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Lysyl oxidase and endothelial dysfunction: mechanisms of lysyl oxidase down-regulation by pro-inflammatory cytokines

Javier F. Alcudia, Jose Martinez-Gonzalez, Anna Guadall, Maria Gonzalez-Diez, Lina Badimon, Cristina Rodriguez

Centro de Investigacion Cardiovascular, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, c/Antoni Mª Claret 167, 08025 Barcelona, Spain

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1. ABSTRACT

Lysyl oxidase (LOX) plays a pivotal role in extracellular matrix (ECM) maturation. Furthermore, novel biological functions has been ascribed to LOX, among them cell differentiation, migration, transformation and regulation of gene expression. In this context, it has been suggested that abnormalities of LOX expression could underlie the development of multiple pathological processes including cardiovascular diseases. LOX seems to be crucial in the preservation of endothelial barrier function. In fact, accumulating evidences suggest a role of this enzyme in atherogenesis and endothelial dysfunction triggered by atherosclerotic risk factors and pro-inflammatory cytokines. Indeed, cytokines such as tumour necrosis factor-alpha (TNF-alpha) modulate vascular LOX expression. This cytokine decreases LOX expression and activity in endothelial cells through a transcriptional mechanism that involves TNF receptor-2 and protein kinase C activation. Interestingly, *in vivo* studies reveal that TNF-alpha causes a down-regulation of vascular LOX expression. Thus, LOX down-regulation seems to be associated to the endothelial dysfunction elicited by multiple pathological factors. LOX rises as a promising target gene for the development of therapeutic strategies in the treatment of cardiovascular diseases.

2. INTRODUCTION

Endothelial dysfunction is an early condition associated with the development of cardiovascular diseases (1). Endothelium is a key regulator of vascular homeostasis through its vasodilator, anti-inflammatory and anti-thrombotic properties. However, the endothelial damage elicited by atherosclerotic risk factors disturb the vasoconstriction/vasodilatation and thrombosis/fibrinolysis balances, increases endothelial permeability and promotes leukocyte recruitment leading to atherosclerosis development (2-4). Endothelial function is also impaired by pro-inflammatory cytokines such as TNF-alpha. TNF-alpha induces endothelial dysfunction in both humans and animal models (4, 5). Indeed, this cytokine was detected in endothelial and vascular smooth muscle cells (VSMC) in the early phases of the atherosclerotic process and has also been associated with plaque instability and rupture (6). The mechanisms related with the ability of TNF-alpha to impair endothelial function include an increase in endothelial permeability and a decrease on nitric oxide (NO) bioavailability among other deleterious effects (3, 7-9).

The vasoprotective properties of the endothelium underlie its potential as a target for the development of therapeutic approaches in cardiovascular diseases.

However, this task will require a better understanding of the mechanisms involved in endothelial dysfunction. In this regard, the activation of endothelium involves an early modulation of gene expression. In search of new genes modulated by atherosclerotic risk factors, we identified LOX as a gene regulated by hypercholesterolemia in endothelial cells (10). This enzyme is a copper containing semicarbazide-sensitive amine oxidase that initiates the covalent cross-linking of collagen and elastin (11). LOX activity is essential to maintain ECM structural integrity and normal connective tissue properties and could participate in vascular remodelling associated to atherosclerosis. Here we describe how TNF-alpha and other atherosclerotic risk factors are able to down-regulate endothelial LOX expression and discussed its relationship with endothelial dysfunction and atherosclerosis development.

3. LOX PROPERTIES AND CELLULAR FUNCTIONS

3.1. LOX isoenzymes: mechanism of action and substrate specificity

LOX is a copper-dependent enzyme that oxidatively deaminates peptidyl lysine residues of collagen and elastin fibres leading to the synthesis of peptidyl aldehydes and producing hydrogen peroxide and ammonia as by-products. These highly reactive aldehydes condense spontaneously to form both inter- and intra-chain covalent bounds that allow the synthesis of the mature and insoluble ECM. Copper and lysyl tyrosyl quinone (LTQ) are required cofactors in this reaction. LTQ is a covalently bound cofactor that confers LOX with specific properties different than other aminooxidases. LOX is synthesized as a pre-proLOX that is post-translationally modified in the endoplasmic reticulum and Golgi to yield a 50 kDa pro-enzyme. This pro-enzyme is secreted into the extracellular space where is proteolytically cleaved to release the mature 32 kDa form of LOX and its propeptide (Figure 1). Regarding LOX substrates and besides collagens and elastin, it has been considered that basic globular proteins with pI> 8.0 could be potential substrates of LOX, at least *in vitro* including histones H1 and H2 (for review see ref 11).

