Tetrahydrobiopterin attenuates superoxide-induced reduction in nitric oxide

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1. ABSTRACT

NADPH oxidase, a source of superoxide anion ($^\cdot O_2^-$), can be stimulated by oxidized low-density lipoprotein (OxLDL). We examined whether tetrahydrobiopterin (BH4) could reduce OxLDL-induced $^\cdot O_2^-$ production by NADPH oxidase, increasing nitric oxide (NO) synthesis. Endothelial cells incubated with OxLDL produced more $^\cdot O_2^-$ (35-67%) than untreated cells, with the highest increase 1 hour after OxLDL addition. The elevated $^\cdot O_2^-$ production correlated with the translocation of the $p47^{phox}$ subunit of NADPH oxidase from the cytosol to the membrane. Cells exhibited a marked decrease in both BH4 (83%) and NO (54%) in the same hour following exposure to OxLDL. An NADPH oxidase inhibitor, apocynin, or antioxidant, N-acetyl-L-cysteine, substantially attenuated the reduction in both BH4 and NO. The $^\cdot O_2^-$ production was increased when cells were pretreated with an inhibitor of BH4 synthesis and decreased following pretreatment with a BH4 precursor, suggesting that NADPH oxidase-induced imbalance of endothelial NO and $^\cdot O_2^-$ production can be modulated by BH4 concentrations. BH4 may be critical in combating oxidative stress, restoring proper redox state, and reducing risk for cardiovascular disease including atherosclerosis.

2. INTRODUCTION

Endothelial cells contribute to vascular homeostasis by constitutive production of nitric oxide (NO) from the reaction of the amino acid L-arginine with oxygen via the endothelial NO synthase (eNOS) enzyme. This reaction is absolutely dependent upon adequate levels of the enzymatic cofactor *6R*-L-5,6,7,8-tetrahydrobiopterin (BH4). Physiological levels of NO have many diverse anti-atherogenic actions, including inhibition of platelet adherence/aggregation, leukocyte adhesion/infiltration, and proliferation of vascular smooth muscle (1). Conversely, atherosclerosis is associated with reduced bioavailability of NO.

Hypercholesterolemia is a dominant risk factor associated with atherosclerosis. Chronic elevations in plasma cholesterol levels bring about morphological and functional changes in blood vessels in a manner consistent with an inflammatory response. As such, the development of cardiovascular disease, including atherosclerosis, is modulated by the balance between pro-inflammatory stimuli on the one hand, and anti-inflammatory and anti-oxidative defense mechanisms on the other hand. Atherosclerotic lesion development occurs in large arteries

in response to months or years of hypercholesterolemia. In contrast, hypercholesterolemia-induced changes in microvessels can be demonstrated in animal models within a few days after placing the animals on a cholesterol-enriched diet (i.e., long before the appearance of fatty streak lesions in large arteries) (2).

Oxidatively modified low-density lipoprotein (OxLDL) is considered to be more important than native-LDL in stimulating atherogenesis. OxLDL leaves the circulation and accumulates within the extracellular matrix of the subendothelial space where it contributes to the inflammatory state of atherosclerosis and plays a key role in its pathogenesis (3,4). OxLDL is also taken up by the cells of the vessel wall via receptor-mediated pathways involving a family of scavenger receptors (5,6). Since endothelial cells lining blood vessels are the first cells affected by OxLDL and endothelial cell dysfunction is a very early step in atherogenesis, studying the effects of OxLDL binding, internalization and signal transduction is of critical importance in designing therapeutic strategies for preventing and/or treating atherosclerosis.

OxLDL has been shown to cause endothelial activation, dysfunction and apoptosis (7,8). It stimulates expression of chemokines and adhesion molecules by endothelial cells, facilitates the adhesion of monocytes to endothelial cells and activates the inflammatory process (7). Importantly, OxLDL has been shown to induce the production of reactive oxygen species in endothelial cells, leading to the development of oxidative stress. Oxidative stress is a major determinant of endothelial dysfunction and increased production of reactive oxygen species, especially superoxide anion ($^{\cdot}O_2^-$), is implicated in the development and progression of atherosclerosis and many other cardiovascular diseases (9,10). Recent studies suggest that the major source of $^{\cdot}O_2^-$ in OxLDL-stimulated human endothelial cells is a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (8). Five major components comprise the endothelial NADPH oxidase enzyme: gp91^{phox} and p22^{phox} in the membrane, as well as p47^{phox}, p67^{phox}, and the GTPase Rac in the cytosol. NADPH oxidase activation is initiated by translocation of p47^{phox}, p67^{phox} and Rac to the membrane leading to a conformation change in gp91^{phox} and the induction of electron flow (11).

