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FORUM ORIGINAL RESEARCH COMMUNICATION

Translation-Linked mRNA Destabilization Accompanying Serum-Induced Nox4 Expression in Human Endothelial Cells

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Abstract

NADPH oxidase is involved in cell signaling, regulating proliferation of vascular cells, especially in endothelium. The Nox4 catalytic subunit has a major role in endothelial cells, but growth arrest of cultured endothelial cells following serum deprivation paradoxically increases mRNA for Nox4. We investigated the relationships between Nox4 mRNA stability and protein expression in human microvascular endothelial cells. Serum starvation increased the steady-state level of Nox4 mRNA but paradoxically diminished Nox4 protein expression. mRNA transcription in the absence of serum is maintained by the p38MAP kinase pathway, for inhibition of p38MAP kinase reduced both Nox4 mRNA and Nox4 promoter activity. In serum-starved cells, reintroduction of serum increased Nox4 protein levels within 30 min and up to 24 h. In contrast, the mRNA decreased equally rapidly after serum stimulation. Inhibition of Nox4 translation by cycloheximide blocked serum-induced mRNA degradation and Nox4 protein synthesis, and actinomycin-D also delayed Nox4 mRNA decay. Therefore, Nox4 mRNA level falls after serum stimulation because of a translation-initiated mRNA destabilization program. Clearly Nox4 mRNA is regulated at both transcriptional and post-transcriptional levels, and the steady state level of Nox4 mRNA does not accurately reflect Nox4 protein abundance and functions, with implications for regulation of cell proliferation and survival. *Antioxid. Redox Signal.* 11, 2399–2408.

Introduction

The NADPH oxidase Nox4 catalytic subunit is highly expressed in a wide range of cells, including vascular (13, 22, 33) and airway smooth muscle cells (41), endothelial cells (1, 23), cardiac fibroblasts (10), kidney cells (14, 17), adipocytes (29), neurons (42), osteoclasts (48), chondrocytes (18), certain tumor cells (9), and embryonic stem cells (28). This expression pattern indicates that this enzyme may have a fundamental role in modulating basic cellular functions. Indeed, several lines of evidence suggest that Nox4 is involved in modulating cell biological processes such as proliferation and differentiation.

For example, in both human airway and pulmonary artery smooth muscle cells, transforming growth factor-β1-induced cell proliferation was partially inhibited by Nox4 gene silencing (40, 41). In aortic smooth muscle cells, Menshikov and colleagues demonstrated that knockdown of Nox4 expression significantly inhibited urokinase plasminogen activator-stimulated reactive oxygen species (ROS) production and cell proliferation (32). We and others have observed similar effects of Nox4 on cell proliferation and migration in vascular endothelial cells (8, 12, 35). All these data suggest that Nox4-dependent redox mechanisms may have important roles in promoting cellular proliferation and angiogenesis (8, 12, 47). Moreover, Nox4 might also be involved in modulating cellular differentiation (11, 28).

Gene expression may be regulated at the transcriptional level and repression of translation is an important level of gene regulation. Due to translation inhibition, certain mRNAs exist at the cellular level without expression of protein product, for translation can be regulated by the untranslated region (UTR) of specific mRNA. It has been reported in previous studies that there was an inverse relationship between the mRNA level for Nox4 and the presence of serum in the culture medium. Specifically, Ago *et al*. found that when serum was removed from the culture medium for 24 h, the Nox4 mRNA increased 2- to 3-fold, whereas addition of serum reduced the Nox4 mRNA level to the basal level in 12 h (1). Similarly, in vascular smooth muscle cells, it was observed that incubation with 10% serum for 4 h decreased the Nox4 mRNA by 32%

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(27). We also observed a similar phenomenon in human microvascular endothelial cells (HMECs; see below). Given that serum is a potent mitogen, these results seem paradoxical to the pro-proliferating role of Nox4 in vascular cells, as discussed above.

Therefore, we have now examined the relationship between Nox4 transcription of mRNA and protein expression in response to serum in HMECs. Our results suggest that serum is an inducer of Nox4 expression in endothelial cells by promoting protein translation or reducing its degradation. Although serum may also enhance Nox4 transcription, the steady state level of Nox4 falls after serum stimulation because of a translation-initiated mRNA destabilization program.

Methods

Cell culture

HMECs are a gift from Professor Philip Hogg (University of New South Wales, Sydney, Australia). Cells were cultured in EGM-2 Bullet Kit with 10% fetal calf serum (Cambrex Corporation, Lonza, MD) in a 5% CO_2 incubator at 37°C.

Experimental condition

Cells were starved overnight and then treated with serum as described in figure legends.

Western blot analysis

Cells (5×10^5 cells/dish) cultured in 100 mm dishes were washed with cold PBS and lysed with 100 μl of cell lysis buffer (pH 7.5) containing 100 m*M* NaCl, 1% Triton X-100, 10 m*M* Tris, 2 m*M* EDTA, and a protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were centrifuged at 13,000 rpm at 4°C for 15 min and the supernatants were mixed with (6X) Laemmli buffer and boiled for 5 min. Equal amounts of protein were separated by 10% SDS-PAGE and transferred to Hybond nitrocellulose membranes (Amersham, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk powder in TBS (pH 7.5) and hybridized overnight at 4°C with primary antibody Nox4 (Santa Cruz, Santa Cruz, CA, 1:200) Nox2 (upstate, 1:1,000), β-actin (Sigma-Aldrich, 1:4,000), and SOD (Sigma-Aldrich, 1:1,000). Proteins were detected using enhanced chemiluminescence (ECL) with horseradish peroxidase conjugated to appropriate secondary antibodies (Amersham).

