Inhibition of inducible nitric oxide synthesis by oxidized lipoprotein(a) in a murine macrophage cell line

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Abstract Increased plasma levels of human lipoprotein(a) (Lp(a)) are highly correlated with the development of atherosclerotic lesions. During our study, we investigated the effects of native and hypochlorite oxidized lipoprotein(a) (ox-Lp(a)) on nitric oxide production by the inducible nitric oxide synthase (iNOS) in lipopolysaccharide/interferon stimulated mouse macrophages (J774A.1). Ox-Lp(a) (0–2 μg/ml) induces a dose dependent inhibition of inducible nitric oxide synthesis. iNOS protein expression showed a dose dependent reduction as revealed by immunoblotting when cells were incubated with increasing amounts of ox-Lp(a). Ox-Lp(a) decreases iNOS mRNA synthesis as shown by reverse transcription-polymerase chain reaction. Ox-Lp(a) induced iNOS inhibition might contribute to the development of atherosclerotic lesions by reducing the anti-atherogenic effects of nitric oxide.

Key words: Nitric oxide; Lipoprotein(a); Mouse macrophage; Atherosclerosis; Inducible nitric oxide synthase

1. Introduction

The plasma concentration of human lipoprotein(a) (Lp(a)) is highly correlated with coronary artery disease [1]. Lp(a) consists of low density lipoprotein (LDL) with the additional protein component, apolipoprotein(a) (apoLp(a)), a homolog of plasminogen [2]. The protein moiety of Lp(a), apoLp(a), consists of two apoproteins, apo(a) and apoB-100, linked by one or more disulfide bonds(s). Apo(a), the protein unique to Lp(a), exists in polymorphic forms that exhibit different apparent molecular weights in the range of 419–838 kDa [1].

It has been shown that Lp(a) accumulates in the arterial wall, partly in the form of lipoprotein-like particles, therefore contributing to plaque formation and coronary heart disease [3–5]. The amount of apo(a) deposition in cerebral vessels correlated well with the degree of cerebral atherosclerosis [6]. Lp(a) has been detected both extracellularly and within foam cells [7]. Oxidative modification of Lp(a) results in its recognition and uptake by scavenger receptors on macrophages [8–10]. As these receptors are not down-regulated by the intracellular cholesterol level, the macrophages transform to foam cells [11].

The mechanisms by which Lp(a) may accelerate the development of atherosclerotic lesions remain obscure. Lp(a) and apoLp(a) enhance proliferation of human vascular smooth muscle cells (VSMC) in culture [12]. This has been explained by inhibiting activation of plasminogen to plasmin, thus blocking the proteolytic activation of transforming growth factor β (TGF-β), an autocrine inhibitor of human VSMC proliferation. Oxidized Lp(a) (ox-Lp(a)) has been shown to impair nitric oxide (NO) mediated, endothelium dependent dilation in renal arteries, probably by increasing superoxide anion production and by enhanced inactivation of NO [13].

NO is synthesized from L-arginine by the L-arginine–NO pathway [14]. A family of enzymes, termed the nitric oxide synthases (NOS), catalyze the formation of NO and citrulline from L-arginine, O_2, and NADPH [15]. The inducible isoform of NOS (NOS-2 or iNOS) generates large amounts of NO over a prolonged period of time through a Ca^{2+} independent pathway [16]. iNOS expression has been observed in many cells, including murine macrophages [17], endothelial cells [18], smooth muscle cells [19], and cardiac myocytes [20]. NO inhibits proliferation of VSMCs, mesangial cells, and fibroblasts [21].

The development of atherosclerotic lesions as a consequence of decreased NO synthesis has been reported [22,23]. The anti-atherogenic properties of NO include antiproliferative actions such as the inhibition of smooth muscle and T-cell proliferation [24], reduced neutrophil adhesion [25,26], inhibition of platelet activation [27], and the reduction of endothelial hyperpermeability [28]. The low release of NO, both basal and stimulated, has been reported for atherosclerotic vessels [29]. Atherosclerosis associated factors with the ability to reduce iNOS include oxidatively modified LDLs [30] and heat shock proteins [31]. In situ hybridization confirmed that the iNOS is present in macrophages, foam cells, and VSMCs of atherosclerotic lesions [32–35]. However, the effects of ox-Lp(a) on inducible NO synthesis have not yet been investigated.

