Tumor Necrosis Factor α Decreases Nitric Oxide Synthase Type 3 Expression Primarily via Rho/Rho Kinase in the Thick Ascending Limb

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Abstract—Inappropriate Na⁺ reabsorption by thick ascending limbs (THALs) induces hypertension. NO produced by NO synthase type 3 (NOS3) inhibits NaCl reabsorption by THALs. Tumor necrosis factor α (TNF-α) decreases NOS3 expression in endothelial cells and contributes to increases in blood pressure. However, the effects of TNF-α on THAL NOS3 and the signaling cascade are unknown. TNF-α activates several signaling pathways, including Rho/Rho kinase (ROCK), which is known to reduce NOS3 expression in endothelial cells. Therefore, we hypothesized that TNF-α decreases NOS3 expression via Rho/ROCK in rat THAL primary cultures. THAL cells were incubated with either vehicle or 1 nmol/L of TNF-α for 24 hours, and NOS3 expression was measured by Western blot. TNF-α decreased NOS3 expression by 51±6% (P<0.002) and blunted stimulus-induced NO production. A 10-minute treatment with TNF-α stimulated RhoA activity by 60±23% (P<0.04). Inhibition of Rho GTPase with 0.05 μg/mL of C3 exoenzyme blocked TNF-α–induced reductions in NOS3 expression by 30±8% (P<0.02). Inhibition of ROCK with 10 μmol/L of H-1152 blocked TNF-α–induced decreases in NOS3 expression by 66±15% (P<0.001). Simultaneous inhibition of Rho and ROCK had no additive effect. Myosin light chain kinase, NO, protein kinase C, mitogen-activated kinase, and p38 kinase were involved, as were NOS3 and the signaling cascade. TNF-α also plays a role in the elevation of blood pressure caused by insulin resistance and systemic lupus erythematosus.

Key Words: eNOS ■ hypertension ■ kidney ■ cytokines

Thick ascending limbs (THALs) reabsorb 20% to 30% of the filtered NaCl load and contribute to the maintenance of the renal corticomedullary osmotic gradient. Increased NaCl reabsorption by this nephron segment induces salt-sensitive hypertension. Conversely, NO produced by NO synthase type 3 (NOS3, or endothelial NO synthase) inhibits CI⁻ transport by THALs and thus leads to natriuresis. Activation of NOS3 by physiological stimulators, such as luminal flow, endothelin 1, and angiotensin II, is mediated by phosphatidylinositol 3-kinase and phosphatidylinositol 3,4,5-triphosphate (PIP3). NOS3 activity is also modulated by hormones, cytokines, mechanical stress, and oxygen tension. Changes in NOS3 activity can be caused by changes in NOS3 expression, phosphorylation, or both. Because reduced NOS3 levels result in enhanced Na⁺ retention by the kidney and elevated blood pressure, studying the factors that decrease NOS3 expression is of physiological relevance.

Tumor necrosis factor α (TNF-α) is a proinflammatory cytokine elevated in hypertension, and heart failure. Angiotensin II stimulates TNF-α release by THALs, and TNF-α mediates part of the increase in blood pressure in angiotensin II–dependent hypertension. TNF-α also plays a role in the elevation of blood pressure caused by insulin resistance and systemic lupus erythematosus. Part of the prohypertensive actions of chronic elevations of TNF-α appears to be attributed to reductions in NO produced by NOS3. TNF-α decreases NOS3 expression in adipocytes, myocytes, and endothelial cells. However, whether TNF-α reduces NOS3 expression in the THAL has not been studied.

TNF-α activates several intracellular signaling cascades. These include protein kinase C (PKC), myosin light chain kinase, mitogen-activated protein kinase kinase (MAPKK), c-Jun amino terminal kinase (JNK), and Rac-1. Contrary to the chronic effects, acute treatment with TNF-α increases NO production and enhances the activity of Rho GTPase and Rho kinase (ROCK). The latter 2 pathways can reduce NOS3 expression. On one hand, we have shown that angiotensin II...
decreases NOS3 expression in THALs via peroxynitrite, and thus, its inhibition depends on NO. On the other hand, hypoxia and thrombin reduce NOS3 expression via Rho/ROCK in endothelial cells. Reductions in NOS3 expression by high glucose were prevented by blocking both peroxynitrite and ROCK activity in endothelial cells. Therefore, we hypothesized that TNF-α decreases NOS3 expression via Rho/ROCK in THALs.