LOX is the most extensively-studied member of a family that includes other four structurally-related isoenzymes named LOX-like (LOXL) 1, LOXL2, LOXL3 and LOXL4, whose specific substrates remains unclear. These enzymes have a conserved C-terminal region corresponding to the catalytic domain that includes the LTQ cofactor, the copper binding site and the cytokine receptor like-domain. Although, most LOX isoenzymes posses LOX catalytic activity, these enzymes show different tissue expression pattern (12) and the phenotypic disparities exhibited by the knockout models for LOX and LOXL-1 suggest that each of them should play different biological roles (13,14).

3.2. Novel biological functions of LOX

Surprisingly, in the last few years novel cellular functions beyond its structural role has been ascribed to LOX (Figure 1). In VSMC and monocytes LOX has demonstrated chemotactic properties due to an increased production of hydrogen peroxide (H_2O_2), a LOX by-product, that causes focal adhesion assembly and formation of stress fibres (11). Furthermore, LOX is a phenotypic suppressor of the *ras* oncogene (15) and facilitates cell migration and adhesion in breast cancer cells by the H_2O_2-mediated activation of Src/FAK, kinases critically involved in both cell migration and adhesion. (16). Interestingly, LOX controls gene expression of collagen and elastin (17, 18), ability that has been related with its effect on chromatin condensation and with the use of Histones H1 and H2 as enzyme substrates (11). Finally, even LOX propeptide has demonstrated biological activity. Indeed, it is able to inhibit *ras*-dependent transformation (19). The fact that some of these functions are elicited by cytosolic and nuclear catalytically active forms of LOX (11) and its broad spectrum array of potential substrates, increase the complexity of the potential roles that this enzyme could fulfil on cell homeostasis.

3.3. LOX in pathological processes: role in cardiovascular diseases

Due to this multiple range of biological functions it has been proposed that abnormalities on LOX expression could underlie the development of pathological processes, not solely of those related with an imbalance in ECM synthesis/degradation such as fibrotic disorders (20), but also of neurodegenerative processes (21), tumour progression and metastasis (22) and cardiovascular diseases (13, 10, 23).

The dramatic consequences on vascular structure of LOX inactivation, that even compromises $LOX^{-/-}$ mice viability, supported its key role in the development and function of the cardiovascular system (13), and hence, an altered pattern of LOX expression could be associated to cardiovascular diseases. LOX down-regulation could contribute to the extensive ECM disorganization characteristic of vascular aneurysms and arterial dissections (24, 25). Moreover, different studies have addressed its potential involvement in atherosclerosis. Indeed, catalytically active LOX is chemotactic for VSMC and monocytes (11) and it could induce ECM deposition favouring neointimal thickening associated to plaque progression and restenosis (26). Furthermore, it has been suggested that the insufficient ECM cross-linking due to defects on LOX activity could also be associated to plaque instability and rupture (27). Finally, recent findings showed that a reduction of LOX expression could be related with the endothelial dysfunction triggered by atherosclerotic risk factors (10, 23).

3.3.1. LOX regulation by hypercholesterolemia

Previous mRNA differential display studies looking for genes involved in the earlier stages of the atherosclerotic process allow us to identify LOX as a gene regulated by hypercholesterolemia in endothelial cells and in the vascular wall (10). This approach was successfully used to identify other genes potentially involved in the initiation of atherosclerosis, including the sterol regulatory element binding protein-2 (SREBP-2), lanosterol 14-alpha-