Using interferon γ (IFN-γ)/lipopolysaccharide (LPS) stimulated macrophages we examined the effects of native Lp(a) and hypochlorite (HOCl) oxidized Lp(a) on inducible NO synthesis. During our experiments HOCl oxidized Lp(a) was used because HOCl might be released from activated neutrophils in vivo [36], and the occurrence of HOCl oxidized lipoprotein within atherosclerotic lesion has been demonstrated [37]. The inhibition of inducible NO synthesis by ox-Lp(a) might initiate macrophage proliferation since NO decreases cell proliferation by increasing the rate of apoptosis [38]. Thus, ox-Lp(a) induced iNOS inhibition might be relevant for the development of atherosclerotic lesions.

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2. Materials and methods

2.1. Materials

[^{3}H]L-Arginine (68 Ci/mmol) was supplied by Amersham Corp., Arlington Heights, IL, USA. Recombinant mouse IFN-γ was purchased from Gibco BRL, Gaithersburg, MD, USA. Rabbit anti-iNOS polyclonal antibody and purified iNOS protein was supplied by Calbiochem, San Diego, CA, USA. Cell culture materials, *Escherichia coli* LPS serotype 055:B5, Lp(a), and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Cell culture

The mouse monocyte/macrophage cell line J774A.1 (ATCC TIB 67) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2, and 95% humidity. Cells were studied between passages 7 and 20. Cells were seeded in 24 well dishes at a density of 2×10^5 cells/well, stimulated by IFN-γ (10 U/ml) and LPS (2 μg/ml), and incubated either with or without the indicated lipoproteins for 24 h.

2.3. Nitrite analysis

Nitrite was determined spectrophotometrically using the Griess reagent (0.5% sulfanilic acid, 0.002% *N*-1-naphthyl-ethylenediamine dihydrochloride, 14% glacial acetic acid) in supernatants. Absorbance was measured at 550 nm with baseline correction at 650 nm and nitrite concentration was determined using sodium nitrite as a standard [39].

2.4. Oxidation of Lp(a)

The preparation of HOCl oxidized Lp(a) was performed as described previously [36,40]. The molar excess of HOCl over Lp(a) was in the range of 400–600. After allowing the HOCl reagent to react with Lp(a) for 15 min on ice, Lp(a) was passed over a size exclusion column (Bio-Rad 10DG) equilibrated with phosphate buffered saline containing 100 μM EDTA to remove excess reagent. Agarose gel electrophoresis showed increased electrophoretic mobility (1.4-fold) of ox-Lp(a) particles.

2.5. Arginine transport

Arginine uptake by macrophages was measured by adding [^{3}H]L-arginine (68 Ci/mmol, 0.2 μCi/well) directly to the medium after a 24 h incubation with or without ox-Lp(a) as indicated. After 60 min the medium was aspirated and the cells were washed twice with 500 μl ice-cold phosphate buffered saline. Cells were lysed with 200 μl of 300 mM sodium hydroxide containing 1% sodium dodecyl sulfate (SDS). Samples were aspirated and subjected to liquid scintillation counting.

2.6. Protein determination

Protein was determined according to the method of Bradford [41] using bovine serum albumin as standard.

2.7. Western blotting for iNOS

Cells were lysed in ice-cold buffer containing 25 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 20 μg/ml aprotinin, and 10 μg/ml trypsin inhibitor and centrifuged at $50\,000\times g$ for 20 min at 4°C. The cytosolic proteins (20 μg per lane) were separated by 8% SDS–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose filters, and then immunoblotted with a rabbit anti-iNOS polyclonal antibody at a 1:1000 dilution. Anti-rabbit horseradish peroxidase conjugated antibody was used as a secondary antibody at a dilution of 1:2500. The blots were detected with the enhanced chemiluminescence method and exposed to photographic film.

