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Hypoxia-induced ROS signaling is required for LOX up-regulation in endothelial cells

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1. ABSTRACT

The adaptive response of endothelial cells to hypoxia involves a substantial remodeling of extracellular matrix (ECM). In endothelial cells hypoxia up-regulates lysyl oxidase (LOX), a key enzyme in ECM assembly, relevant to vascular homeostasis. However, the mechanism underlying this response has not been established. Hypoxia up-regulated LOX expression in endothelial cells (HUVEC and BAEC) and concomitantly increased LOX enzymatic activity. This effect was independent of autocrine factors released by hypoxic cells and relies on a transcriptional mechanism. Both mTOR blockade and HIF-1alpha knockdown slightly prevented LOX up-regulation by hypoxia, suggesting that HIF-1alpha is only partially responsible for this effect. In fact, serial promoter deletion and mutagenesis studies indicated a limited contribution of the previously described hypoxia response element (-75 bp). Interestingly, Smad over-expression further increased LOX transcriptional activity in endothelial cells exposed to hypoxia. Moreover, the increase in LOX expression triggered by hypoxia was significantly reduced by reactive oxygen species (ROS) inhibitors. Thus, our data support a role of Smad signaling and ROS in the up-regulation of LOX by hypoxia in endothelial cells.

2. INTRODUCTION

Hypoxia is associated with pathological conditions such as cancer, ischemic disorders, chronic inflammation and atherosclerosis. The decrease in oxygen tension alters vascular function and affects endothelial cell physiology triggering and adaptive response that promotes cell survival and angiogenesis, modulates cell metabolism, and involves extracellular matrix (ECM) remodeling (1-3). This latter process entails changes in the composition, postraslational modification, assembly and deposition of the ECM, which affect not only its structure and mechanical properties but also modify growth factor availability and thereby severely disturb cell homeostasis (3).

The hypoxia-inducible factor (HIF) 1 is the master regulator of oxygen homeostasis. HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed beta-subunit (known as aryl hydrocarbon-receptor nuclear translocator [ARNT or HIF-1beta]) and an oxygen-regulated alpha subunit (2). The molecular mechanism underlying oxygen sensing is not entirely understood, but recent advances support the role of reactive oxygen species (ROS) in hypoxia signaling (4-6).

Lysyl oxidase (LOX) plays a pivotal role in ECM scaffolding. LOX is an extracellular copper enzyme that initiates the covalent cross-linking of collagen and elastin allowing normal ECM assembly (7,8). There is growing evidence that LOX plays a relevant role in vascular homeostasis. This enzyme assures vascular wall integrity, and participates in endothelial dysfunction triggered by atherosclerotic risk factors such as hypercholesterolemia, hyperhomocysteinemia and proinflammatory cytokines (9-12). LOX expression is highly up-regulated in tumor cells exposed to hypoxic stress (13) and, although high-throughput screenings have demonstrated that hypoxia enhances endothelial LOX expression (14), the molecular mechanisms that underlie the hypoxic up-regulation of this enzyme in endothelial cells have not been well-characterized. This study aims to clarify the mechanisms involved in LOX regulation by hypoxia in endothelial cells.

3. MATERIALS AND METHODS

3.1. Cell culture

Bovine aortic endothelial cells (BAEC; Clonetics) were cultured in RPMI, supplemented with 10% FCS (Biological Industries), antibiotics (0.1 mg/mL streptomycin, 100 U/mL penicillin G) and 2 mM L-glutamine as previously described (10). Human umbilical vein endothelial cells (HUVEC), kindly provided by Dra M Camacho (Hospital de la Santa Creu i Sant Pau, Barcelona) were obtained by collagenase digestion and were cultured in medium M199 supplemented with 20 mM HEPES, pH 7.4, 30 μg/mL endothelial growth factor supplement, 100 μg/mL heparin, 20% FCS and antibiotics (15). Mouse lung endothelial cells (MLEC) were isolated from lungs of C57BL/6 mice by collagenase A (Roche) digestion followed by selection with intercellular adhesion molecule 2 (ICAM-2) coated magnetic beads (Invitrogen) as previously described (15). Endothelial cells were used between third and fifth passage. Cells were maintained in standard culture conditions (21% O_2, 5% CO_2, 95% humidity) until 80% confluent. Hypoxic exposure was carried out under 1% O_2, 5% CO_2 (balanced with N2) for the times indicated (Whitley H35 hypoxystation). The mTOR (mammalian target of rapamycin) inhibitor, rapamycin (100 nM; Sigma), the inhibitor of mitochondrial electron transport, rotenone (2 μM), DPI, an inhibitor of flavoenzymes (5 μM), catalase (3000 U/mL) and apocynin a NADPH oxidase inhibitor that behaves as a ROS scavenger in vascular cells (16) (100 μM) were added 1 h before hypoxia exposure. In some experiments the effect of recombinant human VEGF (vascular endothelial growth factor)-A (50 ng/mL; R&D Systems) was assessed. No cytotoxicity, analyzed by the trypan blue exclusion test and the XTT based assay for cell viability (Roche), was observed after the treatment with either of these compounds.

3.2. Real-time PCR

Total RNA was isolated using UltraspecTM (Biotecx) following manufacturer's instructions. RNA (1 μg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in the presence of random hexamers. Quantification of mRNA levels was performed by Real-time PCR using an ABIPRISM 7000 sequence detection system (Applied Biosystems) and specific primers and probes provided by the Assay-on-Demand system for LOX (Hs00184700_m1 for HUVEC and Rn01491829_m1 for BAEC) and VEGF-A (Hs00173626_m1). TATA-binding protein (TBP) was used as an endogenous control (Hs99999910_m1) (17). The efficiency of PCR reactions was 90-100% based on the slope of calibration curves (slope between -3.6 and -3.1).

3.3. Immunostaining and confocal microscopy

Endothelial cells were plated at a density of 2 x 10^4 cells onto 12 mm diameter glass-bottom dishes (Willco Wells B.V.) coated with gelatin. Cells were maintained in normoxia or exposed to hypoxia as described above. Afterwards, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Tween-20 and incubated with a rabbit polyclonal antibody against LOX (Abcam Ref: ab31238; 1:100) for 1 h. Then cells were washed and incubated for 1 h with a fluorescence-conjugated secondary antibody (goat anti-rabbit Alexa Fluor 488; Molecular Probes). Nuclei and actin fibers were stained with Hoeschst 33342 and Alexa Fluor 633-phalloidin respectively (Molecular Probes).

