Tumor necrosis factor-α reduces argininosuccinate synthase expression and nitric oxide production in aortic endothelial cells

Bonnie L. Goodwin, Laura C. Pendleton, Monique M. Levy, Larry P. Solomonson, and Duane C. Eichler

Department of Molecular Medicine and Johnnie B. Byrd, Sr. Alzheimer’s Center and Research Institute, University of South Florida, College of Medicine, Tampa, Florida

Submitted 9 October 2006; accepted in final form 9 May 2007

Goodwin BL, Pendleton LC, Levy MM, Solomonson LP, Eichler DC. Tumor necrosis factor-α reduces argininosuccinate synthase expression and nitric oxide production in aortic endothelial cells. Am J Physiol Heart Circ Physiol 293; H1115–H1121, 2007. First published May 11, 2007; doi:10.1152/ajpheart.01100.2006.—Endothelial dysfunction associated with elevated serum levels of TNF-α observed in diabetes, obesity, and congenital heart disease results, in part, from the impaired production of endothelial nitric oxide (NO). Cellular NO production depends absolutely on the availability of arginine, substrate of endothelial nitric oxide synthase (eNOS). In this report, evidence is provided demonstrating that treatment with TNF-α (10 ng/ml) suppresses not only eNOS expression but also the availability of arginine via the coordinate suppression of argininosuccinate synthase (AS) expression in aortic endothelial cells. Western blot and real-time RT-PCR demonstrated a significant and dose-dependent reduction of AS protein and mRNA when treated with TNF-α with a corresponding decrease in NO production. Reporter gene analysis demonstrated that TNF-α suppresses the AS proximal promoter, and EMSA analysis showed reduced binding to three essential Sp1 elements. Inhibitor studies suggested that the repression of AS expression by TNF-α may be mediated, in part, via the NF-κB signaling pathway. These findings demonstrate that TNF-α coordinately down-regulates eNOS and AS expression, resulting in a severely impaired citrulline-NO cycle. The downregulation of AS by TNF-α is an added insult to endothelial function because of its important role in NO production and in endothelial viability.

The endothelium plays a crucial role in the maintenance of vascular tone and structure. The major endothelium-derived vasoactive mediator is nitric oxide (NO), which is formed from the amino acid precursor l-arginine by NO synthase. NO is involved in a wide variety of regulatory mechanisms of the cardiovascular system, including vascular tone (e.g., it is the major mediator of endothelium-dependent vasodilation), vascular structure (e.g., inhibition of smooth muscle cell proliferation), and cell-cell interactions in blood vessels (e.g., inhibition of platelet adhesion and aggregation, inhibition of monocyte adhesion) (14, 23, 31). Because of these functions, NO has been designated as an endogenous antithrombotic molecule (17, 21, 25).

Dysfunction of the endothelial citrulline-NO cycle is a common mechanism by which several cardiovascular risk factors mediate their deleterious effects on the vascular wall (17, 21, 25, 39). These risk factors include hypercholesterolemia, hypertension, smoking, diabetes mellitus, homocysteine, and vascular inflammation (17, 21, 25, 39). The multifunctional cytokine TNF-α has been linked to endothelial dysfunction in Type 2 diabetes (35), obesity (41), and congenital heart failure (1, 12). Clinical studies have shown that elevated levels of plasma TNF-α in patients with Type 1 diabetes are associated with cardiological risk factors (24). In vivo studies have also revealed that administration of TNF-α depresses endothelium-dependent relaxation (40) and reduces levels of endothelial NO (20). The impairment of endothelial NO production by TNF-α was initially linked to the reduction in endothelial nitric oxide synthase (eNOS) expression in bovine aortic endothelial cells (BAECs) (2, 4, 27, 44). Therefore, it is not surprising that impairment in endothelial NO production leads to endothelial dysfunction. In fact, loss of endothelium-derived NO is associated with the prothrombotic and hyperproliferative states present in hypertension, diabetes, and atherosclerosis (10).

Although the production of NO is directly related to the enzyme eNOS, overall production of NO by endothelial cells has been shown to depend on a functional citrulline-NO cycle (16, 18, 42, 43). Both NO and citrulline are generated from arginine by eNOS. NO is utilized in signaling, whereas citrulline is recycled back to arginine by two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase, to complete the cycle. AS catalyzes the rate-limiting step in the arginine regeneration side of the citrulline-NO cycle (42) and appears to be coordinately regulated with eNOS activity (10, 30).