**Methods**

**Primary Cultures of Medullary THALs**

All of the protocols involving animals were approved by the institutional animal care and use committee of Henry Ford Hospital. The composition of physiological saline used was (in mmol/L) 130.0 NaCl, 2.5 NaH2PO4, 4.0 KCl, 1.2 MgSO4, 6.0 L-alanine, 1.0 trisodium citrate, 5.5 glucose, 2.0 calcium dillactate, and 10.0 HEPES. The solution was adjusted to 320±3 mosmol/kg of H2O with mannitol and was pH 7.4 at room temperature. Rat medullary THAL (mTHAL) primary cultures were generated as described previously. In the first protocol, 40 hours after cells were seeded, they were treated either with vehicle (DMEM/F12 medium) or 1 nmol/L of TNF-α (Sigma) for 24 hours. In subsequent experiments, mTHALs were seeded in 4 wells, including vehicle, TNF-α, inhibitor alone, and inhibitor plus TNF-α. Cells were preincubated with the desired inhibitor or vehicle 1 hour before adding vehicle or TNF-α. When Rho was inhibited, cells were pretreated with exoenzyme C3 transferase or vehicle for 12 hours before stimulating them with TNF-α.

**Drugs Concentration and Source**

Please see online-only Data Supplement.

**Western Blot Analysis**

NOS3 expression was measured as described previously with some modifications (please see the online-only Data Supplement). One set of samples (ie, vehicle, TNF-α, inhibitor, and inhibitor plus TNF-α) was loaded using a single gel so that each experiment had its own control.

**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 mTHAL suspensions were seeded in 24-well plates (150 μg per well) in DMEM/F12 medium. Tubules were incubated at 37°C and 95%5% O2/CO2 for 4 hours. Tubules were then treated for 0 or 10 minutes with 1 nmol/L of TNF-α, and RhoA activity was measured as described by the manufacturer protocol and detailed in the online-only Data Supplement.

**Measurement of NO Production by Fluorescence Microscopy**

NO was measured before and after treatment with PIP3 in mTHAL cells cultured on glass coverslips and treated previously with either vehicle or TNF-α for 24 hours (please see the online-only Data Supplement).

In experiments where the acute effect of TNF-α on NO production was measured, mTHALs were isolated from 100- to 150-g male Sprague-Dawley rats, as described previously. 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 and detailed in the online-only Data Supplement.

**Statistical Analysis**

Results are expressed as percentage of control±SE. Data were analyzed by the Henry Ford Hospital Biostatistics and Research Epidemiology Department. In some experiments, ANOVA was used with post hoc testing. When multiple pairwise comparisons were done, a procedure for multiple tests of significance was applied using Hochberg significance limits.

**Results**

To begin testing our hypothesis that TNF-α decreases NOS3 expression in mTHALs, we first treated rat mTHAL primary cultures with either vehicle or 1 nmol/L of TNF-α for 24 hours. TNF-α reduced NOS3 expression by 51±6% (Figure 1; n=5; P<0.002) compared with controls. When corrected by β-tubulin, the effect of TNF-α was 51±8% (n=5, P<0.003). These data indicate that TNF-α decreases NOS3 expression in mTHALs.

Next, we tested whether TNF-induced decreases in NOS3 expression resulted in impaired NO production. PIP3 was used to stimulate NOS3. In vehicle-treated cells, NO production increased from 4.85±0.56 fluorescence units (FU)/min to 7.01±0.68 FU/min in response to PIP3 (P<0.02; n=6; Figure 2A). In contrast, in cells treated for 24 hours with TNF-α, PIP3 did not significantly increase NO production (basal: 5.24±0.75 versus PIP3: 5.69±0.86; n=6; Figure 2B). These data indicate that chronic exposure to TNF-α reduces stimulus-induced NO production by mTHALs.