3.4. Lysyl oxidase activity

LOX activity was measured by a high sensitive fluorescent assay as previously described (9). Briefly, endothelial cells were seeded in a six-well plate (10^5 cells/well), and after 48 h media was replaced by phenol red-free M199 (Gibco) depleted of serum, antibiotics and glutamine. Then cells were maintained under normoxia or exposed to hypoxia for the indicated times. LOX activity was measured in cell supernatants. An aliquot of the media (200 μL) was incubated in the presence and absence of 500 μmol/L beta-aminopropionitrile (BAPN) at 37ºC for 30 min with 1 U/mL of horseradish peroxidase, 10 μM Amplex red (Molecular Probes) and 10 mM 1,5-diaminopentane in 1.2 M urea, 0.05 M sodium borate pH 8.2. The reaction was stopped on ice and differences in fluorescence intensity (excitation wavelength: 563 nm; emission wavelength: 587 nm) between samples with and without BAPN were determined.

3.5. Constructs of LOX promoter

The luciferase reporter vector containing 821 bp of LOX promoter (from -821 to + 83 related to the first translation start codon ATG) was amplified by PCR and cloned into the pGL3 basic luciferase reporter vector (Promega) using the *Kpn*I-*Hind*III restriction sites as previously described (pLOX-821) (10). Two serial deletion constructs (-631 to + 83 and -405 to +83) were generated by PCR amplification (pLOX-631 and pLOX-405 respectively). The hypoxia response element (HRE) site located at position -75 (13) was mutated using the Quick-Change II Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions (18). The HRE mutation was introduced with primers: 5'-GGTTGAAGATTTCTCCTTCCCTCtttTGATTTGAGCC

CCG-3' and 5'-CGGGGCTCAAATCAaaaGAGGGAAGGAGAAATCTTCAACC-3' (putative sites are underlined and changes are indicated in lower case letters). The pLOX-821 vector was used as a template. The HRE mutation introduced by this strategy is identical to that described by Erler et al (13) and does not generate any novel element as derived from *in silico* analysis (MatInspector sofware; Genomatix). Mutations were confirmed by DNA sequencing.

3.6. Transient transfections and Luciferase assays

Transient transfections of BAEC were performed with Lipofectin™ (Invitrogen) and the pLOX luciferase constructs described above together with the pSVbeta-gal (Promega), as previously reported (11). Briefly, transfections were carried out with 1 μg/well of the pLOX luciferase construct, 0.3 μg/well of pSVbeta-gal and 3 μl of Lipofectin. In co-transfection experiments expression vectors for Smad2, Smad3 or Smad4 (19) (provided by Dr J Yanagisawa, University of Tsukuba, Japan) were used together with the corresponding empty vector. The complexes DNA/liposome were added to the cells for 7 h. After 24 h, transfected cells were exposed to hypoxia for 18 h. Luciferase activity was measured in cell lysates using the Luciferase assay kit (Promega) and a luminometer (Orion I, Berthold detection systems) according to the manufacturer. Results were normalized by beta-galactosidase activity using the Enzyme Assay System™ (Promega). Beta-galactosidase activity was not significantly modified by hypoxia exposure.

3.7. HIF-1alpha siRNA transfection

HUVEC were transfected with a HIF-1alpha Silencer predesigned siRNA (ID# 42840; Ambion) or a Silencer™ Negative Control1# using an Amaxa Nucleofector™ and the HUVEC Nucleofector kit according to the manufacturer's instructions (Amaxa) as previously described (20). Electroporation was carried out with 1 x 10^6 cells and 1 μg of siRNA with program U-001. After electroporation cells were resuspended in 500 μL of pre-warmed cell culture medium, seeded in 6-well plates (350.000 cells/well) for 24 h and then subjected to hypoxic or normoxic conditions. HIF-1alpha knockdown was verified by Western-blot.

3.8. Western blot analysis

Whole cell protein extracts were resolved by SDS-PAGE and transferred to nitro-cellulose filters (Bio-Rad). Blots were incubated with an antibody directed against HIF-1alpha (NB100-449A, Novus; 1:500). Bound antibody was detected after incubation with a HRP-conjugated goat anti-rabbit IgG and using the SuperSignal West Dura Extended Duration Substrate (Pierce). Equal loading of protein in each lane was verified by Ponceau staining and by beta-actin signal (21).

3.9. Statistical analysis

Data are expressed as mean+/-SEM. Means were compared by one-factor ANOVA followed by Fisher PLSD to assess specific group differences. Differences were considered significant at $p < 0.05$.

4. RESULTS

4.1. Hypoxia induces LOX expression in endothelial cells

To determine whether hypoxia induces LOX expression in endothelial cells, HUVEC, BAEC and MLEC were incubated under hypoxic conditions (1% O_2) up to 24 h. As previously described, in our culture conditions hypoxia significantly induced HIF-1alpha protein levels (data not shown) (18). A similar temporal pattern of LOX up-regulation was observed in HUVEC and BAEC exposed to hypoxia (Figure 1A). LOX mRNA levels reached a maximum after 18 h of hypoxia (3-fold over controls). This response was also observed in MLEC (data not shown). The induction of LOX expression in both HUVEC and BAEC was confirmed in immunohistochemical studies. As shown in Figure 1B, hypoxia enhanced intracellular LOX immunostaining. Furthermore, LOX activity significantly increased in cell supernatants from cells exposed to hypoxia (control: 100±3.3 *vs* hypoxia 48 h: 145±13.8; $p<0.05$).

4.2. The up-regulation of LOX by hypoxia is independent of autocrine factors

Hypoxia is a potent inducer of growth factor secretion in vascular endothelial cells. For this reason, we analyzed whether autocrine factors released by cells exposed to hypoxia mediate LOX up-regulation. Under our culture conditions hypoxia strongly increased VEGF expression (Figure 2A); however, stimulation of endothelial cells with VEGF (50 ng/mL) did not modify LOX mRNA levels (Figure 2B). Furthermore, HUVEC cultured with conditioned media from hypoxic endothelial cells did not show changes on LOX expression (Figure 2C). Thus, we exclude autocrine factors released by endothelial cells in response to hypoxia as responsible for the up-regulation of LOX.