2.8. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the guanidinium thiocyanate method [42]. To determine the RNA concentration the absorption at 260, 280, and 320 nm was measured photometrically (UV/Vis Spectrophotometer Lambda 2, Perkin-Elmer, Norwalk, CT, USA). Single stranded cDNA synthesis was carried out on 2 μg of total RNA primed with oligo(dT)$_{12-18}$ (Pharmacia, Freiburg, Germany) using murine leukemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Vilnius, Lithuania) at 37°C for 60 min. Reactions were stopped by heating for 5 min at 70°C. iNOS cDNA was subjected to DNA amplification by PCR using 0.5 units of Taq DNA polymerase (MBI) with oligonucleotide primers complementary to murine iNOS cDNA (MWG-Biotec, Ebersberg, Germany) at a final concentration of 0.25 μM. Reaction mixtures were subjected to the following conditions in a PE 2400 DNA thermal cycler (Perkin-Elmer): denaturing at 94°C for 30 s, annealing at 55°C for 35 s, and extension at 72°C for 35 s. After 35 cycles, the reaction mixture was cooled down to 4°C. The primers for iNOS were 5′-CTA AGA GTC ACC AAA ATG GCT CCC-3′ (sense) and 5′-ACC AGA GGC AGC ACA TCA AAG C-3′ (antisense). The expected product length was 775 bp. As a control, we used the following primers for the 'housekeeping gene' β-actin: 5′-ATG GTG GGA ATG GGT CAG AAG GAC-3′(sense) and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (antisense). PCR conditions were as above with the exception that only 30 cycles were run. The expected product length was 513 bp. All PCR reactions were in linear range. Final PCR products were separated on a 1.2% agarose gel and detected by ethidium bromide staining. Semiquantitative estimation was done by comparing mRNA expression of iNOS to β-actin represented by the amount of the PCR product formed.

2.9. Data analysis

Each experimental result as shown in the figures is the mean ± S.D. of at least three measurements. When S.D. is not displayed, it is smaller than the size of the symbol. Statistical analyses were performed by use of ANOVA followed by Student's *t*-tests for unpaired data. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Inhibition of inducible NO synthesis by ox-Lp(a)

Activated J774A.1 cells released large amounts of nitrite into the culture medium (71 ± 8.3 nmol nitrite/mg protein within 24 h). Incubation of activated J774A.1 cells with ox-Lp(a) (0–2 μg/ml) was associated with a dose dependent reduction in NO production (Fig. 1). Incubations with native Lp(a) revealed no inhibition of NO synthesis. Measurements of cytotoxicity were performed since toxic effects of oxidized lipoproteins towards various cell species have been described. At the concentrations of ox-Lp(a) or native Lp(a) used during our study, cell viabilities were >95% as measured by trypan

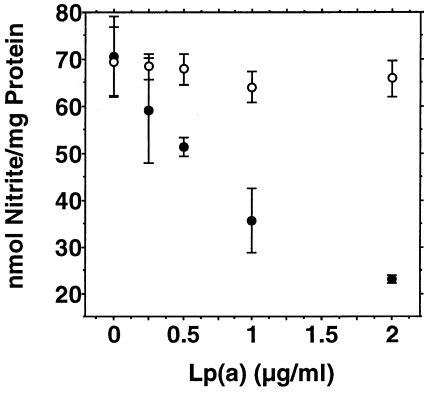

Fig. 1. Dose dependent inhibition of NO production by HOCl ox-Lp(a) in J774A.1 macrophages. Cells were activated with 10 U/ml IFN-γ and 2 μg/ml LPS and incubated with increasing amounts (0–2 μg/ml final concentration) of oxidized (●) or native (○) Lp(a) as indicated for 24 h. After 24 h the culture media were collected and assayed for nitrite as described in Section 2. Each data point shows the mean of triplicate measurements. Error bars show the standard deviation. $P<0.01$ or smaller for incubations containing 0.5–2 μg/ml ox-Lp(a) when compared to controls without ox-Lp(a). Differences between the incubations containing native Lp(a) were not statistically significant.

blue exclusion. To exclude that ox-Lp(a) interferes with detection of nitrite by the Griess reaction or influences the stability of NO, we incubated ox-Lp(a) (0–2 μg/ml) with the NO donor linsidomine (2 mM) for 24 h at room temperature. Ox-Lp(a) had no statistically significant effect on the nitrite measured (122 ± 11 μM nitrite without ox-Lp(a) vs. 131 ± 12 μM nitrite for 1 μg/ml ox-Lp(a) and 138 ± 8 μM nitrite for 2 μg/ml ox-Lp(a)). We conclude, therefore, that ox-Lp(a) does not scavenge NO or interfere with the detection of nitrite by the Griess reaction.

