ONLINE SUPPLEMENT

TUMOR NECROSIS FACTOR ALPHA DECREASES NOS3 EXPRESSION PRIMARILY VIA RHO/RHO KINASE IN THE THICK ASCENDING LIMB

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Running title: TNF and THAL's NOS3 expression

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Extended Material and Methods

Drugs concentration and source: PIP3 5 μmol/L was from Echelon Biosciences (Salt Lake City, UT), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) 4 mmol/L was from Sigma (St. Louis, MO); GF 109203X 5 μmol/L[1] and ML-7 0.5 μmol/L[2] from Enzo Life Sciences; SP600125 (JNK inhibitor)[3] 20 μmol/L from Alexis Biochemicals; PD 98059[4] 50 μmol/L, U0126[5] 10 μmol/L, H-1152[6,7] 10 μmol/L, 2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one (Janus Protein Tyrosine kinases (JAK) inhibitor) 5 μmol/L and NSC 23766[8] 100 μmol/L from Calbiochem; cell permeable exoenzyme C3 transferase[9] 0.05 μg/mL from Cytoskeleton. Concentration of the inhibitors was selected such that was 10 fold or higher than the Kd and did not affect basal NOS3 expression. The experiments were done in the presence of 5% fetal bovine serum.

Western blot analysis. NOS3 expression was measured as previously described[10] with some modifications. Thirty micrograms of protein from freshly prepared lysates were loaded per lane. One set of samples (i.e. vehicle, TNF-α, inhibitor, inhibitor plus TNF-α) were loaded using a single gel so each experiment had its own control. The membrane was incubated in blocking buffer containing 20 mmol/L Tris, 137 mmol/L NaCl, 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk for 60 min and then with a 1:1,000 dilution of an NOS3-specific monoclonal antibody (BD Transduction Laboratories, San Diego, CA) in blocking buffer for 2 hours at room temperature. The membrane was washed with TBS-T and incubated for 2 more hours with a 1:1,000 dilution of secondary antibody against mouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL). For β-tubulin measurements membranes were incubated with stripping buffer (glycine 0.2 mol/L pH 2.8) fro 30 min and then washed 4 times with TBS-T. Membranes were then incubated for 1 hour with blocking buffer at room temperature, followed for 1 hour incubation with a 1:10,000 dilution of anti β-tubulin antibody (Abcam, Cambridge, MA) in blocking buffer, washed 5 times with TBS-T and then incubated with a 1:5,000 dilution of secondary antibody against rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL).
Before starting our experiments we measured the error associated with NOS3 Western blot running and transferring method by loading the same amount of protein in every lane and calculating the error associated with that. We found that the extreme lanes had lower transference efficiency and that the change in optical density in the 4 central lanes when the same sample was loaded was 0 ± 6 % vs lane #5 (n=3). Therefore, for all the experiments we only used the 4 central lanes for the experimental samples whereas the remaining lanes were only loaded to ensure even running and transference.

RhoA GTPase activation. RhoA GTPase was measured using the colorimetric G-LISA RhoA activation assay biochemical kit from Cytoskeleton Inc (Denver, CO). Briefly, aliquots of THALs suspensions were seeded in 24 wells plate (150 μg/well) in DMEM/F12 media. Tubules were incubated at 37°C and 95/5% O_2/CO_2 for 4 hours. Tubules were then treated for 0 or 10 min with 1 nmol/L TNF-α. After treatment, media was carefully aspirated and tubules lysed by pipetting up and down in ice-cold lysis

buffer containing protease inhibitors. Lysate was collected and centrifuged at 9,300xg for 2 min. An aliquot of the supernatant was used for protein measurement and the remaining lysate was snap-frozen in liquid nitrogen and stored at -80°C. On the day of the assay, 10 µg of protein lysate was used per well in a 96 well plate for RhoA G-LISA assay and the assay performed following manufacturer instructions. Active RhoA was detected with a colorimetric reaction and absorbance at 492 nm was measured using a microplate reader Titertek Multiskan MCC/340 (Titertek Instruments, Inc. Huntsville, AL). Readings from each well were corrected by the blank (lysis buffer alone) and the corrected results were expressed in optical density units (OD).