4.3. Hypoxia increases LOX transcriptional activity in endothelial cells

Next we aimed to establish whether hypoxia could modulate LOX transcriptional activity in endothelial cells. Pretreatment of BAEC with 5,6-dichloro-beta-D ribofuranosyl benzimidazole (DRB), a transcriptional inhibitor, completely prevented the increase in LOX mRNA levels elicited by hypoxia (Figure 3A). In agreement, transient transfection studies revealed that hypoxia induces LOX transcriptional activity about 2-fold (Figure 3B).

4.4. Rapamycin partially prevented the induction of LOX by hypoxia

The mTOR pathway is a positive regulator of HIF-1alpha (22). To characterize the role of this pathway in the induction of LOX by hypoxia, we analyzed the effect of rapamycin (a mTOR inhibitor) on both HIF-1alpha protein and LOX mRNA levels. Western blot analysis confirmed that rapamycin prevented the induction of HIF-1alpha protein levels caused by hypoxia (Figure 4A). However, the increase in LOX mRNA levels observed in hypoxic cells was only partially blocked by rapamycin (Figure 4B).

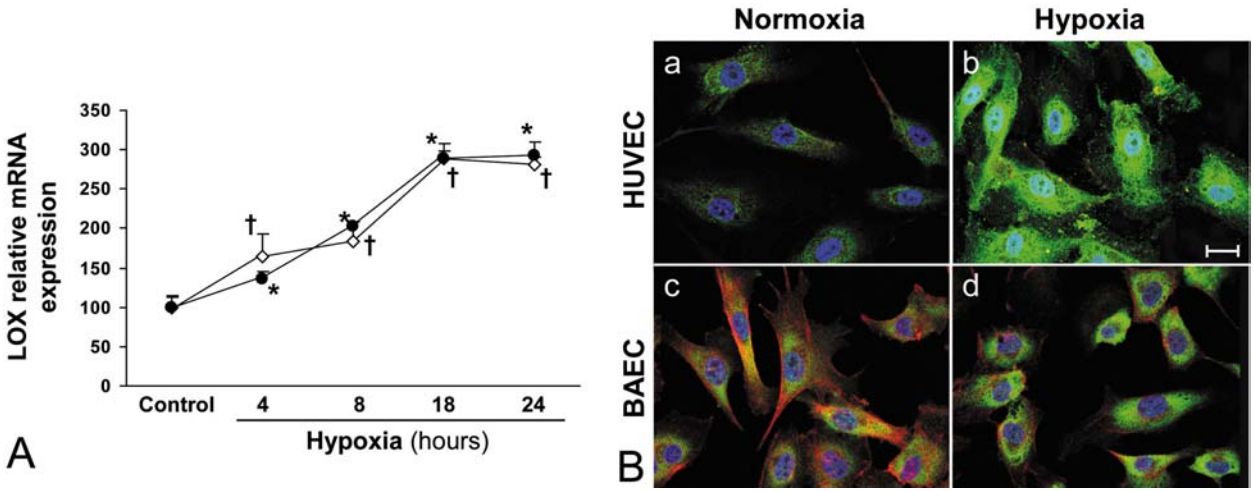

Figure 1. Hypoxia increases LOX expression in endothelial cells. (A) HUVEC (filled circles) or BAEC (empty diamonds) were maintained under normoxic conditions (21% O_2) or exposed to hypoxia (1% O_2) during different times and LOX mRNA levels were evaluated by real-time PCR. Data were normalized by TBP. Results are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs.* control HUVEC [normoxia]; †, *vs.* control BAEC [normoxia]). (B) HUVEC and BAEC were maintained under normoxia or exposed to hypoxia for 48 h and LOX was analyzed by immunocytochemistry. LOX staining is shown in green. Cells were counterstained with Hoescht to highlight nuclei (blue) and with an Alexa Fluor 633-phalloidin antibody to visualize F-actin (red). Bar: 20 μm.

4.5. HIF-1alpha inhibition partially abrogated hypoxia-induced LOX up-regulation

To further evaluate the contribution of HIF-1alpha in LOX regulation, knockdown experiments using siRNA targeting HIF-1alpha were conducted in HUVEC. HIF-1alpha–siRNA suppressed the increase on HIF-1alpha protein levels triggered by hypoxia (Figure 5A) and strongly reduced HIF-1alpha mRNA levels (to a 32.42%; $p<0.001$). The up-regulation of LOX induced by hypoxia was only partially prevented by HIF-1alpha silencing (Figure 5B). Therefore, data from figures 3 and 4 indicate that HIF-1alpha is only partially responsible for the hypoxia-induced up-regulation of LOX in endothelial cells and suggest that other mechanisms should be involved in this process.

4.6. ROS signaling is involved in hypoxia-induced LOX up-regulation

It has been recently demonstrated that ROS play a critical role in hypoxia signaling (4,5,23). In this context, we aimed to characterize the role of hypoxia-induced ROS generation on LOX expression using a pharmacological approach. As shown in Figure 6 the increase of LOX mRNA levels elicited by hypoxia in HUVEC was significantly reduced by DPI, rotenone, catalase and apocynin supporting that ROS signaling is involved in LOX up-regulation by hypoxia in endothelial cells.

4.7. Smad pathway is involved in the increase of LOX transcriptional activity induced by hypoxia

To more accurately establish the contribution of HIF-1alpha to the hypoxic up-regulation of LOX in endothelial cells, site-directed mutagenesis targeting a previously reported HRE located at -75 bp in LOX promoter (HRE/-75) (13) was carried out. As observed in Figure 7A, when HRE/-75 was mutated basal activity of LOX promoter decreased but this strategy only partially reduced hypoxia responsiveness. In fact, hypoxia was still able to significantly increase LOX promoter activity. By serial deletion studies we determined that a promoter region located between positions -821 to -405 is involved in hypoxia-induced LOX up-regulation (Figure 7B). By *in silico* analysis we did not identify any putative HRE in this region, but it contains several putative Smad binding elements (SBEs). Because signaling via Smad proteins mediates hypoxic responses in endothelial cells (24) we analyze their ability to modulate LOX transcriptional activity. Smad2 and/or Smad3 were co-transfected in BAEC with Smad4 under normoxia or hypoxia. Smad over-expression elicited a further increase in pLOX-834 activity in BAEC exposed to hypoxia, while it had not significant consequences on LOX promoter activity in normoxic conditions (Figure 8). These data support a contribution of Smad signaling on LOX up-regulation by hypoxia.