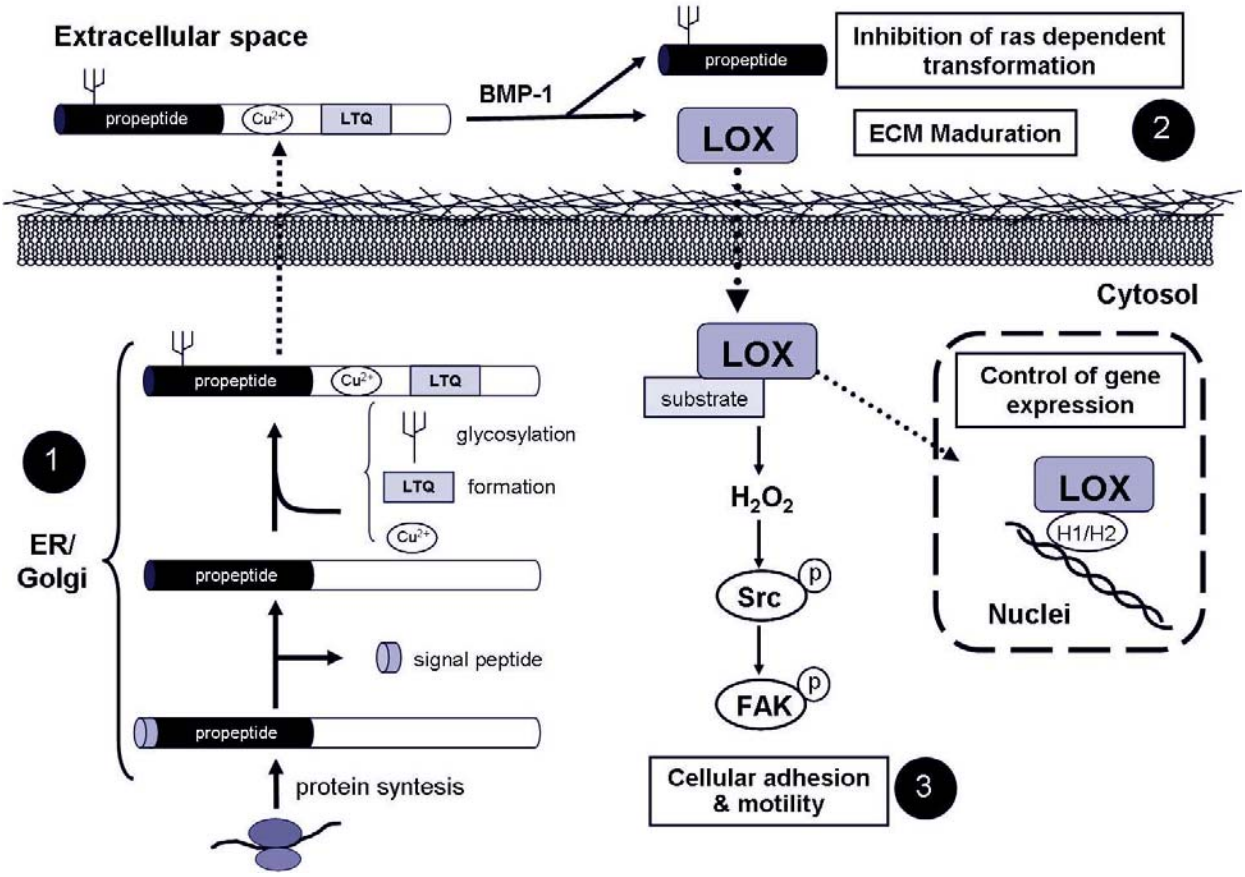

Figure 1. LOX synthesis, and intra and extracellular functions. (1) LOX is synthesized as a pre-proenzyme. In ER/Golgi LOX suffers different post-translational modifications including signal peptide cleavage, glycosylation, copper incorporation and formation of the LTQ cofactor. Then, the protein is transported to the extracellular space, where it is proteolyzed by BMP-1 yielding the mature catalytic form (32 Kda) and LOX propeptide. Mature form acts stabilizing the components of the ECM. LOX propeptide is able to exert biological responses such as the induction of phenotypic reversion of ras-transformed cells (2). Extracellular LOX could translocate to intracellular compartments where elicits multiple functions such as the control of cell adhesion and motility in cancer cells or the regulation of gene expression, probably using histones H1/H2 as substrates (3) (LTQ: Lysyl tyrosyl quinone; BMP-1: Bone morphogenetic protein-1; ECM: extracellular matrix; FAK: Focal adhesion kinase; Src: Src-kinase).