3.2. Effects of ox-Lp(a) on arginine transport

To investigate whether ox-Lp(a) induced inhibition of NO production was associated with a decreased arginine uptake, J774A.1 cells were incubated with [^{3}H]L-arginine as described in Section 2. Arginine transport into the cells was not significantly reduced when incubated with increasing amounts (0–2 μg/ml final concentration) of ox-Lp(a) (data not shown). It can be concluded that inhibition of NO synthesis is not a consequence of reduced cellular arginine uptake.

3.3. Inhibition of iNOS protein expression by ox-Lp(a)

Fig. 2 shows the Western blot analysis of iNOS in J774A.1 cells. IFN-γ/LPS stimulated macrophages were cultured with increasing amounts of HOCl oxidized Lp(a) (0–2 μg/ml) for 24 h. Immunoblotting shows a band with an estimated molecular mass of 130 kDa (the known molecular mass of iNOS) in stimulated J774A.1 mouse macrophages. An identical molecular mass was determined by blotting against purified iNOS protein. iNOS protein showed a dose dependent reduction when cells were incubated with 0–2 μg/ml HOCl oxidized Lp(a). In contrast, actin (43 kDa) levels remained unchanged during incubations of IFN-γ/LPS stimulated J774A.1 mouse macrophages with increasing amounts of ox-Lp(a). This shows that ox-Lp(a) does not cause a generalized decrease in protein expression.

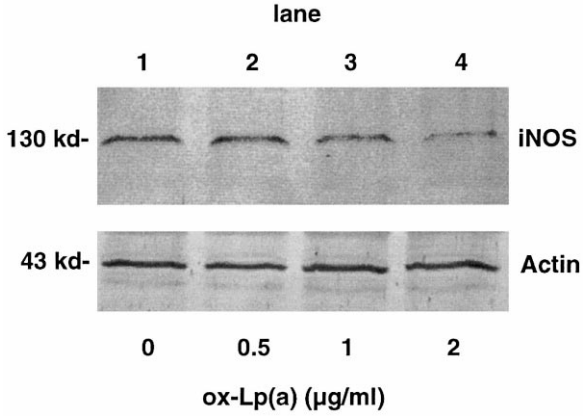

Fig. 2. Immunoblotting against iNOS. IFN-γ/LPS stimulated J774A.1 mouse macrophages were cultured with increasing amounts of HOCl oxidized Lp(a) (0–2 μg/ml) for 24 h. Western blotting was performed as described in Section 2. Lane 1: control incubations of stimulated J774A.1 mouse macrophages without ox-Lp(a); lanes 2–4: incubations containing 0.5–2 μg/ml ox-Lp(a). Immunoblotting identified a band with an estimated molecular mass of 130 kDa (iNOS) in stimulated J774A.1 mouse macrophages. iNOS protein was markedly reduced in cells incubated with ox-Lp(a) (lanes 2–4). In contrast, actin (43 kDa) levels remained unchanged during incubations of IFN-γ/LPS stimulated J774A.1 mouse macrophages with increasing amounts of ox-Lp(a).