Measurement of NO Production in mTHAL primary cultures by Fluorescence Microscopy. mTHAL primary cultures were prepared as explained in Method section with the following modification: cells were grown on sterile glass coverslips in 6 well plates. Forty hrs after cells were seeded, they were treated with either vehicle (medium) or 1 nmol/L TNF-α for another 24 hrs. After treatment, coverslips were lifted, washed with media and mounted in a custom-made, temperature regulated chamber. The bath was started at 0.6 ml/min, and the chamber was warmed to 37.0 ± 0.5 °C. THAL cells were bathed for 15 min in a solution containing (in mM): 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 alanine, 1 Na3 citrate, 5.5 glucose, 2 Ca^{2+} (lactate)2, and 10 HEPES (pH 7.4) (solution A) containing 5 µmol/L 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (Molecular Probes Inc. Eugene, OR) and washed for 15 min with solution A containing 100 µmol/ L-arginine, the substrate for NOS. At this concentration, L-arginine supports but does not stimulate NO production[11]. Ten to 30 THAL cells were imaged using a 40x oil-immersion objective, and the dye was excited with a Xenon arc lamp as a light source filtered with two serial 488 nm excitation filters. The fluorescence emitted by NO-bound dye (>510 nm) was collected through an emission filter (515 long pass) using a CCD camera and measured with a data acquisition and analysis software (Metafluor, Universal Imaging, West Chester, PA). Basal fluorescence readings were taken every 30 seconds during a 12 min period and then the bath solution was changed to one containing 5 µM PIP3 (PIP3 stock was dissolved in Neomycin Neosulfate as a carrier). The slope of fluorescence over time was considered NO production. Basal NO production was calculated for the last 5 min (10 time points) of the basal period. Stimulated NO production was calculated as the maximal 5 min slope observed during a 20 min period subsequent to addition of PIP3. Data are expressed as fluorescence units (FU)/min.

Measurement of NO Production in isolated THALs by Fluorescence Microscopy . THALs were isolated from 100 to 150 g male Sprague Dawley rats as previously described[12-14]. Tubules were held between glass pipettes at 4 °C in a chamber designed for live cell imaging on the stage of an inverted microscope as done routinely in our laboratory[12-14]. The bath was started at 0.6 ml/min, and the chamber was warmed to 37.0 ± 0.5 °C. THALs were bathed for 20 min in a solution containing (in mM): 130 NaCl, 2.5 NaH_2PO_4, 4 KCl, 1.2 $MgSO_4$, 6 alanine, 1 Na_3 citrate, 5.5 glucose, 2 Ca^{2+} $(lactate)_2$, and 10 HEPES (pH 7.4) (solution A) containing 2 µmol/L 4,5-diaminofluorescein diacetate (EMD Biosciences, Gibbstown, NJ) and washed for 20 min with solution A containing 100 µmol/ L-arginine, the substrate for NOS. THALs were

imaged using a 40x oil-immersion objective, and the fluorescence was measured as it was done with the mTHAL cells. Measurements were recorded once every minute for a 5-min control period, and then either 1 nmol/L TNF-α or vehicle was added to the bath. Fluorescence was measured once every minute during 10-min experimental period. Measurements were performed in the absence of luminal flow because we have found that flow stimulates NO production by the THAL[15]. Changes in NO production were calculated as the slope in fluorescence from minute 2 to 7 after adding TNF-α (or vehicle) minus the slope during the control period. Data are expressed as FU/min.

Extended Results

Effect of higher concentrations of Rho inhibitor on TNF-α-induced decreases in NOS3 expression. The effect of C3 exoenzyme on TNF-α-induced decreases in NOS3 expression was not as robust as expected. This could be due to lower than optimal concentrations of the exoenzyme or to the low permeability of this protein. When THALs were treated with 0.1 ug/mL C3 exoenzyme, basal NOS3 expression was increased by 2 fold; however, the ability of C3 to block TNF-α's effect was not enhanced. In addition, when THALs were incubated with 1 μg/mL C3 exoenzyme alone, protein recovery was 49 ± 4 % compared to vehicle treated cells ($p<0.002$) and NOS3 expression was 240 ± 28% compared to vehicle treated cells ($p<0.02$). Higher concentrations resulted in even lower protein recovery. These data suggest that C3 exoenzyme at concentrations of 1 μg/mL or higher induce cytotoxicity.

Effect of concomitant Rho and ROCK inhibition on TNF-α-induced decreases in NOS3 expression. RhoA has been shown to activate signaling cascades other than ROCK[16]. Similarly, ROCK has been reportedly shown to be activated independently of RhoA[17,18]. Because inhibition of RhoA blocked one third and inhibition of ROCK blocked two thirds of TNF-α's effect, we tested whether concomitant inhibition of those signaling molecules had an additive effect. Simultaneous treatment with C3 exoenzyme and H-1152 (RhoA and ROCK inhibitors respectively) blocked TNF-α-induced reductions in NOS3 expression by 56 ± 19 % ($n=5$, $p<0.04$). These data indicate that RhoA and ROCK are part of the same signaling cascade in TNF-α-induced decreases in NOS3 expression.