ROS measurement

ROS production from endothelial cells was measured by 2′,7′- dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) fluorescence, as described previously (34). Cells (10,000 cells/well) were loaded with $DCFH_2$-DA (10 μM) by incubating for 10 min. Fluorescence was then measured with excitation and emission at 480 nm and 530 nm respectively, using a Polarstar microplate reader (BMG Labtech, Offenburg, Germany) at 37°C.

Lucigenin-enhanced chemiluminescence

Complimentary to $DCFH_2$ -DA assay, we also measured superoxide anion by lucigenin-enhanced chemiluminescence as described previously (34).

Immunocytochemistry

HMECs were seeded (5×10^4 cells per well) in Lab-Tek 2-well chamber slides (Nunc, Roskilde, Denmark). After appropriate treatments, the cells were washed and fixed in cold methanol for 15 min, and air dried. The slides were blocked with 3% bovine serum albumin for 1 h at room temperature. Immunofluorescence staining was carried out by incubating slides with the goat anti-Nox4 antibody at 1:50 dilution in the blocking buffer at 4°C overnight. Alexa Fluor® 488 donkey anti-goat IgG (H + L) secondary antibody was used to detect Nox4. Images were taken by an Axioskop-2 fluorescence microscope (Axioskop-2 Zeiss, Gottingen, Germany) equipped with a CCD camera.

Real-time PCR

Cells (1.5×10^5 cells/well in 6-well plate) were washed with PBS and collected in 0.5 ml TriReagent (Ambion, Austin TX). The total RNA was extracted according to the manufacturer's instruction. Total RNA was reverse-transcribed to cDNA using random hexamer and TaqMan reverse transcription reagents (Applied Biosystems) at 48°C for 30 min, followed by 95°C for 5 min. Expression levels of human Nox4, and housekeeping GAPDH mRNAs were determined by using the specific primer as follows: forward Nox4 (5′-CAGAAG GTTCCAAGCAGGAG-3′) and reverse Nox4 (5′-GTTGAG GGCATTCACCAGAT-3′), and forward GAPDH (5′-GAAGG TGAAGGTCGGAGTC-3′) and reverse GAPDH (5′-GAAGA TGGTGATGGGATTTC-3′). The specificity of the products was demonstrated for each fragment by a melting curve analysis and gel electrophoresis. The real-time PCR reactions were performed in a 7300 real-time PCR system (Applied Biosystems) using SYBR Green-based real-time PCR assay with the SYBR Green PCR Master Mix (Applied Biosystems) and in-house designed primers.

Gene silencing with siRNA

To knock down the Nox4 gene expression, we used the Silencer® Pre-designed small interfering RNA (siRNA) obtained from Ambion (Catalogue No. AM16706, sequence ID #118807), which targets exon 2 of the human Nox4 gene. The Silencer® Negative Control #1 siRNA (Catalogue No. AM4611, Ambion) was used as control. Cells were subcultured 24 h before transfection in antibiotic-free medium containing 10% serum. Cells were transiently transfected using Lipofectamine2000 (Invitrogen) and 100 n*M* siRNA in Opti-MEM Reduced Serum Medium (Invitrogen). Experiments were performed 48 h after transfection.

Cloning of Nox4P-pGL3

A 1436 bp-long Nox4 promoter region (−1420 to +16 relative to the ATG start codon) was amplified by PCR using genomic DNA isolated from HeLa cells with the following forward and reverse primers CTCGAGGCTCTCTAACTTCA GAAAGATTTAGGAC (with 5′ XhoI site) and AAGCTTTC CAGGACACAGCGATGC (with 5′ HindIII site), respectively. The PCR product was initially cloned into pCR2.1 plasmid using a TA Cloning Kit (Invitrogen). The Nox4 promoter was then subcloned into pGL3 enhancer (Promega). Transfection efficiency was normalized with the Renilla luciferase containing plasmid, pRL-SV40.

Transfection of luciferase plamids

HMECs were (5×10^4 cells/well) seeded in 12-well plate on the day before transfection. Transfection was performed using Lipofectamine2000. Each well contained 200 ng of either empty pGL3 or Nox4 promoter pGL3 and 50 ng pRL-SV40 mixed with 0.5 μl of Lipofectamine2000 in the presence of 250 μl of Opti-MEM. Cells were transfected for 6 h and then incubated with serum-free medium either in the presence or absence of treatment condition. Luciferase assays were performed using Dual-Luciferase reporter assay system (Promega) and measured on a Polarstar microplate reader (BMG Labtech).

Data and statistics

Data were presented as mean ± standard error of the mean (SEM). The mean data were analyzed with *t* test or one-way analysis of variance (one-way ANOVA) followed by post-hoc Newman–Keuls. A *P* value of less than 0.05 was regarded as statistically significant.

Results

Nox4 mRNA increased in serum-starved cells

Ago *et al.* (1) previously reported that serum starvation of cultured aortic endothelial cells elevated the Nox4 mRNA level. To confirm this finding, HMECs were maintained in serum-free medium for 16 h before measurement of Nox4 mRNA by real-time PCR. We found that, similar to the previous report, the level of Nox4 mRNA significantly increased ~3-fold following serum starvation (Fig. 1a). To clarify the change of Nox4 protein expression following serum starvation, Western blot analysis was performed using a commercial antibody. Serum starvation paradoxically diminished the level of Nox4 protein in contrast to the mRNA levels (Fig. 1b). To confirm that the bands detected by the anti-Nox4 antibody were specific, blocking peptide (1:5 wt/wt; from Santa Cruz) totally removed the Nox4 band (Fig. 1c). To further confirm the identity of the Nox4 band, we overexpressed the wild-type human Nox4 using adenoviral vectors (12). This