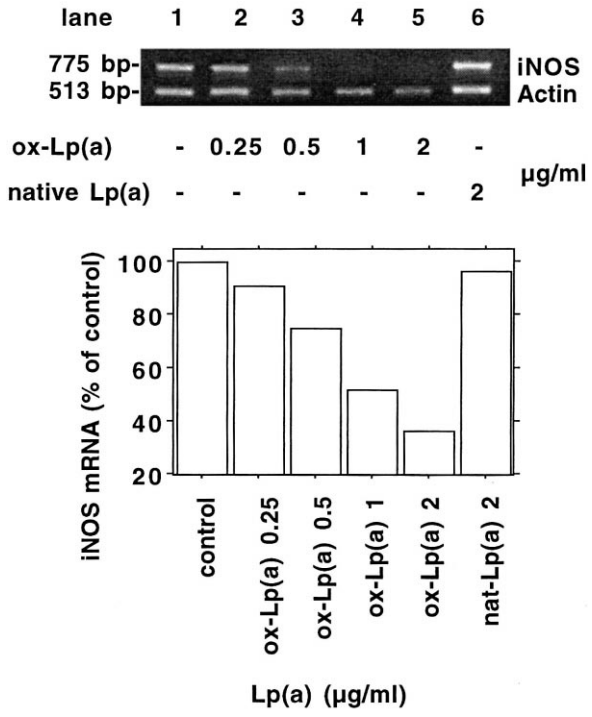

Fig. 3. Effects of ox-Lp(a) on iNOS mRNA expression (RT-PCR). J774A.1 mouse macrophages were incubated with 0–2 μg/ml HOCl oxidized Lp(a) or 2 μg/ml native Lp(a) and IFN-γ/LPS for 24 h. RNA was extracted and analyzed as described in Section 2, single stranded cDNA synthesis was performed, and DNA was amplified by PCR using specific primers for iNOS and actin. iNOS mRNA (775 bp) was reduced by ox-Lp(a) in a dose dependent manner (lane 1–5). Native Lp(a) (lane 6) had no effect on iNOS mRNA level. Semiquantitative estimation was done by comparing mRNA expression of iNOS to β-actin represented by the amount of the PCR product formed.

3.4. Effects of ox-Lp(a) on iNOS mRNA expression

Fig. 3 shows the RT-PCR analysis of iNOS mRNA in J774A.1 cells. IFN-γ/LPS stimulated mouse macrophages were incubated with 0–2 μg/ml HOCl oxidized Lp(a) for 24 h. iNOS mRNA (775 bp) was reduced by ox-Lp(a) in a dose dependent manner (lanes 1–5). Native Lp(a) (lane 6) had no effect on iNOS mRNA level. Semiquantitative estimation was done by comparing mRNA expression of iNOS to β-actin represented by the amount of the PCR product formed. Our data show that ox-Lp(a) inhibits iNOS expression on a transcriptional level.

4. Discussion

This paper shows that HOCl oxidized Lp(a), but not native Lp(a), inhibits inducible NO synthesis in IFN-γ/LPS stimulated J774A.1 mouse macrophages in a dose dependent manner. The ox-Lp(a) induced iNOS (EC 1.14.13.39) inhibition was associated with a dose dependent reduction of iNOS protein as revealed by immunoblotting when cells were incubated with 0–2 μg/ml HOCl oxidized Lp(a). In contrast, actin levels remained unchanged during incubations of IFN-γ/LPS stimulated J774A.1 mouse macrophages with increasing amounts of ox-Lp(a). This shows that ox-Lp(a) does not cause a generalized decrease in protein expression. iNOS mRNA was reduced by ox-Lp(a) in a dose dependent manner. Native Lp(a) had no

effect on iNOS mRNA level. This shows that ox-Lp(a) inhibits iNOS expression on a transcriptional level.

During our experiments HOCl was used for Lp(a) oxidation because its physiological relevance has been emphasized [37]. HOCl is an oxidant produced from H_2O_2 and chloride by myeloperoxidase (MPO, EC 1.11.1.7). MPO is present in its active form in human atherosclerotic lesions and might be released from activated neutrophils in vivo [43]. HOCl oxidized Lp(a) and HOCl oxidized LDL are both effective ligands for loading mouse peritoneal macrophages in vitro [36]. HOCl oxidized proteins have been demonstrated in human atherosclerotic lesions using monoclonal antibodies and were predominantly found in monocytes/macrophages, smooth muscle cells, and endothelial cells [37]. Oxidative modification of Lp(a) results in its recognition and uptake by the macrophage scavenger receptor system [8]. As these receptors are not down-regulated by the intracellular cholesterol level, the macrophages transform to foam cells [11].