Effect of Myosin light chain (MLC) inhibitor on TNF-α-induced decreases in NOS3 expression. Since the inability of the ROCK inhibitor to completely block the effect of TNF-α did not appear to be due to using too low of a concentration, we examined other possibilities. MLC phosphorylation is enhanced after TNF-α treatment[19]. This effect seems to depend upon a) activation of myosin light chain kinase (MLCK) and b) ROCK-induced inhibition of MLC phosphatase[20]. Thus, the effect of ROCK inhibition could ultimately be explained by its effects on MLC. To study whether the remaining effect of TNF-α on NOS3 expression observed after ROCK inhibition was due to a concomitant activation of MLCK, and thus MLC phosphorylation, we incubated cells with the MLCK inhibitor ML-7. Figure S1 shows that TNF-α alone decreased NOS3 expression by 56 ± 8 % ($n=5$, $p< 0.001$) whereas MLCK inhibition did not affect TNF-α-induced decreases in NOS3 expression (Δ= -54 ± 11 %, $n=5$). MLCK blockade did not affect basal NOS3

expression (Δ= 14 ± 9 %, n=5). These data indicate that MLCK does not mediate TNF-α-induced decreases in NOS3 expression in THALs.

Additional pathways tested. We tested a number of other signaling cascades. These pathways have been shown to: 1) be activated by TNF-α; or 2) play a role in NOS3 expression. First we tested PKC. In control cells, TNF-α decreased NOS3 expression by 55 ± 14% (n=4 p<0.02 vs vehicle). PKC blockade with the general PKC inhibitor GF 109203X did not affect TNF-α-induced reductions in NOS3 expression (Δ= -56 ± 9%, n=4 p<0.05 vs GF109203X alone). These data indicate that PKC is not involved in the effect of TNF-α on NOS3 expression. Next, we studied whether inhibition of MAPKK blocked the effect of TNF-α on NOS3 expression. Incubation of cells with 50 μmol/L PD98059 or 10 μmol/L UO126, two different inhibitors of MAPKK, did not prevent TNF-α from decreasing NOS3 expression (Δ=-60 ± 4%, n=3 p<0.005 vs PD98059 alone and Δ= -70 ± 11%, n=3 p<0.03 vs UO126 alone). These data suggest that MAPKK is not required for TNF-α to reduce NOS3 expression. Because TNF-α activates JNK and JAK in other cell types we also tested the inhibitors of those kinases; however, neither of them prevented TNF-α from decreasing NOS3 expression.

Given that none of the cascades we tested could account for the Rho/ROCK-independent portion of TNF-α's actions, we next tested whether either TNF-α itself or ROCK inhibition activated a pathway that enhances NOS3 expression. Rac-1 has been shown to antagonize Rho under certain conditions[21] and to increase NOS3 expression[22]. Furthermore, inhibition of ROCK can activate Rac-1[23]. Thus, we tested whether Rac-1 inhibition exacerbated TNF-α-induced decreases in NOS3 expression. Contrary to what we expected, Rac-1 inhibition with 100 μmol/L NSC 23766 did not affect basal NOS3 expression or TNF-α-induced decreases in NOS3 expression (TNF-α alone Δ= -48±6% p<0.001 vs vehicle and TNF-α + Rac-1 inhibitor Δ= -47±10, n=5, Figure S2A). These data suggest that Rac-1 does not counteract Rho/ROCK when the latter are activated. To test whether inhibition of ROCK activates Rac-1 and Rac-1 then stimulates NOS3 expression, we tested the simultaneous inhibition of ROCK and Rac-1 on NOS3 expression. Figure S2B shows that TNF-α alone decreased NOS3 expression by 49±6% (n=7, p<0.001) and that incubation with both Rac-1 and ROCK inhibitors together blocked TNF-α's effect on NOS3 expression by 37±11% (n=7 p<0.05 vs TNF-α alone). These results were similar to those found with the ROCK inhibitor alone (Figure 5) implying that the blockade of TNF-α-induced reduction in NOS3 expression seen under ROCK inhibition was not due to a parallel activation of Rac-1.