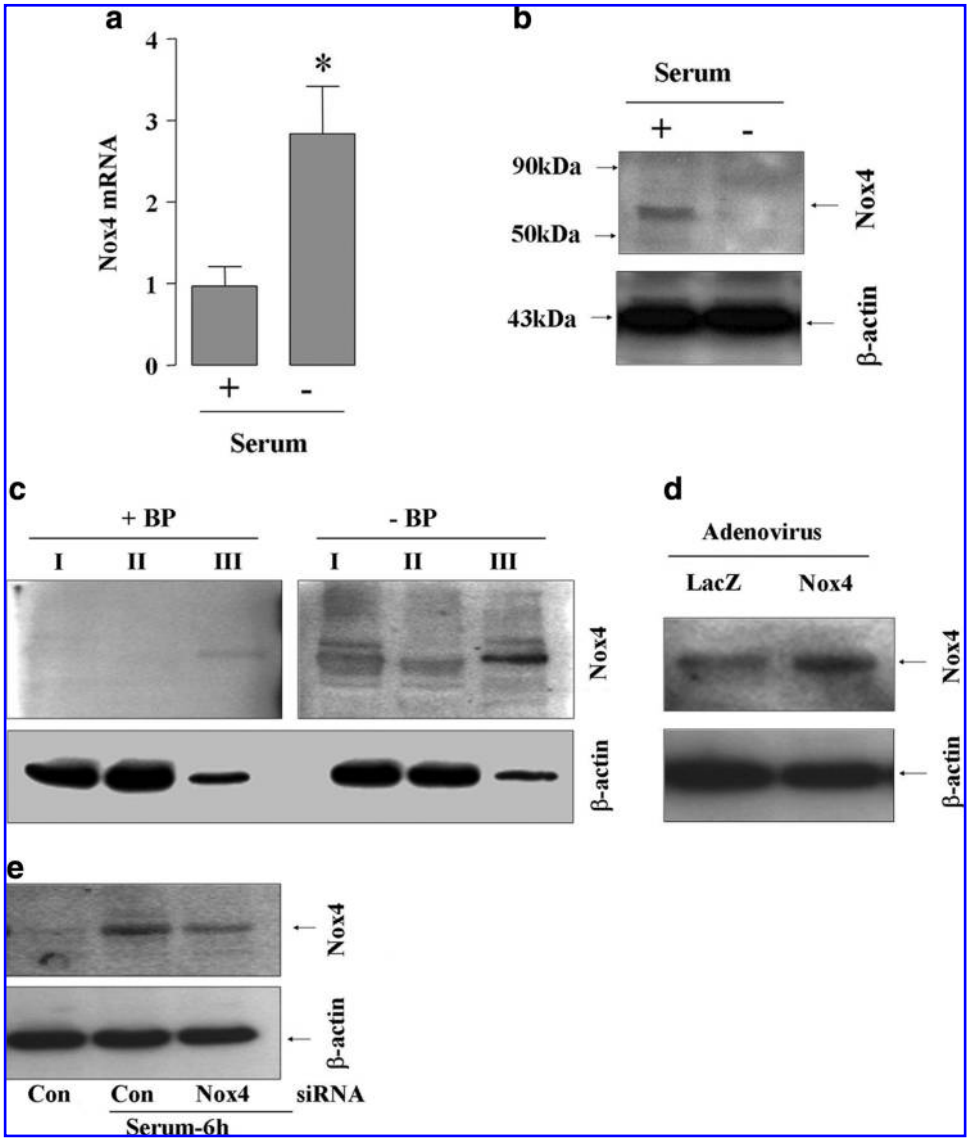

FIG. 1. The effect of serum starvation on Nox4 mRNA and protein expression. (**a**) Nox4 mRNA was measured using real-time PCR. Data were normalized to GAPDH and expressed as fold changes over that in serum-containing medium. (**b**) Nox4 protein analyzed by Western blot analysis. (**c**) shows specificity of Nox4 antibody by using blocking peptide. *Lane I:* Serum-treated HMECs; *lane II*: overexpression of Nox4 in HMECs in absence of serum; *lane III*: mouse kidney sample. (**d**) shows that Nox4 protein expression was enhanced when HMECs were treated with an adenovirus containing Nox4 but not when the adenovirus contains LacZ. Following treatment of HMEC-1 with siRNA specific to Nox4 (**e**), the effect of serum addition (10% for 6 h) was reduced compared to control siRNA treated cells. $N=3$, $^*p<0.05$ following an unpaired *t*-test.

antibody could readily detect the overexpressed Nox4 protein (Fig. 1d), and Nox4 protein expression was attenuated in cells treated with the Nox4 siRNA (Fig. 1e), further validating the performance of the Nox4 antibody.