TNF-α has been shown to activate RhoA GTPase in tubular and endothelial cells. Therefore, we next tested whether TNF-α increased RhoA activity in mTHALs. Basal RhoA activity was 0.520±0.038 OD, and acute treatment with TNF-α (10 minutes) increased RhoA activity to 0.816±0.090 OD (Δ=0±0.23%; n=4; P<0.04; Figure 3). These data indicate that TNF-α stimulates RhoA GTPase activity in mTHALs. Therefore, we tested whether TNF-α decreased NOS3 expression via Rho by incubating cells with 0.05 μg/mL of exoenzyme C3 transferase. Figure 4 shows that Rho inhibition blocked TNF-α–induced decreases in NOS3 expression by 30±8% (n=7; P<0.02 versus TNF-α), whereas treatment with C3 exoenzyme did not significantly affect basal NOS3 expression (Δ=14±15% versus vehicle; n=7). These data suggest that TNF-α reduces NOS3 expression via Rho GTPase.

Active Rho binds and activates ROCK; thus, we tested whether ROCK mediated the effect of TNF-α on NOS3 expression.

**Figure 1.** Effect of tumor necrosis factor α (TNF-α) in NO synthase (NOS) 3 expression in medullary thick ascending limbs (mTHALs). Top, Representative Western blot for NOS3 and the loading control β-tubulin. Bottom, Cumulative data (n=5).
expression. Figure 5 shows that TNF-α alone decreased NOS3 expression by 56±7% (n=6; P<0.001). Inhibition of ROCK with 10 µmol/L of H-1152 blocked TNF-α–induced reductions in NOS3 expression by 66±15% (n=6; P<0.01 versus TNF-α alone). ROCK inhibitor alone did not significantly affect basal NOS3 expression (Δ=19±11% versus vehicle; n=6). Lower and higher concentrations of the inhibitor were tested; however, the ability to blunt TNF-α–induced inhibition was the same. These data indicate that TNF-α reduces NOS3 expression in part via activation of ROCK. In addition, concomitant inhibition of RhoA and ROCK did not have an additive effect, indicating that RhoA and ROCK are part of the same signaling cascade (please see the online-only Data Supplement).

NO can decrease NOS3 expression via peroxynitrite formation.31 Therefore, we next tested whether TNF-α acutely increased NO production by isolated mTHALs. Treatment with TNF-α increased NO production from −0.153±0.116 FU/min to 0.408±0.071 FU/min (Figure 6; n=5; P<0.02), whereas vehicle treatment did not significantly affect NO production (basal NO production, −0.020±0.103 FU/min; vehicle treatment, 0.065±0.040 FU/min; n=4). These data indicate that, similar to angiotensin II, TNF-α acutely stimulates NO production by mTHALs.

Next, we tested whether the TNF-α–induced reductions in NOS3 expression were mediated by NO by inhibiting NO production with Nω-nitro-l-arginine methyl ester. TNF-α alone decreased NOS3 expression by 48±6% (n=4; P<0.001 versus vehicle); however, Nω-nitro-l-arginine methyl ester (4 mmol/L) did not block the effect of TNF-α on NOS3 expression (Δ=−43±5% versus vehicle; Figure 7). Nω-nitro-l-arginine methyl ester treatment did not affect basal NOS3 expression. Thus, NO, and, therefore, peroxynitrite, did not appear to mediate TNF-α–induced reductions in NOS3 expression.

Finally, we tested a number of other signaling cascades. These pathways have been shown to be activated by TNF-α or to play a role in NOS3 expression. Inhibition of myosin light chain kinase, PKC, MAPKK, JNK, Janus kinase, or Rac-1 did not prevent TNF-α from decreasing NOS3 expression (please see the online-only Data Supplement).