Recent results from our laboratory using small interfering RNA knockdown of AS expression demonstrated that AS is important for both basal and stimulated endothelial NO production, even in the presence of excess arginine, as well as for maintenance of endothelial viability (16). A recent study of transgenic rat blood-brain barrier endothelial cells supported our conclusions that regeneration of citrulline from intracellular arginine via AS provides the major arginine pool for stimulated NO production (37). Collectively, these results demonstrated the key role of AS in endothelial NO production and endothelial cell viability. Thus our work and the work from other laboratories have developed strong evidence supporting the proposal that substrate availability, governed by arginine regeneration as part of the citrulline-NO cycle, plays an important and possibly essential role in NO production, thus affecting vascular endothelial function and viability (16, 18, 42, 43).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
In this report, we demonstrate that TNF-α, which represses NO production in endothelial cells, does so not only by downregulating eNOS expression but also by suppressing the availability of arginine. Moreover, evidence is provided that the mechanism by which TNF-α transcriptionally represses eNOS expression is mimicked in the downregulation of AS expression through similar transcriptional factors. Thus TNF-α depresses endothelial NO production via the coordinate downregulation of both eNOS and AS.

EXPERIMENTAL PROCEDURES

Cell culture. BAECs were cultured in DMEM (1 g/l glucose; Mediatech) containing 10% FBS (Hyclone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech) at 37°C and in an atmosphere of 5% CO2. For cytokine treatment, cells in control medium were treated with the indicated concentrations of TNF-α up to 48 h.

Cell lysate preparation and immunoblotting. Cell lysates were prepared as previously described (16). Protein (10 μg) was resolved on 4–15% polyacrylamide gels (Bio-Rad) and blotted onto polyvinylidene difluoride membranes (Immobilon-P) as previously described (33). Primary antibodies used included: 1:2,500 anti-AS (BD Transduction Laboratories), 1:1,000 anti-eNOS (Abcam), 1:7,500 anti-Actin (Sigma), and 1:1,000 anti-eGAPDH (Transduction Laboratories).

RNA isolation and quantitative RT-PCR. Total RNA was isolated with TriReagent according to the manufacturer’s recommendations (Sigma). RNA was treated with DNase (Ambion) and quantitated with TriReagent according to the manufacturer’s recommendations.

Luciferase assay analysis. Luciferase and renilla activities were measured as described (33). Experiments were carried out three times in triplicate.

Nuclear extract preparation. Nuclear extracts were prepared from BAECs as described previously (45) with the following modifications. Cells were plated in 10-cm dishes and treated once they reached confluence. The culture monolayer was rinsed twice with PBS, once with PBS containing 1 mM Na3VO4 and 5 mM NaF, and once with 1× hypotonic buffer [20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na3VO4, 1 mM Na2PO4, 1 mM DTT, and 1× protease inhibitors (Calbiochem)]. Cells were scrapped into 1× hypotonic buffer containing 0.2% Nonidet P-40 and resuspended by gentle pipetting. Samples were centrifuged for 20 s at 12,000 g at 4°C. The nuclear pellet was resuspended in high-salt buffer [in mM: 420 NaCl, 20 HEPES, pH 7.9, 1 EDTA, 1 EGTA, 20% glycerol, 20 NaF, 1 Na3VO4, 1 Na2PO4, 1 DTT, and 1× protease inhibitors (Calbiochem)] and rotated for 30 min at 4°C. Samples were centrifuged at 12,000 g, 4°C for 20 min.

EMSA. Nuclear extracts were combined with or without cold competitors or specific antibodies and incubated for 20 min at room temperature. Probes were labeled by combining equimolar amounts of complementary oligonucleotides (2 × 10-10 mol of each), which were heated to 70°C and allowed to cool to room temperature slowly. The oligonucleotides were labeled with 10 μl of [α-32P]dCTP (3,000 Ci/mmol) and Klenow enzyme, and unincorporated label was removed by Nuc Away (Ambion). The reaction mixture, composed of

serum-free medium (33). Luciferase and renilla activities were measured as described (33). Experiments were carried out three times in triplicate.