Serum- induced Nox4 protein expression in endothelial cells

To clarify the direct effects of serum on Nox4 expression, subconfluent HMECs were cultured in serum-free medium for 16 h before serum (10% final concentration) was reintroduced into the culture medium for varying periods. As shown in Fig. 2a, serum potently induced Nox4 protein expression as early as 30 min, and this was maintained up to 24 h. This effect of serum was concentration dependent (Fig. 2b). In contrast to Nox4 proteins, Nox2 and Cu-Zn SOD proteins were not responsive to serum stimulation (Fig. 2b). Serum had a similar enhancing effect on Nox4 protein expression in other cells, including human umbilical vein endothelial cells (HUVEC), aortic smooth muscle cells (HASMC), and dermal fibroblasts (HDF; see online supplement Fig. S1). To determine whether the increased Nox4 expression after serum treatment was associated with enhanced NADPH oxidase activity, we measured intracellular ROS production using $DCFH_2$-DA fluorescence. As shown in Fig. 2c, 10% serum induced a consistent increase in ROS production from 30 min to 24 h, with a similar pattern to Nox4 protein expression. This effect of serum also showed concentration-dependent increases in ROS production in endothelial cells (Fig. 2d). The increased ROS production induced by serum was abolished by the flavin-containing oxidase inhibitor diphenyleneiodonium (DPI, 10 μM) and a dominant negative Nox4 (Fig. 2e). Furthermore, overexpression of Nox4 under serum-free conditions significantly increased ROS formation in endothelial cells (see online supplement Fig. S2b). The overexpression of Nox4 and dominant negative Nox4 were confirmed by real-time PCR (see online supplement Figs. S2a and S3). Nox4 may produce superoxide anion or H_2O_2 or both. To test this, we used NADPH-dependent lucigenin-enhanced chemiluminescence method to measure superoxide. Similar to $DCFH_2$-DA, serum-induced superoxide formation in endothelial cells was inhibited by acute treatment with either the SOD-mimetic MnTmPyP or DPI (1 μM) as shown in Fig. 2f. These findings suggest that Nox4 induced both superoxide and H_2O_2 for signaling in endothelial cells.

We also confirmed the effect of serum on Nox4 protein accumulation using an immunofluorescence method. Again serum or overexpression of Nox4 endothelial cells showed higher immunofluoresence (Fig. 3B and D) compared to their respective controls (Fig. 3A and C).

Serum reintroduction decreased the level of Nox4 mRNA

Paradoxically, Nox4 protein induction by serum is accompanied by a decrease in the steady state level of Nox4 mRNA: Serum-starved HMECs were treated with serum which reduced Nox4 mRNA level within 15 min, and this was further reduced up to 24 h, as shown in Fig. 4.

Serum initiates protein translation of Nox4

Why mRNA and protein expression of Nox4 change in opposite directions in serum is unclear. To address this issue, we used two classical inhibitors, actinomycin D and cycloheximide, to inhibit transcription and translation, respectively. Serum-starved HMECs were pretreated with actinomycin D (5 μg/ml) to block *de novo* mRNA synthesis before stimulation with serum. Surprisingly, pretreatment with actinomycin D for 1 h significantly reduced Nox4 mRNA levels under serum-free conditions ($49 \pm 1.55\%$ of control). This finding suggests that induction of Nox4 mRNA by serum starvation is blocked by actinomycin D and remaining Nox4 mRNA is rapidly degraded. Likewise, pretreatment of HMECs with cycloheximide (5 μg/ml) for 1 h blocked serum-induced Nox4 protein levels as expected (Fig. 5b). Inhibition of serum-induced Nox4 proteins by actinomycin D and cycloheximide (Fig. 5a and b) also reduced serum-stimulated ROS production in endothelial cells (Fig. 5c and d).

Serum induced a translation-linked destabilization of Nox4 mRNA

Previous studies on the expression of certain proto-oncogenes and cytokines revealed that mRNA stability might be regulated by the translation process. We therefore assessed the Nox4 mRNA half life in HMECs in the presence and absence of serum. As shown in Fig. 6a, after blocking *de novo* mRNA synthesis with actinomycin D, the Nox4 mRNA levels remained significantly higher in the presence of serum compared to serum-free conditions. This finding suggests that during translation, Nox4 mRNA is stabilized in the presence of serum and may be degraded after translation. To confirm translation-linked mRNA degradation, translation was inhibited by cycloheximide. The inhibition of translation blocks the serum-induced Nox4 mRNA degradation (Fig. 6b). This finding suggests that Nox4 mRNA was regulated at the translational level by serum in HMECs.

p38 MAPK is involved in Nox4 mRNA regulation in serum-starved cells

Prolonged serum deprivation is a major cellular stress for endothelial cells under culture conditions. We therefore chose to examine the role of mitogen-activated protein kinase (MAPK) p38, a stress-responsive kinase in mRNA transcription of Nox4 serum-starved cells. Phosphorylation of p38 MAPK was obvious following serum starvation and was rapidly decreased following reintroduction of serum (Fig. 7a). To clarify whether endogenously-activated p38 MAPK has a role in modulating Nox4 transcription, serum-starved cells were treated with the p38 inhibitor SB202190 (10 μM), which significantly decreased the Nox4 mRNA level (Fig. 7b). Finally, to confirm that serum starvation induced Nox4 transcription, we cloned the 1,436 bp promoter region of the human Nox4 gene into a pGL3 luciferase reporter vector (Nox4P–pGL3) and tested the effects of serum starvation. Indeed upon the serum starvation, HMECs exhibited Nox4P-pGL3 promoter activity (Fig. 7c) which was significantly suppressed by SB202190 or addition of serum.