Additional discussion

The role of TNF-α on regulation of blood pressure is controversial. Neutralization of TNF-α blunted the increase in blood pressure in Ang II-induced hypertension[24,25] and prevented the increase in blood pressure in a model of lupus erithematosus[26]. Similarly, administration of angiotensin II to TNF-α knock out mice failed to elicit the increase in blood pressure observed in wild type animals[25]. In addition, patients with rheumatoid arthritis receiving anti-TNF therapy showed reduced blood pressure and improved endothelial dependent vasodilatation[27]. However, infusion of TNF-α in anesthetized rats induced natriuresis and a decrease in blood pressure[28] and infusion of recombinant

TNF-α in cancer patients (an old treatment) resulted in a decrease in blood pressure within a couple of hours[29]. These data suggest that TNF-α by itself does not increase blood pressure but rather acts as an important mediator of hypertension in certain physiological settings.

Our data show that maximal inhibition of TNF-α's effect on NOS3 expression using the Rho inhibitor C3 exoenzyme was about 30%. Other investigators have used concentrations of C3 exoenzyme ranging between 0.03 μg/mL [30,31] to 50 μg/mL[32]. In this study we used 0.05 μg/mL for two reasons: 1) concentrations of 0.1 μg/mL increased basal NOS3 expression by about two fold without improving the blockade and 2) concentrations of 1 μg/mL or higher resulted in cell death (50% reduction in protein recovery) and an increase in basal NOS3 expression. Although these data could suggest hat RhoA is involved in basal NOS3 expression levels, such conclusion can not be drawn without further studies due to the cytotoxicity induced by the high concentrations of the compound.

Rho GTPases are small GTP-binding proteins that act as molecular switches and cycle between active (GTP bound) and inactive (GDP bound) states. This cycling is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-disociation inhibitors (GDIs)[33]. Activation of RhoA by TNF-α has been shown in renal cell lines. In LLC-PK and Madin Darby canine cells TNF-α augmented paracellular permeability by stimulating RhoA/ROCK and increasing MLC phosphorylation[19]. In LLC-PK cells, GEF-H1 mediates TNF-α-induced activation of RhoA and increases in parecellular permeability[19]. However, whether TNF-α activates RhoA via GEF-H1 in mTHALs remains unknown.

When ROCK is inactive, the carboxy-terminal regulatory domain, composed of a pleckstrin homology domain (PH) and a Rho binding domain (RBD), inhibit the catalytic domain[34]. Binding of RhoA-GTP to RBD causes a conformational change that relieves the autoinhibition and increases ROCK activity. This change in conformation of ROCK can also be achieved by binding of arachidonic acid to the PH domain[17] or by cleavage of the carboxy terminus by caspase-3[18,35]. To test whether ROCK was activated independently of RhoA we simultaneously inhibited Rho and ROCK. Concomitant blockade of Rho and ROCK had no additive effect over inhibiting ROCK alone. These data suggest that TNF-α reduces NOS3 expression by activating ROCK via RhoA GTPase.

We did not study the molecular mechanisms by which TNF-α reduces NOS3 protein levels. However, in endothelial cells both, decreases in NOS3 mRNA stability and in promoter activity have been reported. After TNF-α treatment, cytosolic proteins bind to the 3' untranslated region (UTR) of NOS3 mRNA decreasing its half life[36-38]. In particular, translation elongation factor 1 a (EF1-a), a ribosomal protein, has been identified as a modulator of NOS3 mRNA stability[38]. ROCK has been shown to phosphorylate EF1-a and to decrease EF-1a binding to filamentous actin[39] whereas in pulmonary endothelial cells ROCK reduce NOS3 mRNA half-life[40]. Therefore activation of Rho/ROCK by TNF-α could lead to EF1-a phosphorylation, enhanced EF1-a binding to NOS3 mRNA, decreased NOS3 mRNA stability and reduced NOS3 protein levels. TNF-α has also been shown to reduce NOS3 promoter activity. In pulmonary microvessel endothelial monolayers it has been demonstrated that TNF-α increases Sp3 and decreases GATA-4 binding to the -370CACC and -231GATA site respectively,

resulting in reduced promoter activity[41]. In addition, a decreased Sp1/Sp3 binding to the upstream -109/-95 site in bovine aortic endothelial cells has also been shown to be responsible for the impaired NOS3 promoter activity after TNF-α treatment[42]. However, to our knowledge a role of Rho/ROCK in decreasing NOS3 promoter activity has not been shown.