demethylase (CYP51) and C-4 sterol methyl oxidase (ERG25) (28, 29). Given the importance of LOX in ECM maturation and the relevance of ECM remodelling in cardiovascular diseases subsequent studies were focused on the involvement of this enzyme in atherosclerosis and endothelial dysfunction. In this regard, we have observed that LOX inhibition increases the exchange of macromolecules across an endothelial monolayer, suggesting that the alteration of ECM structure due to LOX down-regulation causes a dysfunction of endothelial barrier (Figure 2A). Our studies revealed that LOX expression and activity in endothelial cells in culture were reduced by atherogenic concentrations of low-density lipoproteins (LDL) (Figure 2B) and that a decrease on LOX transcriptional activity precedes LOX down-regulation. The *in vivo* relevance of these results was confirmed in the porcine model of hypercholesterolemia, a model of early atherosclerosis, in which a hypercholesterolemic diet reduces vascular LOX expression about 3-fold (Figure 2C). Conversely, it has been previously reported no changes or even increases in vascular LOX expression under hypercholesterolemia in other animal models (30), discrepancies that could be explained by differences in the type of lesions in each animal model. Furthermore, sera from type IIA hyperlipoproteinemic patients inhibited collagen synthesis but not LOX activity in VSMC (31), although there are no data about the expression level of LOX in the vascular wall of these patients. Nowadays a method to determine circulating levels of LOX is not available, tool of high interest in order to establish the clinical relevance of our experimental data. Hence, our results suggest that vascular LOX down-regulation could be an early phenomenon underlying endothelial dysfunction triggered by hypercholesterolemia and support a relevant role of LOX in the maintenance of endothelial barrier integrity.

3.3.2. Regulation of LOX by hyperhomocysteinemia

Hyperhomocysteinemia is a well-established inducer of endothelial dysfunction that is associated with