5. DISCUSSION

We have previously demonstrated that LOX, a key enzyme for the maintenance of ECM stability, regulate endothelial cell function and seems to play a key role in vascular homeostasis (9,10,12,25,26). In fact, LOX is strongly expressed in the endothelium of healthy coronary arteries (12), suggesting that this cell monolayer significantly contributes to vascular LOX activity, although the relative contribution of endothelial cells *versus* vascular smooth muscle cells (VSMC) has not been analyzed. Interestingly, disturbed LOX activity has been associated with cardiovascular diseases as well as with tumor progression (26-28) pathologies in which hypoxia is a

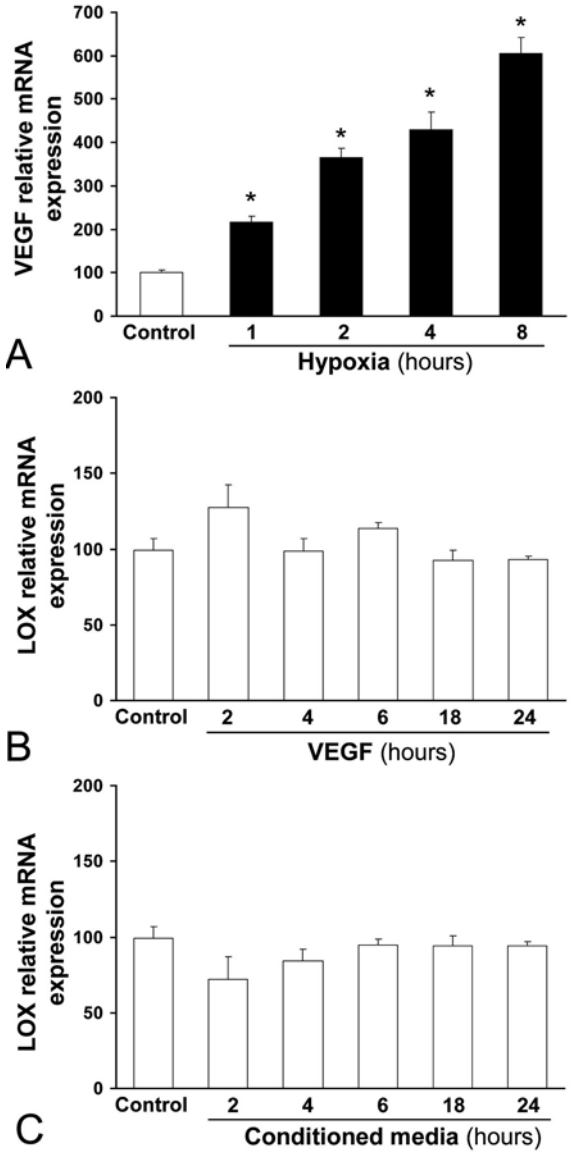

Figure 2. The increase in LOX expression elicited by hypoxia is not mediated by autocrine mechanisms. (A) HUVEC were maintained under normoxic conditions or exposed to hypoxia during the times indicated and VEGF mRNA levels were analyzed. (B) LOX mRNA levels were evaluated in HUVEC cells stimulated with VEGF (50 ng/mL) during different times. (C) LOX mRNA levels from HUVEC stimulated during the times indicated with conditioned media from cells maintained under normoxia or hypoxia for 18 h. Data are expressed as mean ± SEM, (n=9; $p<0.05$: *, *vs.* control cells).

common enviromental factor that promotes vascular remodeling and triggers a metastatic phenotype respectively (29-32). HIF-1 is the master regulator of hypoxic responses (1,2). HIF-1 could also be activated in an oxygen-independent manner by hormones and growth factors further contributing to the control of vascular function (33, 34). Among many other effects HIF-1 modulates the expression of ECM proteins in endothelial cells (14). Furthermore, HIF-1 controls epithelial-mesenquimal transition and fibrogenesis (35), at least in part, through the regulation of LOX and LOX-like 2 (LOXL2), both identified as hypoxia targets in tumor cells (13,36). In the present study we demonstrated that both HIF-1-dependent and independent mechanisms underlie the induction of LOX expression by hypoxia in endothelial cells and that ROS participate in the signaling pathways involved in such effect.

Our data show that hypoxia increases LOX mRNA levels in endothelial cells as it has been previously demonstrated by high-throughput microarray approaches (14). It should be noted that the increase in LOX expression induced by hypoxia in endothelial cells (3-fold) is markedly lower than that reported in breast cancer cells (120-fold) or in Hep3B cells (13-fold) (36,37), suggesting that hypoxia regulates LOX