Under pathophysiological conditions, excessive $^{\cdot}O_2^-$, generated by OxLDL interaction with the endothelial cell, reacts rapidly with NO, producing peroxynitrite and reducing NO bioavailability (12). Peroxynitrite can oxidize BH4 to dihydrobiopterin (BH2). This in turn leaves eNOS (the enzyme responsible for NO generation from the reaction of L-arginine and oxygen) in an uncoupled state in which electrons are transferred from the heme center of eNOS to oxygen rather than to L-arginine, the substrate for NO. The result is the formation of $^{\cdot}O_2^-$ rather than NO. Thus, under pathological conditions of OxLDL excess, eNOS-mediated synthesis of NO may be downregulated and eNOS becomes a direct source of $^{\cdot}O_2^-$.

In the present study we investigated OxLDL-induced NADPH oxidase-mediated production of endothelial $^{\cdot}O_2^-$ and its ability to decrease NO bioavailability. We focused on the interaction of $^{\cdot}O_2^-$ and BH4. The purpose of this study, therefore, was to assess the relationship between OxLDL-induced $^{\cdot}O_2^-$ production and BH4 levels while examining whether inhibition of NADPH oxidase and/or antioxidant therapy could directly attenuate the decrease in NO bioavailability.

3. METHODS AND MATERIALS

3.1. Oxidation of low-density lipoprotein

Human low-density lipoprotein (LDL, Sigma Aldrich, St. Louis, MO) was oxidized as previously described (13,14). Briefly, native LDL (6.5 mg/ml) was incubated with CuCl$_2$ (20 μM) in phosphate-buffered saline for 5-24 hours at 23°C. Oxidation was denoted as a color change from a yellow to a clear solution. Oxidation of LDL was stopped by adding 100 mM EDTA to the oxidized LDL/CuCl$_2$ mixture and the degree of oxidation was assessed using ThioBarbituric Acid Reactive Substances (TBARS) analysis (15). OxLDL samples were compared to malondialdehyde (MDA, Sigma Aldrich) standards using spectrophotometric methods and oxidation was expressed as nmol MDA equivalents/mg of OxLDL protein. Native LDL ranged from 0.2-1.0 nmol MDA/mg protein while the degree of oxidation for OxLDL ranged from 18.3 to 23.5 nmol MDA/mg protein. OxLDL was dialyzed against phosphate buffered saline for 24 hours at 4°C to remove Cu^{2+} ions. It was stored at 4°C in the dark and freshly prepared every 2 weeks.

3.2. Endothelial cell culture

Human mesenteric microvascular endothelial cells were cultured at 37°C under 10% CO$_2$ in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 2 mM L-glutamine, 4.5 g/L D-glucose, 10 units/ml sodium heparin, 1 mM sodium pyruvate, and 0.5 mg/ml gentamicin. Cultures of confluent endothelial cells were switched to the same Dulbecco's medium containing low serum and low glucose (0.5% and 1 g/L, respectively) for 48 hours. Cells were then pretreated with or without the following inhibitors or supplements: apocynin (100 μM), a proposed NADPH oxidase inhibitor (Frinton Laboratories, Vineland, N.J.); N-acetyl-L-cysteine (1 mM, Sigma), an antioxidant superoxide inhibitor; 2,4-diamino-6-hydroxypyrimidine (DAHP, 10 mM, Sigma), a GTP cyclohydrolase-I inhibitor; or sepiapterin (10 μM, Sigma), a BH4 precursor (via the salvage pathway for BH4 synthesis). Following a 2-hour pretreatment period, endothelial cells were incubated for 1-18 hours with OxLDL (50-100 μg/ml). No phenotypical changes were noted in the cells prior to OxLDL treatment.

