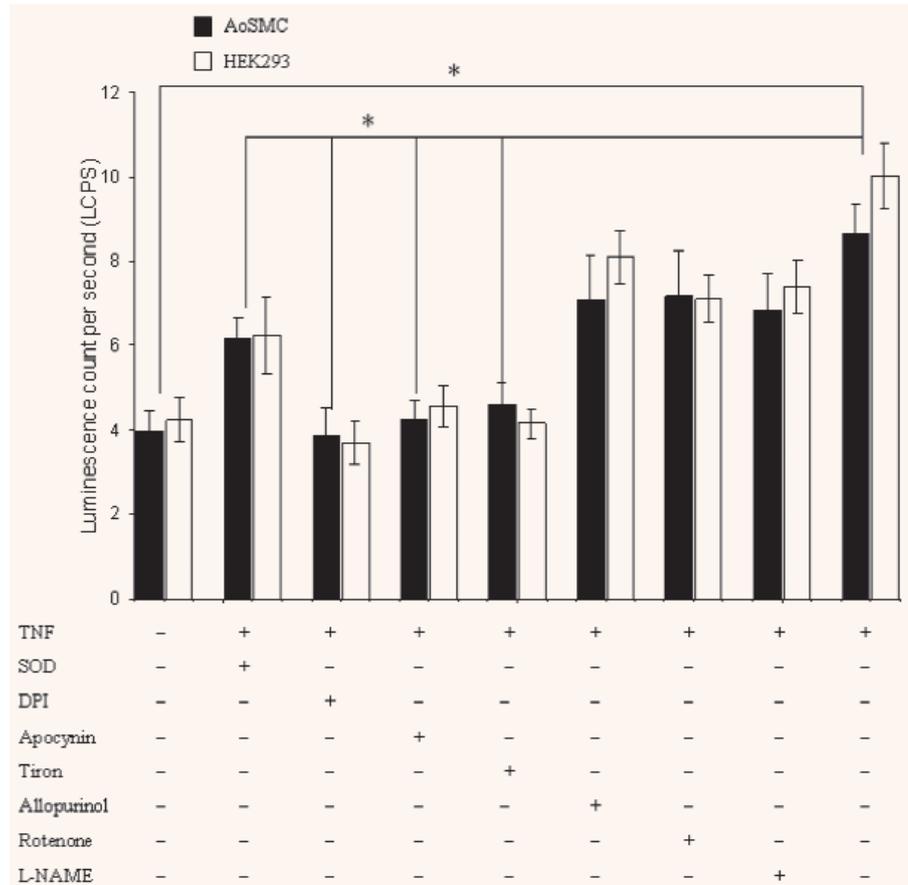
membrane, suggesting differential regulation by growth factors and hormones [39]. This might explain the reported downregulation of Nox4 and upregulation of Nox1 by platelet derived growth factor and angiotension II in vascular smooth muscle cells [40]. We found that Nox4 could be transcriptionaly upregulated by up to 5 fold after incubation with TNF-$\alpha$ for 6h, with 40% increase of the protein level at 12h, accounting for increased TNF-$\alpha$-induced superoxide production at 12h in AoSMC, suggesting that Nox4 might be exclusively involved in redox-sensitive signaling in vascular cells in response to cytokine stimulation.

Phagocytic NADPH oxidase is activated via Nox2 in several ways. One proposed mechanism is stimulus-induced membrane translocation of cytosolic proteins including small GTPase Rac and the two specialized cytosolic proteins p67phox and p47phox, each containing two SH3 domains [41, 42]. However, modulation of the expression Nox2 and its homologues, and the underlying mechanisms have been little explored in vascular cells. Studies aiming to determine the specific transcriptional factor(s) involved in modulation of Nox expression in vascular cells are in progress. Our study has shown that TNF-$\alpha$-induced, NADPH oxidase-mediated superoxide production in AoSMC and HEK293 is similarly modified by conventional inhibitors of NADPH oxidase. We demonstrated striking cell specific differences of Nox isoform expression in response to TNF-$\alpha$. Our work highlights the potential of identifying isoform specific Nox modulators as targets for therapies in cardiovascular disease.

# Acknowledgement

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