**Discussion**

We hypothesized that TNF-α decreases NOS3 expression via RhoA GTPase and its associated ROCK in mTHALs. We found the following: (1) chronically, TNF-α decreases NOS3 expression and NO production in rat mTHAL cells; (2) acutely, TNF-α activates RhoA GTPase; (3) two thirds of the TNF-α–induced decrease in NOS3 expression was mediated by the Rho/ROCK pathway; and (4) none of the several other signaling molecules that we tested could account for the remaining third, including NO, myosin light chain kinase, PKC, MAPKK, JAK, JNK, and Rac-1.
We used primary cultures of mTHALs to avoid the systemic effects of TNF-α that could confound our interpretation of the data. The concentration of TNF-α in the interstitium of the outer medulla under physiological and pathophysiological conditions is unknown. Thus, we used 1 nmol/L of TNF-α, which is approximately the concentration produced by THALs stimulated with angiotensin II in vitro. 

All of the experiments using inhibitors had their own paired controls to account for anticipated variability in the effect of TNF-α on NOS3 expression in primary cultures generated from different rats at different times.

The effect of TNF-α on NOS3 expression in mTHAL cells is similar to those shown by other investigators studying different tissues. Valerio et al.22 reported that obesity caused a reduction in NOS3 expression in white and brown fat tissue, as well as in skeletal muscle attributed to TNF-α. Agnoletti et al.16 demonstrated that serum from patients with severe heart failure decreased NOS3 expression and NO production by mTHALs stimulated with angiotensin II in vitro.17

In mTHALs, increases in NO production by luminal flow, endothelin 1, and angiotensin II are mediated by NOS3 and depend on phosphatidylinositol 3-kinase, PIP3, and Akt.5–7 In line with the reduced NOS3 expression, PI3K-induced increases in NO production were blunted in mTHAL cells treated with TNF-α for 24 hours. These data indicate that chronic exposure to elevated levels of TNF-α decreases NOS3 expression and blunts the ability of physiological stimuli to increase NO production. These results are in agreement with those found by Valerio et al.22 and Goodwin et al.32 in which TNF-α decreased NOS3 expression and NO production in fat, muscle, and endothelial cells.

Although the effect of TNF-α on NOS3 expression in nonrenal cells has been widely tested, data addressing the signaling pathway by which TNF-α impairs NOS3 expression are scare. Here we show for the first time that Rho/ROCK mediates most of the inhibitory effect of TNF-α on NOS3 expression in mTHALs. This conclusion was supported by 4 lines of evidence. First, TNF-α increased RhoA GTPase activity, as shown by the increase in GTP-bound RhoA levels. Second, exoenzyme C3 transferase, an
inhibitor of Rho, blunted the inhibitory effect of TNF-α. Third, a ROCK inhibitor reduced the effect of TNF-α by ≈70%. Finally concomitant inhibition of RhoA and ROCK had no additive effect.

Our finding that ROCK mediates the effects of TNF-α on NOS3 expression is unique. Although ROCK mediates the decrease in NOS3 expression induced by high glucose, hypoxia, and thrombin in endothelial cells, there have been no reports of this kinase mediating TNF-α–induced reductions in NOS3 expression. Thus, our data suggest that TNF-α, high glucose, hypoxia, and thrombin use ROCK as a common pathway to decrease NOS3 expression.

The fact that ROCK inhibition only accounted for 66% of the effect of TNF-α on NOS3 expression is unlikely to be attributed to too low of a concentration of the inhibitor. We used a concentration that is several times higher than the inhibition constant, and higher concentrations induced no further blockade. To achieve maximal ROCK inhibition, we used the compound H-1152. H-1152 has been shown to be an inhibitor of Rho, blunted the inhibitory effect of TNF-α on NOS3 expression but did not prevent TNF-α from reducing NOS3 expression in mTHALs. These results are similar to those found in endothelial cells, where NO inhibition did not block TNF-α–induced destabilization of NOS3 mRNA nor TNF-α–induced reduction in NOS3 promoter activity. However, El-Remessy et al showed that high glucose and diabetes mellitus reduced NOS3 expression via peroxynitrite and ROCK, and both conditions have been shown to increase TNF-α levels. Therefore, it is possible that high glucose and angiotensin II enhance peroxynitrite formation, leading to an increase in TNF-α release, which, in turn, reduces NOS3 expression primarily via Rho/ROCK.