3.3. Superoxide anion production

Endothelial cells were incubated with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Dojindo Laboratories, Kumamoto, Japan) in order to allow extracellular $^{\cdot}O_2^-$ generated by the cells to reduce the WST-1 to blue formazan. OxLDL (100 μg/ml) was first added to confluent endothelial cells for various periods of time. WST-1 was then added to the medium and incubated for an additional 30 minutes at

37°C. After incubation with WST-1, microscopic examination verified the generation of insoluble formazan as light blue granules. Formazan granules from the medium were solubilized with 1 ml dimethylsulfoxide and 50 µl of 2.0 M potassium hydroxide. Superoxide anion production was expressed as optical density measurements of solubilized formazan read at a wavelength of 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Optical density measurements from the treated cells were compared to those of untreated endothelial cells.

3.4 BH4 analysis

The cellular BH4 content was determined using high performance liquid chromatography, as we previously described (16). Briefly, endothelial cells (3-5 x 10^{6} cells) were trypsinized and washed twice with 1.5 ml of phosphate buffered saline (pH 7.4). The cell pellet was extracted in 0.3 ml of 0.1 M phosphoric acid containing 5 mM dithioerythritol (an antioxidant), to which 35 µl of 2.0 M trichloroacetic acid (TCA) was added. The solution was centrifuged at 12,000xg for 1 minute, and the supernatant was used immediately for analysis of BH4. For oxidation under acidic conditions, 100 µl of cell extract or BH4 standard (50 pmol/ml) was mixed with 15 µl of 0.2 M TCA and 15 µl of 1% I$_2$/2% KI in 0.2 M TCA. For oxidation under alkaline conditions, 100 µl of cell extract or BH4 standard (50 pmol/ml) was mixed with 15 µl of 1 M NaOH and 15 µl of 1% I$_2$/2% KI in 3 M NaOH. After oxidation for 1 hour in the dark at room temperature, excess iodine in the assay mixture was destroyed by the addition of 25 µl of 0.114 M ascorbic acid. The assay mixture was centrifuged (4°C, 12,000xg, 1 minute) and 75 µl of the supernatant was injected into a 5-µm reverse-phase Phenosphere 5 ODS-1 column (25 cm x 4.6 mm) (Phenomenex; Torrance, CA). Biopterin was eluted by an isocratic mobile-phase solution consisting of 5% methanol and 7.5 mM sodium phosphate buffer (pH 6.35) at a flow rate of 1.0 ml/minute and was detected by a fluorometer (excitation 350 nm, emission 440 nm). The amount of BH4 in the endothelial cell extracts was calculated from the difference between the amount of biopterin formed by oxidation under acidic conditions and the amount of biopterin formed by oxidation under alkaline conditions. Recovery of BH4 from endothelial cells was greater than 96%, as determined by addition of a known amount of BH4 standard into the cell suspension.

3.5. Western blotting

Endothelial cells were lysed in an ice-cold hypotonic 20 mM phosphate buffer, pH 7.4, containing 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, and 0.5 mM phenylmethanesulfonylfluoride. For eNOS blots, cells were lysed and equal amounts of protein [determined by the bicinchoninic protein assay] were loaded per lane. For the p47phox blots, the cell lysate was pelleted by centrifugation at 15,000xg for 5 minutes and the supernatant was further centrifuged at 100,000xg for 1.5 hours. The supernatant from this second centrifugation was considered the cytosol, and the insoluble pellet was considered the membrane portion. The membrane portion was resuspended in the hypotonic phosphate buffer containing 1% Triton X-100, incubated for 10 minutes on ice, centrifuged at 2,000xg for 10 minutes, and the supernatant was collected for the final membrane fraction. Western blot analysis was performed on fractions from equivalent numbers of cells. These fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% to 12% gradient gels, 1.5-mm thick) under reducing conditions. The proteins were transferred to nitrocellulose by electroblotting, and the membrane was blocked for 3 hours at room temperature in 5% nonfat dried milk in Tris buffered saline (blocking buffer). Blots were incubated with a 1:2500 dilution (in blocking buffer) of mouse anti-human eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) or rat anti-human p47phox (Upstate Biotech, Lake Placid, NY) on a rocker, overnight at 4°C. After washing, the membrane was incubated with an appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:20,000 - 1:100,000 in blocking buffer. Protein bands were visualized using the West Dura Extended Duration substrate system (Pierce, Rockford, IL) and Kodak Biomax film (Kodak, Rochester, NY). Densitometric analysis was performed on each immunoblot.