Discussion

The Nox4 subunit of NADPH oxidase is a major source of ROS in vascular cells and has important roles in cell signaling (3, 7, 12). In this study, we have clearly demonstrated that serum is a potent inducer of Nox4 protein expression in human endothelial cells. This observation is important since

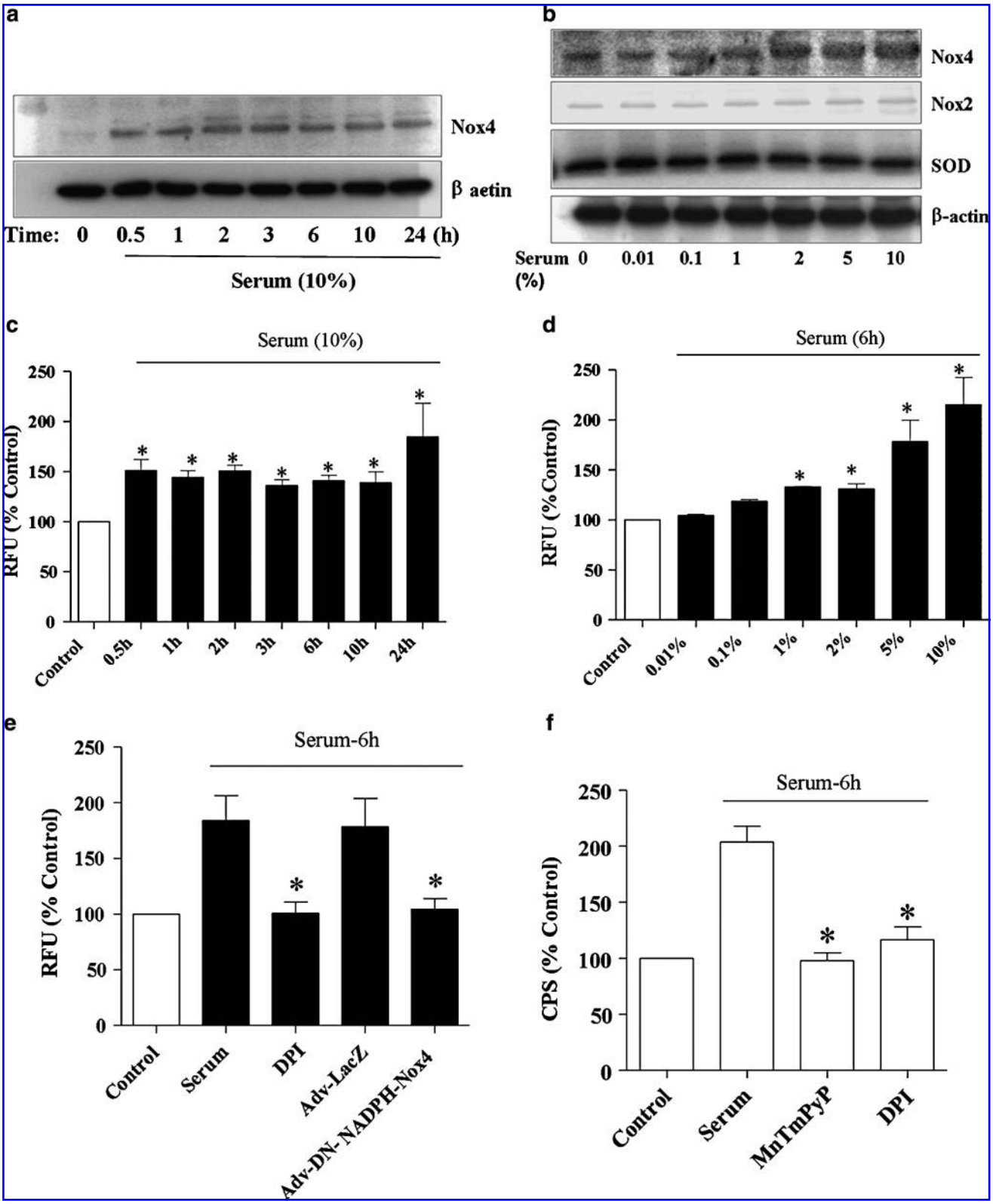

FIG. 2. ROS production and protein expression after application of serum to HMEC-1. Following serum starvation for 16 h, cells were exposed to 10% FCS for increasing periods of time (**a, c**) or increasing concentrations of FCS for 6 h (**b, d**). (**a**) and (**b**): representative Western blots of Nox4 expression. Total ROS production was determined by $DCFH_2$-DA fluorescence (**c, d**). Relative fluorescence units (RFU, mean ± SEM from 3 experiments) were normalized per 10^3 cells and expressed as a percentage of the values obtained in cells following serum starvation. (**e**): total ROS measurements obtained from cells treated with the NADPH oxidase inhibitor DPI (10 μM), adenovirus expressing LacZ or adenovirus expressing dominant negative Nox4 following a 6 h incubation with 10% FCS. (**f**): superoxide measurement by lucigenin-enhanced chemiluminescence from cells following 6 h incubation with 10% FCS in absence or presence of either SOD mimetic MnTmPyP (5 μM) or DPI (1 μM). $N = 3$–5, $^*p < 0.05$ in following one way ANOVA with Newman Keuls post hoc analysis between serum and serum plus inhibitors.

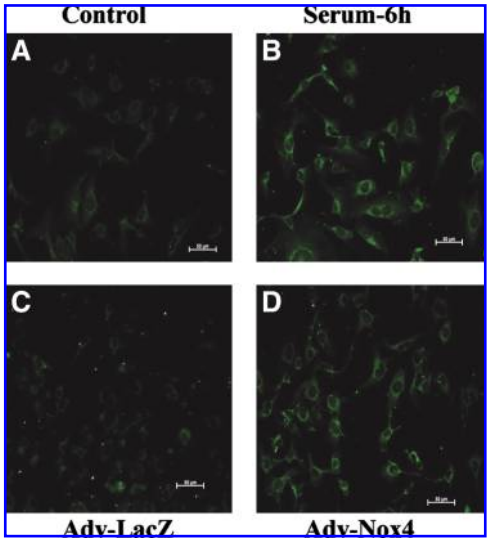

FIG. 3. Nox4 expression, detected by immunocytochemistry, increases in HMECs following serum application. HMECs were serum starved for 16 h (**A**) before addition of 10% FCS for 6 h (**B**). Increased Nox4 expression was also detected in HMECs infected with adenovirus expressing Nox4 (**D**) compared to adenovirus containing LacZ (**C**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

previously we and others have consistently shown that serum treatment decreases the steady state level of Nox4 mRNA. We have also confirmed that induction of Nox4 protein accumulation by serum was not unique to endothelial cells, with similar effects observed in vascular smooth muscle cells and fibroblasts. Our results suggest that the level of Nox4 mRNA does not accurately reflect Nox4 protein abundance and function. Specifically, in cell culture experiments, the presence or absence of serum is a critical factor that needs to be taken into consideration in studies of Nox4 expression or function.