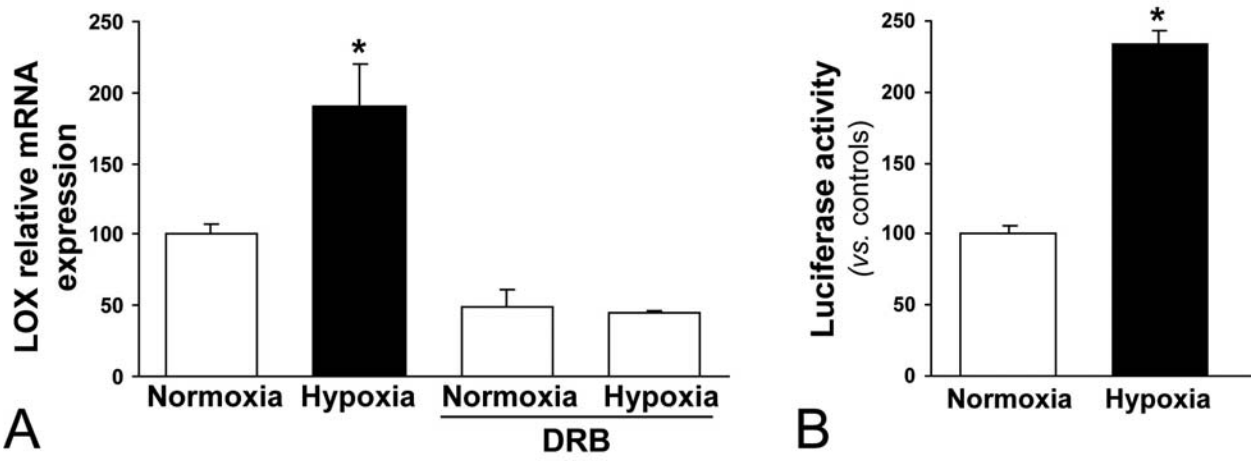

Figure 3. Hypoxia up-regulates LOX transcriptional activity. (A) LOX mRNA levels were evaluated in BAEC exposed to normoxic or hypoxic conditions for 24 h in the presence or in the absence of a transcriptional inhibitor (50 μM DRB). (B) BAEC transfected with the pLOX-834 luciferase construct were exposed to normoxic or hypoxic conditions for 18 h. Luciferase and β-galactosidase activities were determined as described in Methods. Data are expressed as mean ± SEM. (n=9; $p<0.05$: *, *vs.* control cells [normoxia]).

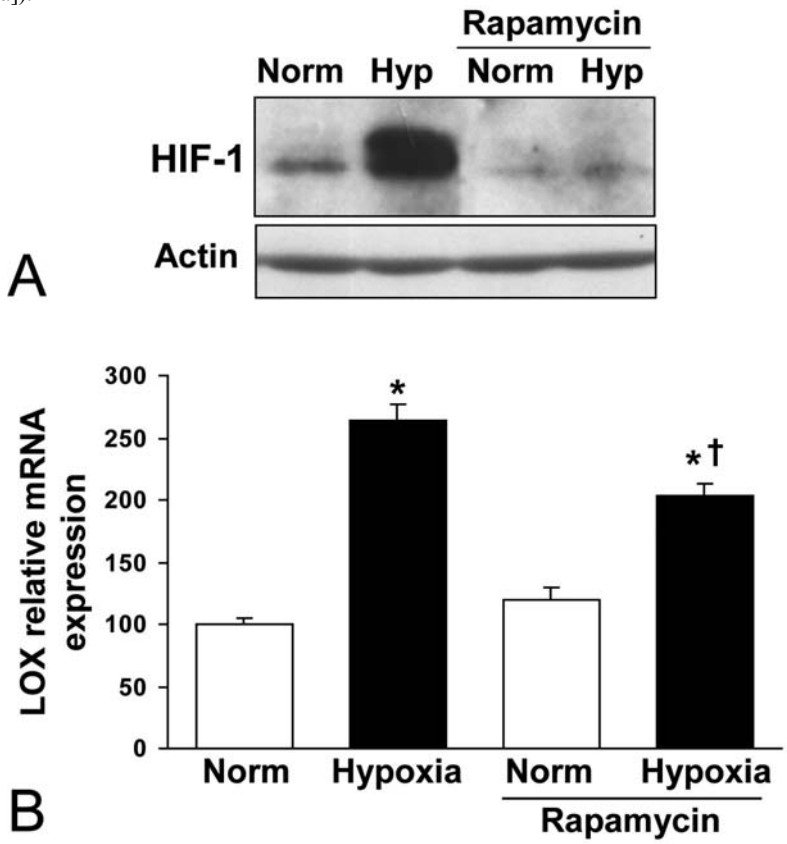

Figure 4. The mTOR pathway is only partially involved in the up-regulation of LOX by hypoxia in endothelial cells. BAEC were preincubated with rapamycin (100 nM) and exposed to normoxia (Norm, controls) or hypoxia (Hyp, 24 h). (A) HIF-1alpha (HIF-1) protein levels were analyzed by Western-blot. Beta-actin (actin) levels were used as a loading control. A representative autoradiogram of three independent experiments performed by duplicate is shown. (B) LOX mRNA levels were determined in these cells. Data are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs*. control cells [normoxia]; †, *vs*. cells exposed to hypoxia in the absence of rapamycin).