Luciferase assay analysis. BAECs were plated at 2 × 104 cells per well in a 24-well plate 24 h before transfection. pRL-TK, a renilla expression vector, was used as an internal transfection control where indicated. Experimental plasmids (200 ng each) and pRL-TK (50 ng) were transiently transfected into BAEC using TransIt-LTI (Mirus) in

AJP-Heart Circ Physiol • VOL 293 • AUGUST 2007 • www.ajpheart.org
TNF-α DOWNREGULATES THE CITRULLINE-NO CYCLE

H1117

binding buffer [10 mM HEPES, pH 7.9, 10% glycerol, 1 mM DTT, 0.1 μg/μl poly(dI:dC), 0.5 μg/μl BSA and 4,000 dpm/μl radiolabeled probe] and nuclear extract (5 μg) in a total volume of 10 μl was incubated at 30°C for 30 min. Samples were loaded onto a 5% polyacrylamide gel and run at 180 V. Gels were dried under vacuum and exposed to film. The oligonucleotides for EMSA analysis included Sp1 site 1 (5'-GCTCCAGGGGCGGGCCGGGCGGGGCGGGC-3'), Sp1 site 2 (5'-GGCCCGGGCCGGCCGGGCGGGTCTGTGGCCGGC-3'), and Sp1 site 3 (5'-CCGGTCAACGCGGCTGCCCCCGGGCCCTG-3'). The antibodies used were Sp1, Sp3, and Egr1, as a control (Santa Cruz).

**Results**

**Coordinate reduction of AS and eNOS expression by TNF-α.** Previous results have indicated that the expression of AS is required for NO production and also for endothelial cell viability (16, 18, 42, 43). Therefore, we investigated whether AS expression was coordinately downregulated with eNOS by TNF-α. Initially, conditions were chosen that were known to suppress eNOS expression in BAECs (4, 36, 46). BAECs were treated with 10 ng/ml TNF-α and subsequently lysed and analyzed by Western blotting. As shown in Fig. 1, A and B, TNF-α treatment resulted in a significant reduction of both eNOS and AS protein expression. This concentration of TNF-α reasonably mimics serum levels found in the inflammatory environment of chronic disease states such as diabetes and obesity and does not reduce endothelial cell viability (17, 19, 29, 47).

To examine whether the decrease in AS protein that resulted from TNF-α treatment correlated with a reduction in steady-state AS and eNOS mRNA levels, total RNA was isolated and quantitated by real-time RT-PCR. As shown in Fig. 1C, TNF-α treatment resulted in a 74% reduction of AS mRNA and a 70% reduction in eNOS mRNA (P < 0.0004).

Consistent with the reduction in both AS and eNOS protein expression, TNF-α reduced bradykinin-stimulated NO levels by 70%, whereas the eNOS inhibitor nitro-L-arginine methyl ester reduced NO levels by 93% (Fig. 2). NO production in treated BAECs was measured as nitrite by a fluorescent assay.
nmol nitrite per million cells were calculated using a standard curve (26), with nitrite levels normalized to the number of cells counted by trypan blue exclusion to account for cell viability.

Regulation of the proximal AS promoter by TNF-α. To assess the effect of TNF-α on AS promoter activity, the reporter construct p3ASP189 was used. Plasmid constructs were transiently transfected into BAECs and treated for 48 h with increasing concentrations (0–10 ng/ml) of TNF-α. Luciferase expression was measured as relative luciferase units, and data are presented as relative change in expression. As shown in Fig. 3, TNF-α treatment resulted in a dose-dependent decrease in luciferase activity.

Anderson et al. (4) showed that TNF-α mediated transcriptional suppression of eNOS via two Sp-binding sites positioned in the proximal eNOS promoter. This finding, in addition to a report that characterized an essential role for Sp in AS expression in the human carcinoma cell line RPMI2650 (3), led us to hypothesize that both AS and eNOS may be coordinately downregulated in endothelial cells by TNF-α through a similar transcriptional mechanism. To support this hypothesis, a detailed reporter gene and mutational analyses were carried out in endothelial cells, focusing on the three Sp sites in the proximal AS promoter. As shown in Fig. 4, mutation of two of the Sp sites (M1 and M2) restored luciferase activity to a small extent, whereas mutation of Sp site 3 (M3) resulted in complete ablation of the TNF-α effects (Fig. 4). It is interesting to note that mutation of this site also reduced the promoter activity to <4% (data not shown). This is similar to the situation that has been identified in the eNOS promoter (4) where TNF-α acted to suppress promoter activity by reducing binding to an Sp1 site that is essential to basal eNOS promoter activity.