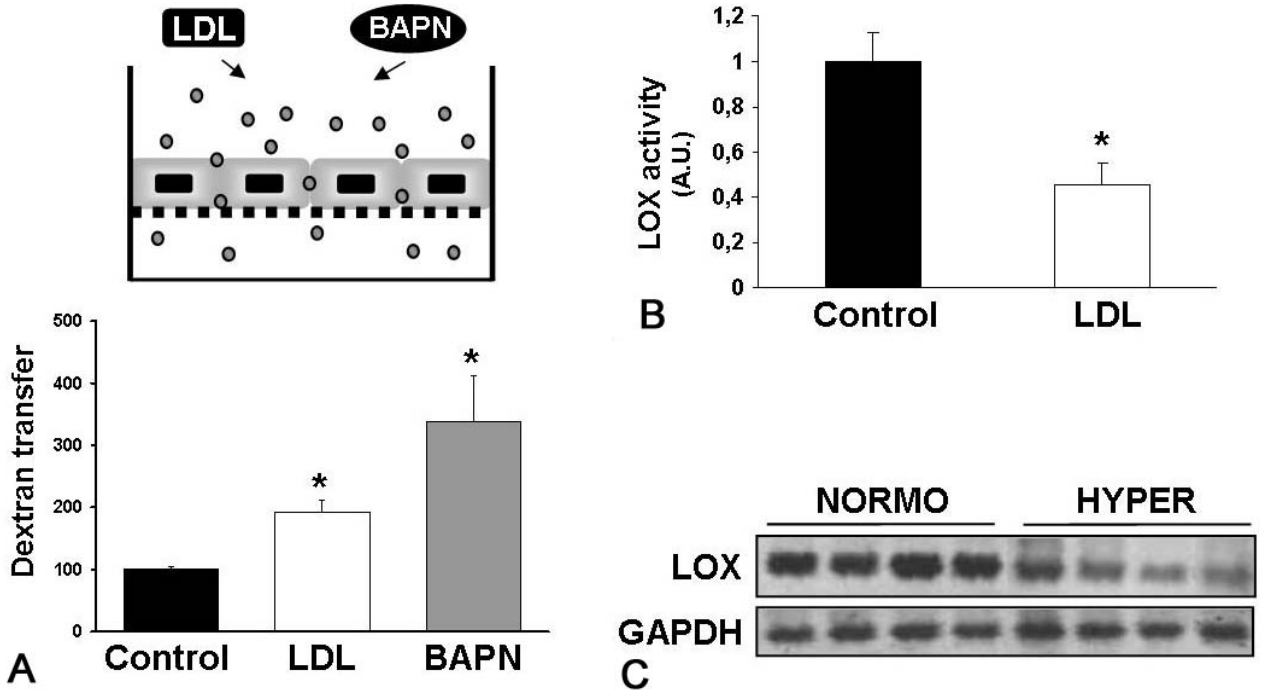

Figure 2. Hypercholesterolemia down-regulates LOX expression and activity in endothelial cells and in the vascular wall. (A) LOX inhibition by BAPN (a specific LOX inhibitor) or LDL increases endothelial permeability. Endothelial permeability was determined by the exchange of FITC-labelled dextran through an endothelial monolayer in a transwell system, as shown in the upper panel ($p<0.01$ **vs.* control cells). (B) LOX activity was assessed by a fluorimetric assay in cell supernatants from porcine aortic endothelial cells (PAEC) stimulated with LDL (180 mg/dL) for 48 h. Briefly, an aliquot of the media was incubated in the presence or in the absence of BAPN at 37ºC for 30 min with 1 U/mL of horseradish peroxidase, 0.01 mmol/L Amplex red (Molecular Probes) and 10 mmol/L 1,5-diaminopentane in 1.2 mol/L urea, 0.05 mol/L sodium borate pH 8.2. Differences in fluorescence intensity (excitation wavelength: 563 nm; emission wavelength: 587 nm) between samples with and without BAPN were determined. (C) Hypercholesterolemic diet reduced LOX mRNA levels evaluated in the abdominal aorta from pigs. LOX expression was determined by RT-PCR analysis using specific oligonucleotides for LOX. GADPH was used as a housekeeping gene (BAPN: Beta-aminopropionitrile; FITC: Fluorescein isothiocyanate; Normo: normolipemic animals, Hyper: hypercholesterolemic animals).

disturbances in the elastic properties of ECM. We have observed that homocysteine (HC) is also able to inhibit LOX activity in endothelial cells, an effect dependent on the presence of the free thiol group of this amino acid and mediated by the oxidative stress induced by HC (23). Indeed, it has been previously reported that HC thiolactone (HCTL) and other HC analogues inhibit LOX activity (32). HC is also able to down-regulate both LOX mRNA and transcriptional activity after time-extended treatments with high concentrations of HC. However, the early inhibition of LOX activity caused by HC (after 3 h of incubation) suggests that the primary effect of this amino acid is exerted on enzymatic activity.

3.3.3. Regulation of LOX by pro-inflammatory cytokines

In this context, our interest was focused on the effect of pro-inflammatory cytokines, specifically of TNF-alpha, on LOX expression (33). We have observed that TNF-alpha administration in rats elicited a down-regulation of vascular LOX expression with a similar magnitude than that caused by hypercholesterolemia in the porcine model (Figure 3A). *In vitro* studies demonstrated that TNF-alpha decreases LOX expression in endothelial cells in a dose- and time-dependent manner, effect that precedes a reduction of LOX activity (Figure 3B and C). An accurate analysis of the mechanisms that contribute to this effect revealed that TNF-alpha-induced LOX down-regulation is mediated through a transcriptional mechanism similarly to LDL. Experiments using specific antibodies with either agonist or antagonist activity to TNF receptors (TNFR) demonstrated that TNFR2 mediates LOX-down-regulation by TNF-alpha (Figure 4A). This result is consistent with the observation that TNFR2 preferentially modulates the responses to low concentrations of TNF-alpha similar to those used in this study (34). Signalling pathways downstream TNFR2 and effectors for this receptor are mostly unknown, however, protein kinase C (PKC) mediates some of the deleterious effects of TNF-alpha on endothelial function (9). Indeed, we have observed that PKC inhibitors counteract the effect of TNF-alpha on either LOX mRNA levels or transcriptional activity (Figure 4B), suggesting the involvement of this family of protein kinases on LOX regulation. Altogether, these data show that TNF-alpha exerts a down-regulatory effect on LOX expression and function in endothelial cells in culture and in the

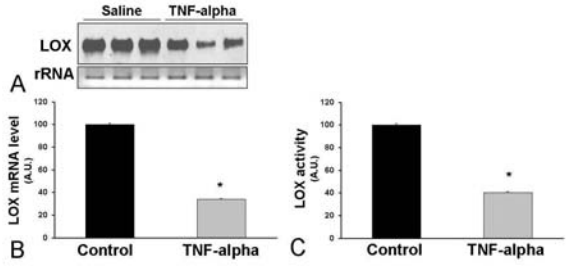

Figure 3. TNF-alpha decreases LOX expression in the vascular wall and in endothelial cells. (A) LOX mRNA levels were evaluated by semi-quantitative RT-PCR in thoracic aorta samples form TNF-alpha–treated rats or control animals (saline). A representative autoradiography is shown. PAEC were incubated with TNF-alpha (1 ng/mL) and both LOX mRNA levels (B) and enzymatic activity (C) were evaluated by real-time PCR using the Assay-on-Demand system (Applied Biosystems) and a fluorimetric assay respectively. (p<0.01 **vs.* control cells).