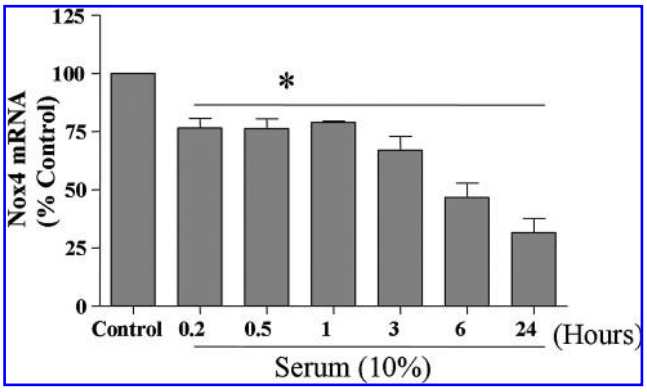

FIG. 4. Nox4 mRNA expression decreases in a time dependent manner following application of FCS. HMECs were serum starved for 16 h before the application of 10% FCS for increasing periods of time. Nox4 mRNA was measured using real-time PCR. Data were normalized to GAPDH and expressed as a percentage of the values obtained following serum starvation (mean ± SEM from 3 experiments). *$p < 0.05$ value following a one-way ANOVA with Newman Keuls post hoc analysis.

Our finding of paradoxical regulation of Nox4 mRNA and protein accumulation in endothelial cells is supported by a recent study in which discrepancies between Nox4 mRNA and protein have been observed in vascular smooth muscle cells in response to insulin-like growth factor (31), and others have suggested that paradoxical regulation of mRNA and protein levels is not unique for Nox4. For example, Hatakeyama and colleagues (21) examined in neurons the changes in mRNA and protein expression levels of the highly conserved transcription factor, CAAT/enhancer binding protein, and found that after neuronal stimulation a significant decrease in the mRNA copy number was accompanied by a significant increase in the protein level (21). Differential regulation of mRNA and protein expression have also been reported for c-myc (15), p53 (36), and the nucleolar phosphoprotein nucleolin (25). Of note, the mechanisms underlying the differential regulation of mRNA and protein expression are diverse, and may include alterations in the rate of transcription and/or translation, as well as changes in the stability of mRNA or protein.

In the present study, we attempted to examine the mechanisms of regulation of Nox4 mRNA and protein in response to serum in human endothelial cells. First, we demonstrated that in endothelial cells in which the *de novo* mRNA synthesis was blocked by actinomycin D, the stability of remaining Nox4 mRNA was higher in the presence of serum compared to serum-free conditions. Second, Nox4 promoter activity was not regulated by serum, whilst serum starvation activates Nox4 promoter activity. Third, inhibition of Nox4 translation reduced the serum-induced Nox4 protein synthesis and blocked mRNA degradation, a phenomenon which is seen with several other mRNA species (2, 6, 20, 37, 44). These observations are in agreement with previous studies that examined the expression of certain proto-oncogenes and cytokines, suggesting a translation-initiated mRNA destabilization mechanism (2, 6, 20, 44). For example, there is evidence that initiation of mRNA translation may accelerate the rate of mRNA decay of the proto-oncogenes c-fos and c-myc (38, 49). Conversely, it has been shown that after inhibition of translation with cycloheximide, mutation of the initiation codon or insertion of a stable secondary structure attenuated the rate of degradation of the mRNAs of c-fos or granulocyte monocyte colony-stimulating factor (reviewed in (4)). Taken together, our results may provide a mechanistic explanation for the unresolved observation that Nox4 mRNA and protein are differentially regulated under certain circumstances in vascular cells (31). Regulation of mRNA takes place at transcriptional, post-transcriptional, and translational levels. Protein synthesis by translation is the ultimate step in gene expression. Translational mechanisms may facilitate rapid changes in protein production without synthesis of new mRNA. Conversely, several mRNAs are expressed in cells but not translated into protein due to translational repression. This mechanism is largely regulated by the 3′ UTR (untranslated region) of a gene (30, 39). The UTR of mRNA can either bind to specific protein(s) which blocks translation or to small microRNAs which also suppress protein synthesis. However, the mechanism of transcription repression depends on the type of transcript as well as the tissues in which the transcript is expressed. For example, tunicamycin-induced endoplasmic reticulum stress inhibits cytochrome C mRNA translation through the binding of T-cell-restricted intracellular antigen 1