Fig. 5. TNF-α reduces binding to Sp1 sites. Supershift analysis of Sp1 sites 1 (A), 2 (B), and 3 (C) show that Sp3 binds to all 3 promoters, whereas Sp1 also binds to site 3. D–F: EMSAs were performed with nuclear extracts prepared from BAECs treated with 10 ng/ml TNF-α for 0, 15, 30, and 60 min and probes for Sp1 site 1, 2, and 3 as indicated. Probe only is presented in lane 1 (P). Oligonucleotide probes used contained the 3 Sp1 elements of the proximal AS promoter sequence: site 1 (A), site 2 (B), site 3 (C). Spot density for each graph is presented in G–I. N, without antibody.

AJP-Heart Circ Physiol • VOL 293 • AUGUST 2007 • www.ajpheart.org
TNF-α reduces Sp1 binding to AS promoter elements.

EMSA was carried out with labeled oligonucleotides for each of the three Sp1 sequences combined with untreated nuclear extracts. Supershift studies using antibodies directed against Sp1, Sp3, and Egr-1 indicated that Sp3 bound to all three elements, whereas Sp1 bound to site 3 only (Fig. 5, A–C). To investigate the effect of TNF-α on the binding of Sp1/3 factors to the basal promoter, BAECs were cultured in the presence or absence of 10 ng/ml TNF-α for 15–60 min, and nuclear extracts were isolated. EMSA was carried out with labeled oligonucleotides for each of the three Sp sequences combined with treated and untreated nuclear extracts. TNF-α reduced formation of the Sp complex at all three sites by 60 min, indicating that a decrease in binding was responsible for the downregulation of the promoter without a change in factor binding to that site. As shown in Fig. 5, there was a rapid decrease in binding to site 1 and an initial increase, followed by a time-dependent decrease in binding to Sp3 site 2. An initial broadening of the band followed by a slower decrease to the essential Sp1/3 binding site 3 probes in BAEC nuclear extracts exposed to TNF-α treatment indicates a second binding factor may be involved in regulation of this essential element. These results show that all three elements that bind Sp1/3 transcription factors (3) are affected by TNF-α treatment.

Role of NF-κB in signaling TNF-α-mediated suppression of AS.

The proinflammatory effects of TNF-α are mediated through activation of the NF-κB (6). The phosphorylation of IkB, an inhibitory protein that complexes with NF-κB in the cytoplasm, targets IkB for degradation (11) and results in an increased nuclear localization of NF-κB and subsequent gene regulation. Previous work with BAECs has demonstrated TNF-α-mediated, time-dependent reduction in IkBα, reflecting degradation of the inhibitor and activation of the NF-κB signaling pathway (4). TNF-α has also been shown to stimulate the translocation of NF-κB to the nucleus in BAEC (15). To test for the involvement of NF-κB in signaling the TNF-α-dependent downregulation of AS expression, endothelial cells were treated with TNF-α in the presence or absence of the NF-κB inhibitor BAY-7082. Whole cell lysates were isolated and subjected to Western blot analysis. Figure 6, A and B, shows that NF-κB inhibitors blocked the downregulation of AS expression by TNF-α. To investigate this further, BAECs were transiently transfected with the vector pGL3-AS189 and treated with TNF-α in the presence and absence of the inhib-
itor. Treatment with BAY-7082 inhibited the suppression of the AS promoter activity by TNF-α (Fig. 6C), further supporting the role of NF-κB in signaling the TNF-α-mediated transcriptional suppression of AS expression.

**DISCUSSION**

Recent reports by our laboratory and others have sparked new interest relative to the function and regulation of AS in endothelial NO production (16, 37, 42). As reported previously (13, 16, 42), AS is important for the regeneration of arginine required for endothelial NO production, even in the presence of excess exogenous arginine. Thus the capacity of endothelial cells to sustain NO production is severely limited not only by the downregulation of eNOS (2, 4, 36) but also by the availability of arginine provided by AS via the citrulline-NO cycle. Coordinate upregulation of AS and eNOS expression has been identified previously in a number of systems (10, 30). For example, AS and eNOS are coordinately induced in the aorta of diabetic rats after streptozotocin treatment (30). TGF-β1 induces both enzymes in human umbilical vein endothelial cells (30). In addition, fluid shear stress induces both AS and eNOS mRNA expression (10). In the present study, both eNOS and AS expression were found to be coordinately downregulated by TNF-α in cultured BAECs. These results support a model whereby TNF-α reduces endothelial NO production by both a downregulation of eNOS expression and also by reduction in substrate availability via the downregulation of AS expression.