Lp(a) plasma levels are strongly correlated with the development of atherosclerotic lesions. It has been shown that Lp(a) accumulates in the arterial wall, partly in the form of lipoprotein-like particles, therefore contributing to plaque formation and coronary heart disease [3–5]. The amount of apo(a) deposition in cerebral vessels correlates well with the degree of cerebral atherosclerosis [6]. Lp(a) has been detected both extracellularly and within foam cells [7]. The mechanisms by which Lp(a) may accelerate the development of atherosclerotic lesions remain obscure. Lp(a) and apoLp(a) enhance proliferation of human VSMCs in culture [12]. This has been explained by inhibiting the activation of plasminogen to plasmin, thus blocking the proteolytic activation of TGF-β, an autocrine inhibitor of human VSMC proliferation.

Formation of NO by the constitutive NOS expressed in endothelial cells is believed to protect against the development of atherosclerotic lesions. Under conditions with decreased constitutive NOS activity, iNOS might substitute the synthesis of NO [44]. iNOS expression in vascular smooth muscle cells and macrophages may be beneficial as a compensatory mechanism for the lack of endothelial NO synthesis, thereby preventing restenosis following angioplasty or heart transplant vasculopathy [45]. Ox-Lp(a) induced iNOS inhibition might contribute to the development of atherosclerotic lesions by reducing the anti-atherogenic effects of NO.

iNOS gene transfer to rats and pigs has been shown to inhibit intimal hyperplasia in response to vascular injury in vivo. iNOS cDNA was used to express iNOS at sites of arterial injury. In vivo iNOS gene transfer to injured rat carotid arteries resulted in a near complete ($>95\%$) reduction in neointima formation even when followed long-term to 6 weeks post injury. This protective effect was reversed by selective iNOS inhibition [46].

The development of atherosclerotic lesions as a consequence of chronic inhibition of NO production by L-NAME, an inhibitor of both inducible and constitutive NOS, has been reported [22,23]. Morphometric analyses revealed a marked enlargement of intimal atherosclerotic areas in aortas from L-NAME treated animals.

iNOS expression within macrophages of atherosclerotic lesions has been described. iNOS was not detected in normal vessels but widespread iNOS protein staining was found in early and advanced lesions in macrophages, foam cells, and VSMCs of atherosclerotic vessels [32–35]. Protective effects of NO towards the oxidation of lipoproteins have been described [47]. iNOS dependent NO release may play protective roles in oxidative modification of LDL during the atherosclerosis process [48]. Ox-Lp(a) induced inhibition of inducible NO synthesis could result in an increased lipoprotein oxidation, thereby establishing a vicious circle by a further decrease of NO synthesis with concomitant increased lipoprotein oxidation.

Accumulation of monocyte derived foam cells in the arterial intima is a major event in the development of atherogenesis. Ox-Lp(a) and ox-LDL have been described to be potent stimuli of monocyte adhesion to endothelial cells [49]. Appearance of macrophages within atherosclerotic lesions has been supposed to be the consequence of an increased recruitment of monocytes via NF-κB dependent expression of vascular endothelial cell adhesion molecule 1 (VCAM-1), macrophage colony stimulating factor (M-CSF) and monocyte chemoattractant protein 1 (MCP-1) by human vascular endothelial cells. NO has been shown to inhibit VCAM-1 [25], M-CSF [50] and MCP-1 expression [26] by inhibiting NF-κB activation. Inhibition of NO synthesis by L-NMA was shown to activate NF-κB with concomitant VCAM-1, M-CSF, and MCP-1 expression [25,26,50]. Inhibition of inducible NO synthesis by ox-Lp(a) might influence macrophage accumulation within atherosclerotic lesions by enhancing NF-κB dependent VCAM-1, M-CSF, and MCP-1 expression, thereby increasing monocyte adhesion to endothelial cells.

Furthermore, the inhibition of inducible NO synthesis by ox-Lp(a) might initiate macrophage proliferation because NO decreases cell proliferation by increasing the rate of apoptosis [38]. This hints to a causative role of ox-Lp(a) induced iNOS inhibition for the development of atherosclerotic lesions.

Numerous anti-atherogenic properties of NO in vitro have been described. NO inhibits LDL oxidation [47], smooth muscle cell proliferation [24], smooth muscle cell migration [51], macrophage proliferation [38], neutrophil adhesion [52], MCP-1 expression [26], and NF-κB activation [26,50]. Ox-Lp(a) might contribute to the development of atherosclerotic lesions by reducing inducible NO synthesis.

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