TNF-α can activate a number of other signaling cascades. Consequently, we tested several of them in a vain attempt to characterize the remaining 34% of the actions of TNF-α that were ROCK independent. MAPKK, PKC, Rac-1, JAK and JNK did not appear to play a role in TNF-α–induced reductions in NOS3 expression.

In conclusion, we found that TNF-α reduces NOS3 expression primarily via RhoA GTPase and ROCK in the mTHAL. Myosin light chain kinase, NO, PKC, MAPKK, JAK, JNK, and Rac-1 were not involved.

Perspectives

A correlation between TNF-α levels and elevated blood pressure has been demonstrated in humans and in animals. In rodents, TNF-α blockade with pharmacological blockers or genetic manipulations blocks or delays the progression of hypertension in angiotensin II–infused animals, systemic lupus erythematosus, insulin resistance, and preeclampsia. TNF-α–neutralizing drugs like etanercept and infliximab are available for patients with chronic inflammatory diseases, and, thus, the effect of TNF-α blockade on hypertension in humans could be studied. On the other hand, ROCK inhibitors like fasudil are currently used to treat pulmonary hypertension and cerebral vasospasm and have beneficial effects on patients with systemic hypertension and chronic heart failure. However, most of the studies using ROCK inhibitors have been focused on the vascular effects of those compounds, whereas the effects on the kidney remain largely unexplored. Our results indicate that these drugs would improve renal function and decrease blood pressure by mitigating the effect of TNF-α on NOS3 expression, elevating NO levels and, thus, enhancing natriuresis.

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Disclosures

None.

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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) for 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 Ca2+ (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 production11. 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 described12-14. 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 laboratory12-14. 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 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 alanine, 1 Na3 citrate, 5.5 glucose, 2 Ca2+ (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\textsuperscript{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\textsuperscript{16}. Similarly, ROCK has been reportedly shown to be activated independently of RhoA\textsuperscript{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\textsuperscript{19}. This effect seems to depend upon a) activation of myosin light chain kinase (MLCK) and b) ROCK-induced inhibition of MLC phosphatase\textsuperscript{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 ($\Delta = 14 \pm 9 \%$, 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 ($\Delta = -56 \pm 9\%$, 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 ($\Delta =-60 \pm 4\%$, n=3 p<0.005 vs PD98059 alone and $\Delta =-70 \pm 11\%$, 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 and to increase NOS3 expression. Furthermore, inhibition of ROCK can activate Rac-1. 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 $\Delta =-48\pm6\%$ p<0.001 vs vehicle and TNF-α + Rac-1 inhibitor $\Delta = -47\pm10$, 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 and prevented the increase in blood pressure in a model of lupus erythematosus. Similarly, administration of angiotensin II to TNF-α knock out mice failed to elicit the increase in blood pressure observed in wild type animals. In addition, patients with rheumatoid arthritis receiving anti-TNF therapy showed reduced blood pressure and improved endothelial dependent vasodilatation. However, infusion of TNF-α in anesthetized rats induced natriuresis and a decrease in blood pressure and infusion of recombinant
TNF-α in cancer patients (an old treatment) resulted in a decrease in blood pressure within a couple of hours\textsuperscript{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\textsuperscript{30,31} to 50 µg/mL\textsuperscript{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 that 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-disassociation inhibitors (GDIs)\textsuperscript{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\textsuperscript{19}. In LLC-PK cells, GEF-H1 mediates TNF-α-induced activation of RhoA and increases in paracellular permeability\textsuperscript{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\textsuperscript{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\textsuperscript{17} or by cleavage of the carboxy terminus by caspase-3\textsuperscript{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\textsuperscript{36-38}. In particular, translation elongation factor 1 a (EF1-a), a ribosomal protein, has been identified as a modulator of NOS3 mRNA stability\textsuperscript{38}. ROCK has been shown to phosphorylate EF1-a and to decrease EF-1a binding to filamentous actin\textsuperscript{39} whereas in pulmonary endothelial cells ROCK reduce NOS3 mRNA half-life\textsuperscript{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\textsuperscript{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-\(\alpha\) treatment\textsuperscript{42}. However, to our knowledge a role of Rho/ROCK in decreasing NOS3 promoter activity has not been shown.
References


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).
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).