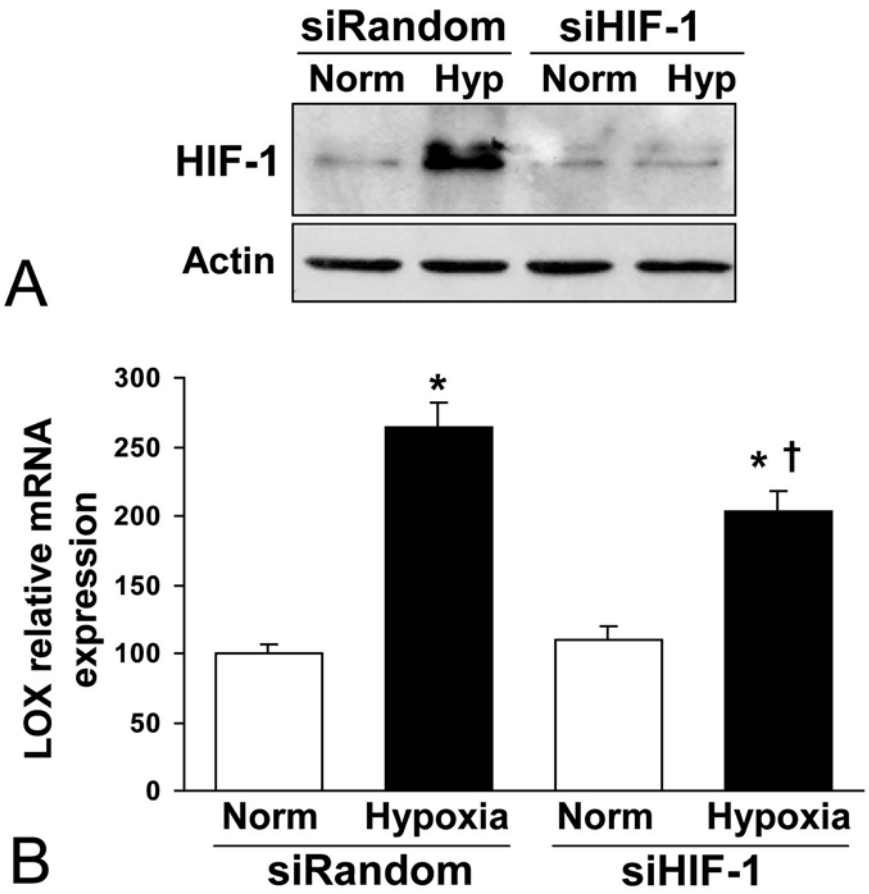

Figure 5. HIF-1alpha silencing partially abrogates hypoxia-induced LOX up-regulation in HUVEC. HUVEC were transfected with a HIF-1alpha specific siRNA (siHIF-1) or a control siRNA (siRandom) and exposed to normoxia or hypoxia during 24 h. (A) Western blot assays confirm the blockade of HIF-1alpha (HIF-1) protein levels by siHIF-1. Beta-actin (actin) levels were used as a loading control. (B) HIF-1alpha silencing slightly prevents the up-regulation of LOX mRNA levels. Results are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs.* normoxic cells transfected with the same siRNA; †, *vs.* hypoxic cells transfected with siRandom).

expression in a cell-type specific manner. In fact, under respiratory hypoxia LOX is up-regulated in white adipose tissue, whereas its expression decreases in other tissues such as muscle (38). Interestingly, the up-regulation of LOX mRNA levels was associated to an increase in both LOX protein levels (analyzed by immunocytochemistry) and activity (evaluated in cell supernatants). In previous studies Postovit *et al.* (37) also reported a marked increase in mature LOX protein levels in tumor cells exposed to hypoxia, but their approach only detected significant changes in LOX activity after cell re-oxygenation, because LOX catalytic activity requires molecular oxygen.

We have shown that in endothelial cells the increase in LOX expression triggered by hypoxia relies on a transcriptional mechanism and seems to be independent of the autocrine secretion of factors such as VEGF. Cell response to hypoxia is mediated by a network of transcription factors (18,39,40), but particularly by HIF-1 (1,2). It has been suggested that besides HIF-1 other transcription factors could participate in the transcriptional up-regulation of LOX under hypoxia, although these factors have not been identified (37). Accordingly, HIF-1alpha silencing or inhibition of the mTOR axis in endothelial cells only slightly prevented LOX up-regulation by hypoxia. In this regard, recent findings indicate that in several tumor cells Notch signaling promotes an increase in HIF-1alpha recruitment to LOX promoter and thereby enhances hypoxia-induced activation of LOX transcription (41). Whether the low up-regulation of LOX in endothelial cells as compared to other cell types is due to a defective interaction between Notch and HIF-1alpha in these cells is currently unknown, however, our data support the participation of other transcriptional mechanisms. In fact, by site-directed mutagenesis we show that the previously reported HRE responsible for hypoxia-dependent LOX up-regulation in tumor cells (HRE/-75) is only responsible for a part of the observed effect. Furthermore, by serial deletion studies we located a hypoxia-responsiveness region between 821 to 405 bp

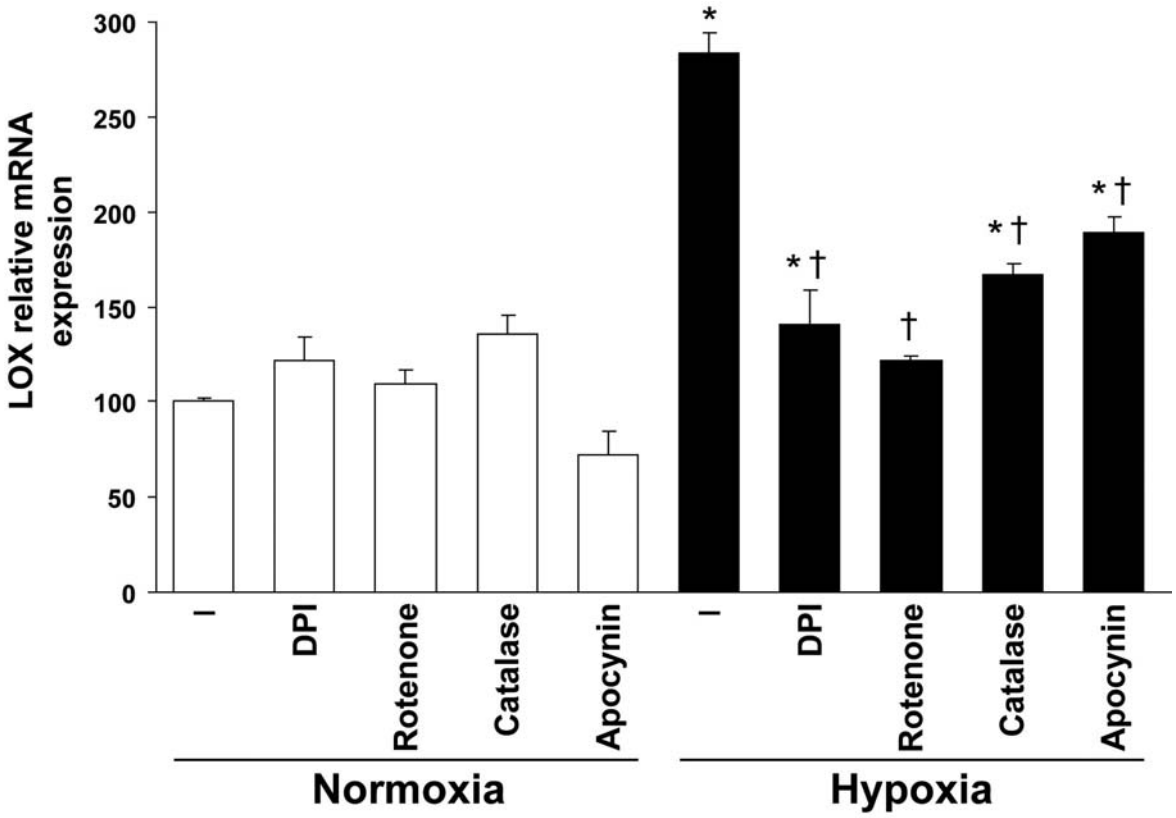

Figure 6. ROS signaling is involved in the up-regulation of LOX by hypoxia in endothelial cells. LOX expression was determined in HUVEC pre-incubated with inhibitors of oxidative stress maintained in normoxia or exposed to hypoxia for 24 h (DPI; 5 μM; Rotenone, 2 μM; Catalase, 3000 U/mL; Apocynin, 100 μM). Data are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs.* control cells; †, *vs*. hypoxic cells).