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Figures

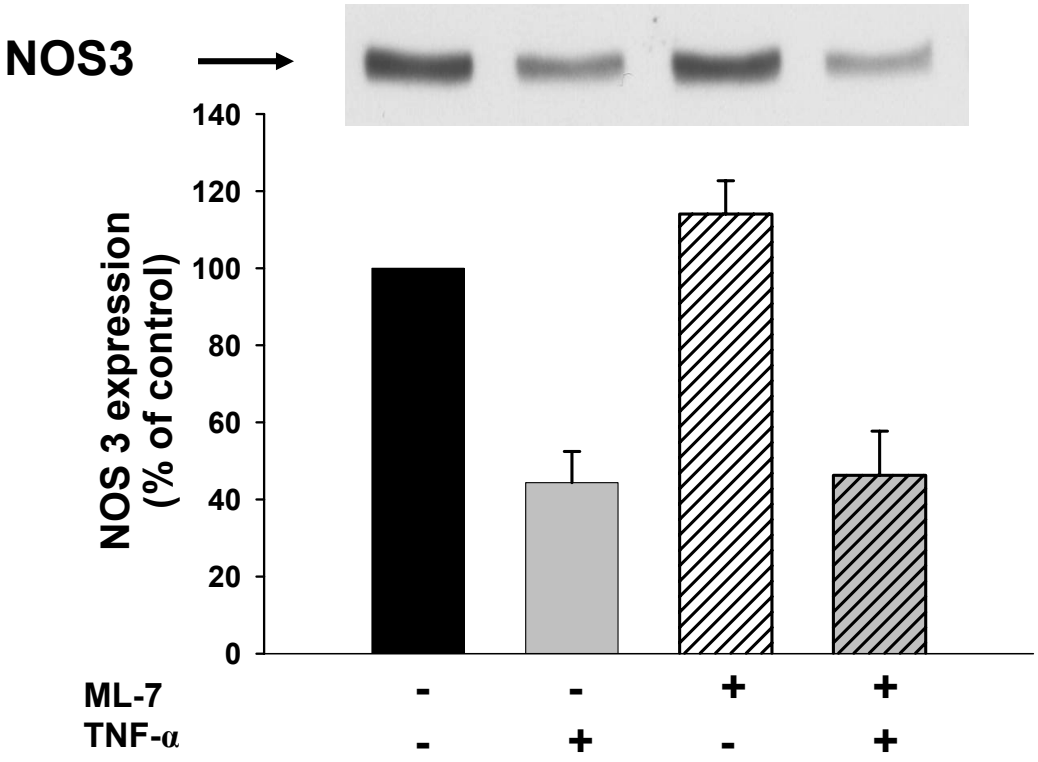
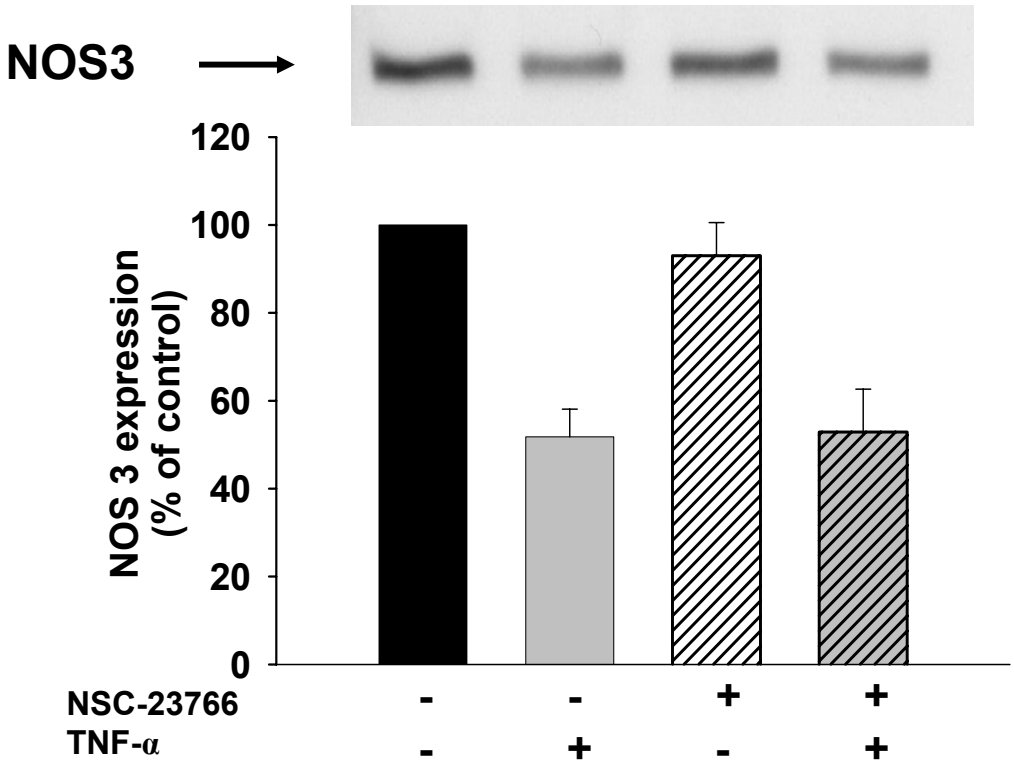