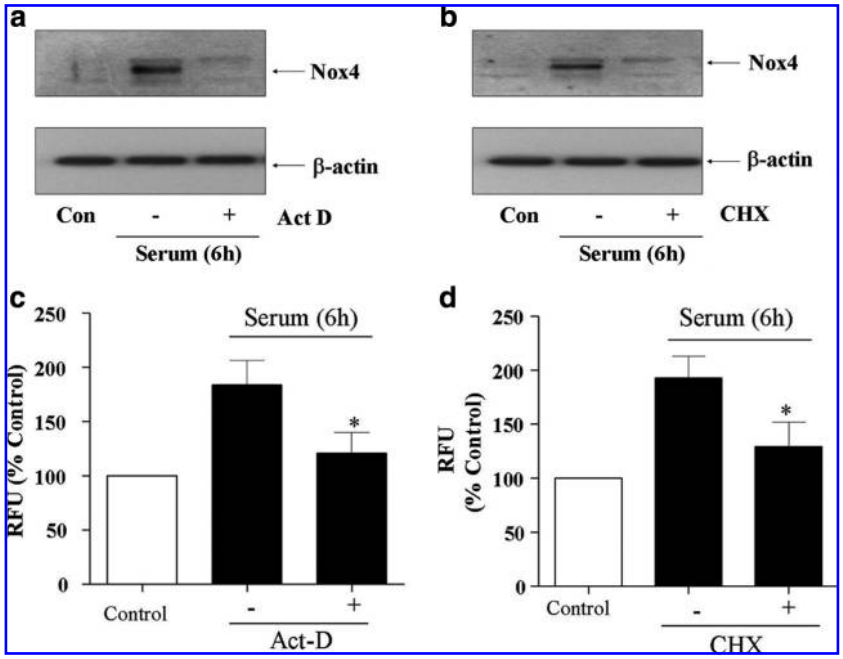

FIG. 5. Nox4 protein levels and total ROS production stimulated by serum are decreased by inhibitors of transcription and translation. After serum starvation for 16 h, HMECs were pretreated with (**a, c**) actinomysin D (5 μg/ml) or (**b, d**) cycloheximide (CHX, 5 μg/ml) for 1 h prior to the addition of 10% FCS for 6 h. (**a**) and (**b**): representative Western blots of Nox4 and β actin expression following serum starvation and addition of serum in the presence or absence of actinomysin D or cycloheximide. (**c**) and (**d**): total ROS production measured by $DCFH_2$-DA where relative fluorescence units (mean $\pm$ SEM from 4 experiments) are normalized per 10^3 cells and expressed as a percentage of the values obtained in serum starved cells. $^*p < 0.05$ value following one-way ANOVA and Newman Keuls post hoc analysis.

(TIA-1) to the 3′UTR (24), whereas amino acid deprivation of Huh7 hepatoma cells leads to suppression of cationic amino acid transporter-1 mRNA translation via microRNA-122. This inhibition was cell specific with the effect being absent in cell lines such as Hela, HEK 293, and HepG2 (5). It is worth mentioning that under both conditions of stress the mRNA was detected, but translation was inhibited via two different mechanisms. Serum starvation is a stressful condition for endothelial cells and it is possible that the Nox4 3′ UTR may be involved in translational regulation of this gene. This hypothesis is currently under investigation in our laboratory.

Serum is a strong mitogen in cultured cells, and Nox4 has an important role in positively regulating endothelial cell proliferation and anti-apoptotic activity (7, 8, 12, 35, 47). It is intriguing that serum starvation, which suppresses cell proliferation and migration, leads to increased Nox4 mRNA in these cells. We attempted to clarify how this occurs by examining the activation of p38 MAPK, an enzyme which is responsive to various cellular stresses, including serum starvation and gamma-irradiation (19, 26). Interestingly, we found that p38 is phosphorylated in the absence of serum and this is rapidly diminished following the reintroduction of serum to the culture medium. These observations suggest

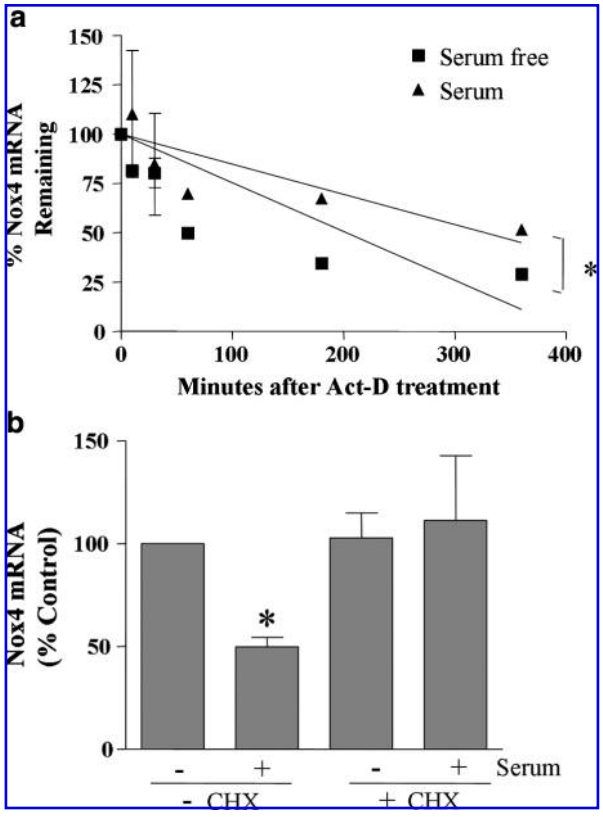

FIG. 6. Serum stabilizes Nox4 mRNA. (**a**) HMECs cultured in serum -free conditions for 16 h were treated with or without 10% serum, in the presence of actinomysin D (5 μg/ml) for the indicated times (0–6 h). Total RNA was isolated at the times shown and analyzed for Nox4 mRNA expression using real-time PCR and normalized to GAPDH expression. The graph shows the average mRNA as a percentage of control (0 h). (**b**) Following serum starvation for 16 h, HMECs were pretreated with cycloheximide (5 μg/ml) for 1 h before the application of 10% serum for 6 h. Nox4 mRNA was measured using real-time PCR. Data (mean $\pm$ SEM from 4 experiments) was normalized to GAPDH and expressed as a percentage of values obtained following serum starvation in the absence of cycloheximide. $^*p < 0.05$ value following one-way ANOVA and Newman Keuls post hoc analysis.