AS expression was significantly reduced at levels of TNF-α found in an inflammatory environment caused by chronic disease states such as diabetes and obesity. Not surprisingly, the loss of eNOS and AS expression was accompanied by a dramatic decrease in the capacity of the bradykinin-stimulated cells to produce NO in the presence of TNF-α. Transcriptionally, eNOS is downregulated via reduced binding to two Sp1 elements, which are required for basal promoter activity (4). A strikingly similar mechanism was identified for AS. TNF-α-mediated suppression of the AS promoter was found to be through Sp1 site 3, which is required for basal AS promoter activity. Site 3 was the most important site regulated by TNF-α, as is the case for eNOS.

We have shown that TNF-α regulates AS transcription through reduced binding to essential Sp1 binding sites consistent with the findings of Anderson and Freytag (3) showing that Sp1 elements in the proximal promoter are required for basal AS expression. More importantly, though, was our finding that the regulation of the AS promoter by TNF-α through an Sp1/3 element was similar to that reported for the TNF-α-mediated downregulation of the eNOS promoter (4). Therefore, the expressions of AS and eNOS, both essential components of the citrulline-NO cycle, are coordinately downregulated by TNF-α through decreased binding at a proximal Sp3 element that is required for basal promoter activity for both genes (4).

In cultured endothelial cells, NF-κB resides in the cytoplasm and is associated with an inhibitory protein, IκB (5, 7). Inflammatory cytokines, such as TNF-α, activate IKKs, which phosphorylate IκB, targeting IκB for degradation. As a result, NF-κB translocates to the nucleus where it transcriptionally provokes a proinflammatory response. Previous work with BAECs showed that TNF-α signaled a time-dependent reduction in IκBα expression in endothelial cells (4), resulting in NF-κB-mediated inhibition of the eNOS promoter (4). More recently, activation of NF-κB by IL-1β was also shown to suppress AS expression at the transcriptional level via a functional NF-κB site (8). Interestingly, we did not find this NF-κB binding site to be necessary for the suppression of the proximal AS promoter by TNF-α, although binding to this element was increased by TNF-α treatment as indicated by gel-shift analysis (data not shown). Rather, we have shown that TNF-α regulates AS transcription through reduced binding of the transcription factors Sp1 and Sp3, consistent with the findings of Anderson and Freytag (3) showing that Sp1/3 elements in the proximal promoter are required for basal AS expression. More importantly, was our finding that the regulation of the AS promoter by TNF-α through an Sp1/3 element was similar to that reported for the TNF-α-mediated downregulation of the eNOS promoter (4). Therefore, the expressions of AS and eNOS, both essential components of the citrulline-NO cycle, are coordinately downregulated by TNF-α through decreased binding at a proximal Sp3 element that is required for basal promoter activity for both genes (4), and preliminary evidence indicates the involvement of the NF-κB signaling pathway in this effect.

TNF-α has been implicated in the pathogenesis of cardiovascular diseases such as congestive heart failure, acute myocardial infarction, myocarditis, and dilated cardiomyopathy (28). Serum TNF-α levels are elevated in patients with congestive heart failure (38). In fact, incubation of endothelial cells with serum from patients with congestive heart failure was shown to downregulate eNOS expression in a TNF-α-dependent manner (1). Other studies have shown that TNF-α administration in vivo depresses endothelium-dependent relaxation (40) and reduces levels of endothelial NO (20). The present results provide important new evidence as to how elevated levels of TNF-α in a disease state may promote endothelial dysfunction. This understanding may also suggest that drugs designed to reduce TNF-α expression may restore endothelial function at two levels: 1) by restoring substrate availability for NO production and 2) by restoring the enzyme, eNOS, which catalyzes the production of NO.

**GRANTS**

This work was supported by American Heart Association, Florida Affiliate, Grant 0455228B.

**REFERENCES**

TFN-α DOWNREGULATES THE CITRULLINE-NO CYCLE

H1121