upstream the transcription start site. In this context, we focused our attention on other transcription factors, specifically in Smad signaling that in endothelial cells participates in cellular responses to hypoxia. Smads are activated by hypoxia in different cell types including HUVEC (24,42,43) and a cooperation between Smad and HIF-1alpha signaling has been extensively reported (44,45). Furthermore, Smads also interact with other transcription factors such as FoxO proteins regulating endothelial cell responses to hypoxia (46). In this context, we have observed that Smad2/3/4 over-expression elicited a further increase in LOX promoter activity in endothelial cells exposed to hypoxia, while baseline LOX transcriptional activity was unaffected by Smad co-transfection under normoxic conditions. This behavior is similar to the Smad-dependent up-regulation of TGF (transforming growth factor) beta-2 by hypoxia reported in HUVEC (24). The mechanism underlying hypoxia-dependent Smad activation is unclear. It has been related with an autocrine process in which bioactivation of TGFbeta-2 by hypoxia results in the induction of the TGFbetaR/Smad axis. As we have indicated above, however, our data ruled out the involvement of autocrine factors. Therefore, since hypoxia activates multiple intracellular kinases able to promote Smad activation by phosphorylation, a TGFbetaR-independent mechanism could not be ruled out.

ROS signaling controls several aspects of the pathophysiology of the vascular wall. ROS alter vascular function and contribute to vascular remodeling and ECM reorganization associated to cardiovascular diseases such as atherosclerosis or hypertension (47). Although the induction of oxidative stress under hypoxia has been surrounded by some controversy, it has been demonstrated that acute exposure to hypoxia results in a sudden increase in ROS generation by mitochondrial complex III. In fact, mitochondrial ROS are required for hypoxia-dependent HIF-1alpha stabilization, probably by direct inhibition of prolyl hydroxylases that target HIF-1alpha for ubiquitination and proteasomal degradation (4,5,23); thus ROS seem to be the initial stimulus that triggers HIF-1alpha -dependent responses under hypoxia. Interestingly, our experiments in the presence of oxidative stress inhibitors evidenced the relevance of ROS signaling in LOX up-regulation by hypoxia. This result is somewhat unexpected given that ROS seem to be essential for HIF-1alpha up-regulation but HIF-1alpha silencing only slightly limited the induction of LOX by hypoxia. In this regard, it should be noted that NADPH oxidase, one of the major sources of ROS in endothelial