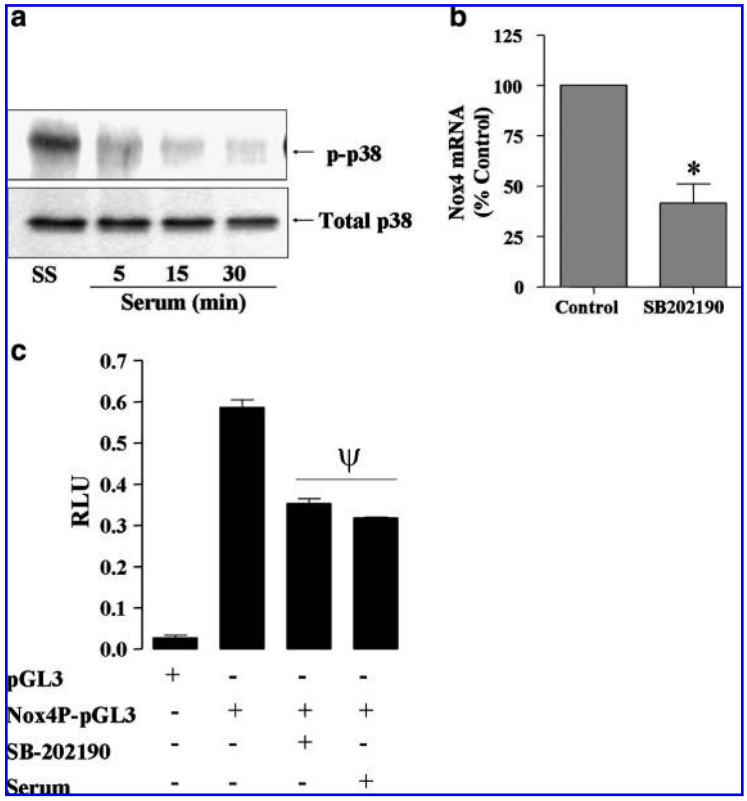

FIG. 7. p38 MAP kinase regulates Nox4 mRNA expression. p38 MAP kinase showed increased phosphorylation in response to serum starvation. This decreased following the application of serum to HMECs without an alteration in total p38 levels (**a**). Nox4 mRNA, detected by real-time PCR, was decreased following incubation with the p38 inhibitor SB202190 (10 μM) for 6 h in serum-free conditions (**b**). Data (mean $\pm$ SEM from 4 experiments) was normalized to GAPDH and expressed as a percentage of the values obtained following serum starvation alone. $^*p < 0.05$ following a Students paired t test. (**c**): Nox4 promoter activity is decreased in the presence of the p38 inhibitor SB202190 to levels similar to those observed following incubation with 10% serum. Luciferase activity is expressed as relative luminescence units (RLU, mean $\pm$ SEM from 3–5 experiments). ψ denotes $p < 0.05$ following a one-way ANOVA with Newman Keuls post hoc analysis.

that p38 may be involved in Nox4 mRNA transcription in serum-starved cells. Supporting this, we demonstrated that inhibition of endogenous p38MAP kinase activity by SB202190 decreased the Nox4 promoter activity as well as Nox4 mRNA expression in serum-starved cells. Similarly, serum addition shuts down the p38MAP kinase pathway and reduces Nox4 promoter activity. However, the downstream mediator of p38MAP kinase pathway involved in Nox4 mRNA stabilization remains to be determined. The downstream target of p38 MAP kinase/MAP kinase-activated protein kinase 2 (MAPKAP-K2) is known to regulate the stability and translation of mRNA's containing AU-rich regions (45, 46), and it is possible that this acts to stabilize the Nox4 mRNA. It would also be interesting to check the involvement of transcription factors in regard to Nox4 regulation under this condition. Previously it has been shown that the transcription factor E2F regulates Nox4 promoter activity in rodent vascular smooth muscle cells (50) and it is known that E2F and its associated activators are involved in growth- promoting activity (16). Whether Nox4 promoter activity is upregulated by E2F in the serum-starved condition remains to be determined in human endothelial cells. On the other hand, addition of serum to pancreatic cancer cell lines was able to induce Nox4 protein expression and ROS formation, which is consistent with the present finding, although the authors did not measure Nox4 mRNA expression in these cells (43). The underlying mechanism may be that the absence of serum strongly induces Nox4 mRNA expression in endothelial cells which is able to accumulate within the cell due to repression of translation. Addition of serum then releases the translation repressors, leading to increased Nox4 protein synthesis and activity.

In conclusion, our results suggest that serum is an inducer of Nox4 protein expression in contrast to its negative effect on the level of Nox4 mRNA. Based on these observations, we propose a model of regulation of Nox4 transcription and translation by serum in cultured endothelial cells. In the absence of serum, Nox4 mRNA is maintained at a relatively high level, at least partly via p38 MAPK activation. When the cells are treated with serum, there is an early Nox4 protein accumulation independent of mRNA transcription by promoting translation of existing mRNA copies or reduced protein degradation. However, this translation process then triggers destabilization of Nox4 mRNA. These findings suggest an additional level of Nox4 transcript regulation in endothelial cells which resolves some of the controversial findings in the published record.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

Act-D = actinomycin D
CHX = cycloheximide
$DCFH_2$-DA = dichlorodihydrofluorescein diacetate
HMECs = human microvascular endothelial cells
p38MAPK = p38 mitogen-activated protein kinase
ROS = reactive oxygen species
SOD = superoxide dismutase
UTR = untranslated region

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