Figure S1

Figure S1. Effect of MLCK inhibition on TNF-α-induced decreases in NOS3 expression in mTHALs. Top: representative Western blot. Bottom: cumulative data (TNF-α vs vehicle $p<0.002$; TNF-α vs ML-7 + TNF-α not significant; vehicle vs ML-7 not significant; n=5).


NOS3
120
100
80
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40
20
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NOS 3 expression
(% of control)
NSC-23766
TNF-α
-
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Figure S2A

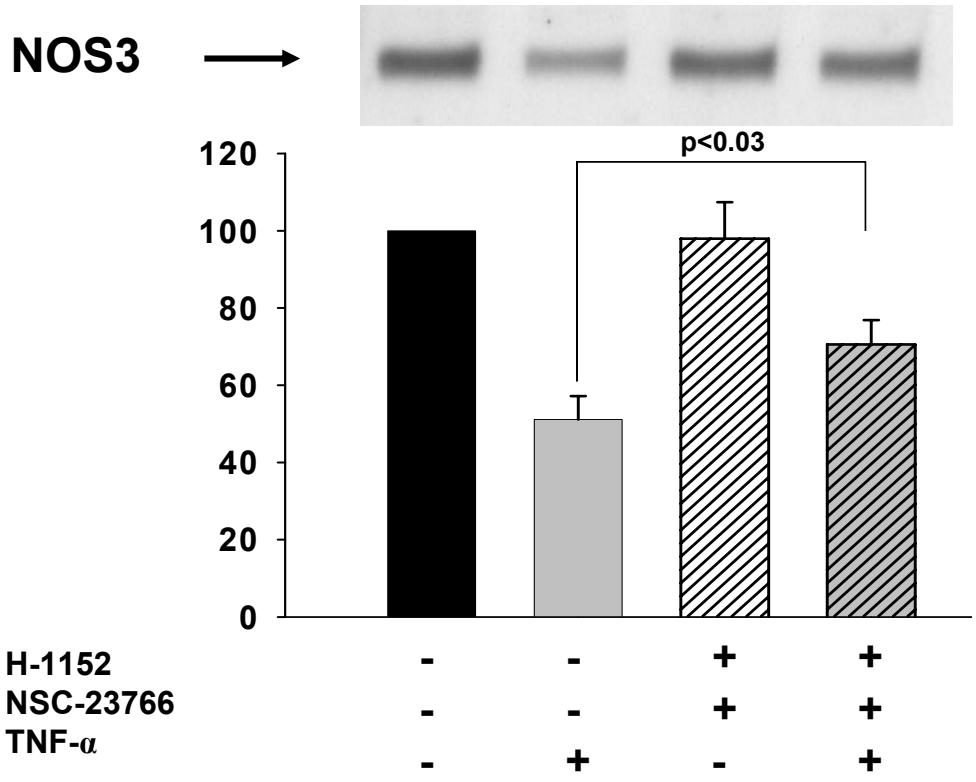

Figure S2B

Figure S2. A) Effect of Rac-1 inhibition on TNF-α-induced decreases in NOS3 expression in mTHALs. Top: Representative Western blot. Bottom: cumulative data (TNF-α vs vehicle $p<0.002$; TNF-α vs NSC 23766+ TNF-α not significant; vehicle vs NSC 23766 not significant; n=4). **B) Effect of concomitant inhibition of Rac-1 and ROCK on TNF-α-induced decreases in NOS3 expression in mTHALs**. Top: representative Western blot. Bottom: cumulative data (TNF-α vs vehicle $p<0.001$; TNF-α vs H-1152 + NSC 23766 + TNF-α $p<0.03$; vehicle vs H-1152 + NSC 23766, not significant; n=7).