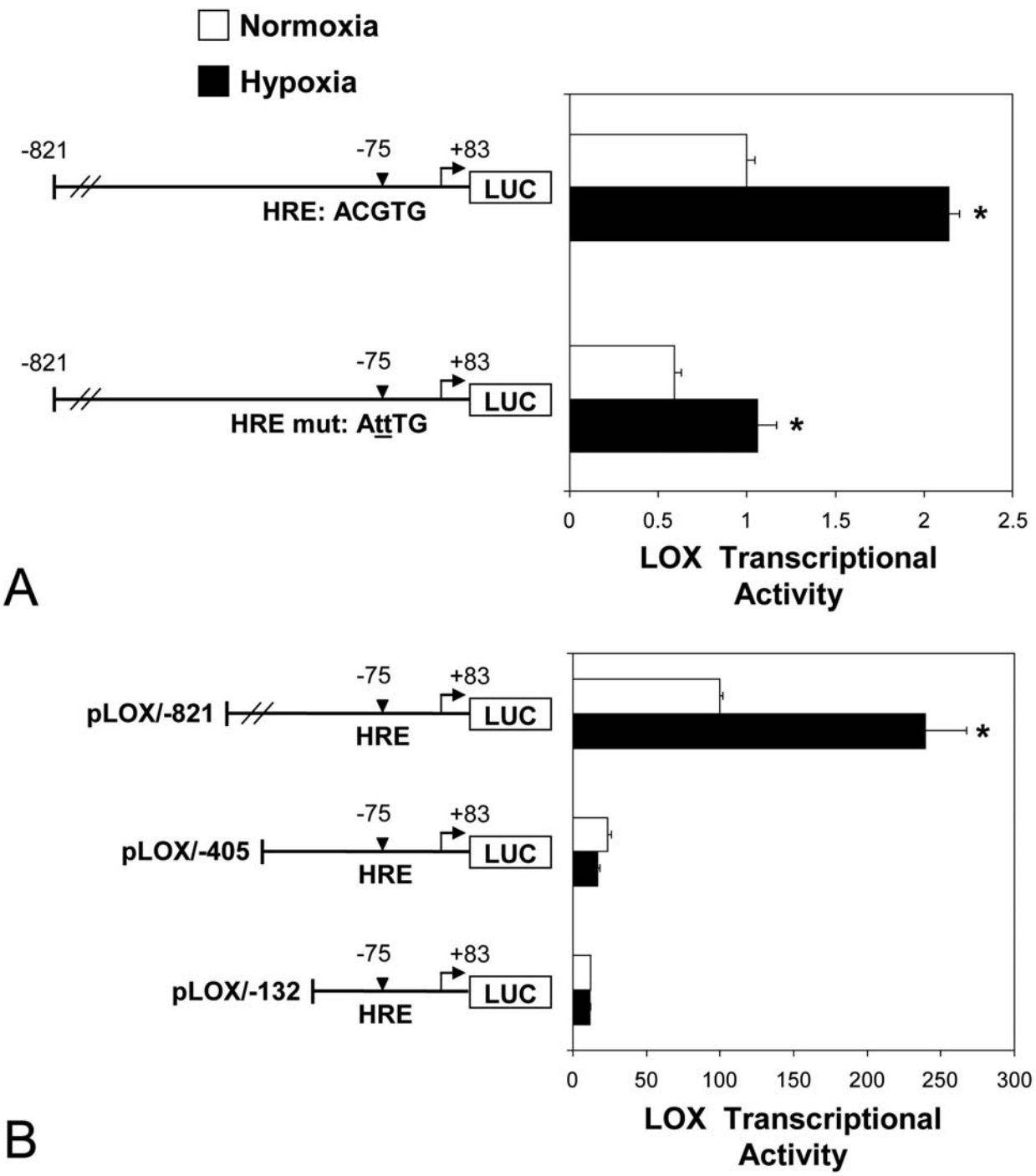

Figure 7. The up-regulation of LOX transcriptional activity by hypoxia is only partially dependent on the hypoxia response element previously identified (HRE/-75). (A) BAEC transfected with the wild-type pLOX-821 luciferase construct or the HRE-mutated vector were cultured under normoxia (white bars) or hypoxia (black bars) and luciferase was determined in cell lysates. Results were normalized by beta-galactosidase activity. The scheme corresponds to the LOX promoter region analyzed in transient transfection studies. The sequence of the putative HRE motif and the changes introduced by mutagenesis are indicated. (B) A promoter serial deletion study was performed using pLOX-821, pLOX-405 and pLOX-132 luciferase constructs. The position of the HRE is indicated. Results are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs.* the same construction under normoxia).

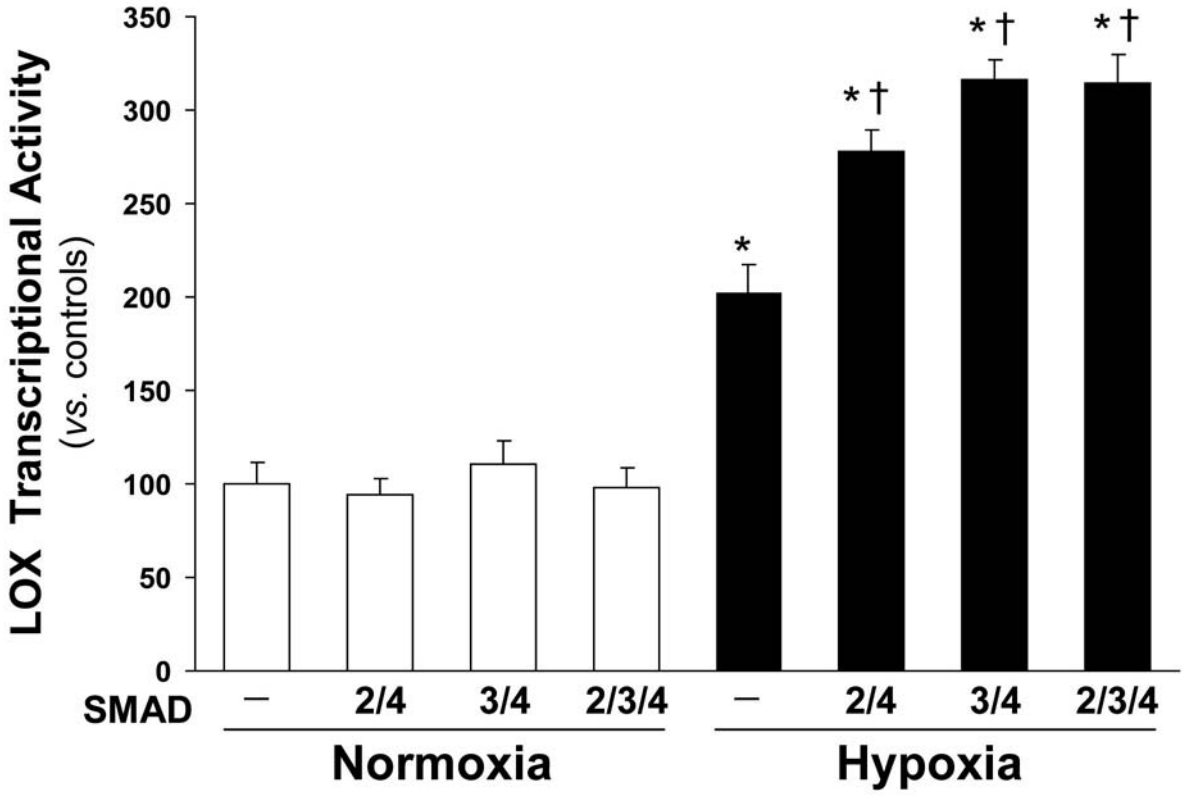

Figure 8. Smad signaling pathway is involved in hypoxia-induced up-regulation of LOX transcriptional activity. BAEC transfected with the wild-type pLOX-821 luciferase construct were co-transfected with the Smad2 and/or Smad3 expression vectors together with the Smad4 construct or the corresponding empty vector (pcDNA3), and were cultured under normoxia (white bars) or hypoxia (black bars). Results are expressed as mean ± SEM (n=9; $p<0.05$: *, *vs.* cells exposed to normoxia; †, *vs.* cells co-transfected with pcDNA3 empty vector (-) and exposed to hypoxia).

cells, could also contribute to hypoxic ROS generation (48) and that its role in hypoxia-dependent signaling has not been clearly established. Furthermore, Smad signaling is also able to induce ROS release (49) and hence, the effect of ROS inhibition on LOX expression should be derived from the blockade of both HIF-1alpha- and Smad-dependent signaling pathways.

In summary, our data support the contribution of Smad and ROS signaling in LOX up-regulation by hypoxia in endothelial cells. Further studies will be required to more accurately define the mechanisms through which the crosstalk between HIF-1, Smad and ROS signaling pathways modulates LOX promoter activity and to establish the functional consequences of LOX up-regulation by hypoxia in endothelial cells.

6. ACKNOWLEDGMENT

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Abbreviations: ECM: extracellular matrix; LOX: lysyl oxidase; LOXL: LOX-like; ROS: reactive oxygen species; HIF: hypoxia inducible factor; ARNT: aryl hydrocarbon-receptor nuclear translocator; BAEC: bovine aortic endothelial cells; HUVEC: human umbilical vein endothelial cells; MLEC: mouse lung endothelial cells; ICAM: intercellular adhesion molecule; mTOR: mammalian target of rapamycin; VEGF: vascular endothelial growth factor; TBP: TATA binding protein;

BAPN: beta-aminopropionitrile; HRE: hypoxia response element; SBE: Smad binding element; TGF: transforming growth factor.

Key Words: Endothelial Cells, Hypoxia, Reactive Oxygen Species, Lysyl Oxidase

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