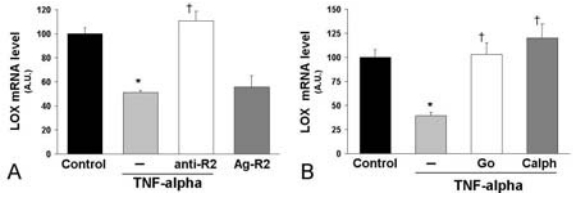

Figure 4. TNF-alpha decreases LOX expression through TNFR2 activation and PKC induction. (A) LOX mRNA levels from HUVEC stimulated with TNF-alpha (1 ng/mL; 21 h) or an agonist antibody to TNFR2 (5 µg/mL) were evaluated by real-time PCR using specific primers and probes provided by the Assay-on-Demand system (Applied Biosystems). In some experiments cells were pre-incubated with neutralizing antibodies against TNFR2 (25 µg/mL) 1 h prior to TNF-alpha addition. Neither an agonist antibody to TNFR1 nor an antagonist antibody against TNFR1 were able to modulate LOX expression or TNF-alpha-dependent LOX down-regulation. (B) The PKC inhibitors, Go6976 (6.2 nmol/L) and calphostin C (200 nmol/L) prevented the TNF-alpha-dependent down-regulation of LOX mRNA levels. (p<0.01 **vs.* control cells; † *vs.* cells incubated with TNF-alpha). (TNFR2: Tumour necrosis factor receptor-2; anti-R2: antagonist antibody against TNFR2; ag-R2: agonist antibody to TNFR2; Go:Go6976; Calph: Calphostin C).

arterial wall, thus emphasizing the role of LOX as a key enzyme in endothelial function and homeostasis.

4. SUMMARY

In summary, we have observed that several atherosclerotic risk factors and pro-inflammatory cytokines that induce endothelial dysfunction down-regulate vascular LOX expression/activity. In this context, the loss of endothelial cell adhesion and the phenotypic changes displayed by endothelial cells observed in LOX knockout animals, the strong expression of LOX in vascular endothelium, its relationship with the maintenance of endothelial barrier function and the reported down-regulation of LOX by multiple factors that impair endothelial function support the hypothesis that LOX inhibition could be an early event underlying endothelial dysfunction. It is evident that further studies must be performed in order to confirm this concept and that multiple questions remains unresolved, including the identity of those transcription factors and signalling pathways that participates in both LDL- and TNF-alpha-dependent LOX down-regulation. Furthermore, it must be established if LOX inhibition could play yet undefined functions in endothelial function beyond the maintenance of endothelial barrier integrity. However, these data suggest that LOX could be a target gene in the endothelium in order to develop suitable therapeutic tools for the treatment of cardiovascular diseases.

5. ACKNOWLEDGEMENTS

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Abbreviations: LOX: lysyl oxidase; LOXL: lysyl oxidase-like; TNF: tumour necrosis factor; ECM: extracellular matrix; VSMC: vascular smooth muscle cells; NO: nitric oxide; LTQ: lysyl tyrosyl quinone; BMP: bone morphogenetic protein; LDL: low density lipoproteins; HC: homocysteine; HCTL; homocysteine thiolactone; TNFR: tumour necrosis factor receptor; PKC; protein kinase C; ER: endoplasmic reticulum; PAEC: porcine aortic endothelial cells; FITC: Fluorescein isothiocyanate; BAPN: beta-aminopropionitrile; GADPH: glyceraldehyde-3-phosphate dehydrogenase; PCR; polymerase chain reaction; RT-PCR: reverse transcriptase-PCR; HUVEC: human umbilical vein endothelial cells; calph: calphostin C; Go: Go6976.

Key Words: Lysyl oxidase, Endothelial Dysfunction, Atherosclerosis, Cytokines, Review

Send correspondence to: Dra. Cristina Rodriguez, Centro de Investigacion Cardiovascular (CSIC-ICCC). Hospital de la Santa Creu i Sant Pau, c/Antonio Mª Claret 167, 08025 Barcelona, Spain, Tel: 34-93-5565897, Fax: 34-93-5565559, E-mail: crodriguezs@csic-iccc.org

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