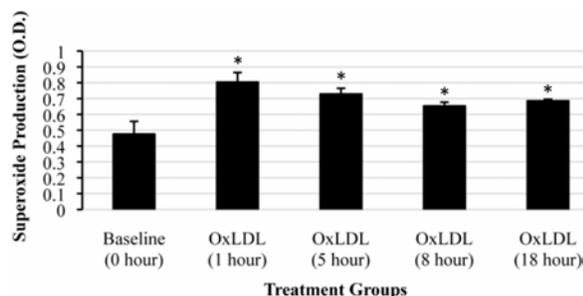### 3.6. Nitric oxide production

The production of NO was assessed by measuring the concentration of nitrite, a stable metabolite of NO, in medium using a sensitive and specific fluorometric technique developed in our laboratory (17). Briefly, 100 µl of conditioned medium was incubated for 10 minutes at room temperature with 10 µl of 316 µM 2,3-diamnonaphthalene (in 0.62 M HCl), followed by addition of 5 µl of 2.8 M NaOH. The 3,3-naphthotriazole formed from the reaction with nitrite was separated on a 5-µm reverse-phase C$_8$ column (150 X 4.6 mm I.D.) using 15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (flow rate = 1.3 ml/minute). The fluorescence of 2,3-naphthatriazole was monitored with excitation at 375 nm and emission at 415 nm.

3.7. Statistical analysis

Data are presented as means ± standard error of the mean of at least 3 different experiments for each condition. A minimum of 3 independent experiments utilizing media and cell lysates was performed for the BH4 and nitrite analyses. Statistical comparisons among groups were performed using one-way analysis of variance with an appropriate post hoc test. Differences were considered statistically significant at p<0.05.

4. RESULTS

The production of O_2^- was assessed in untreated and OxLDL-treated endothelial cells. There was a significant increase in O_2^- production at 1, 5, 8, and 18 hours compared to baseline (0 hour), following incubation with OxLDL (100 µg/ml) (Figure 1). The greatest increase in O_2^- was noted at the 1-hour time point. Optical density measurements, indicative of increased production of O_2^-, increased from 0.48 ± 0.11 at time 0 to 0.81 ± 0.06, 0.73 ± 0.04, 0.65 ± 0.02, and 0.69 ± 0.01 for time periods 1 hour, 5 hours, 8 hours, and 18 hours, respectively. The O_2^- production remained substantially increased over the

Figure 1. OxLDL-Induced Superoxide Production. Comparison of WST-1 reduction [a measure of $\cdot O_2^-$ production] by untreated endothelial cells and endothelial cells exposed to 100 µg/ml OxLDL (for the times indicated) measured spectrophotometrically at 450 nm. Data are means ± standard error of the mean, n = 4, * p < 0.05 compared to untreated endothelial cells (baseline control).
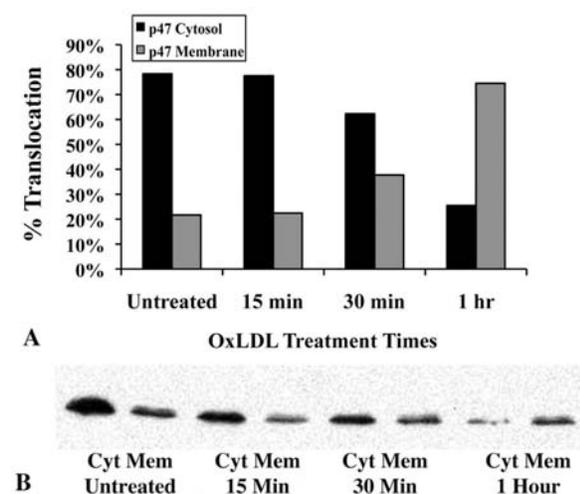
Figure 2. OxLDL-Induced Translocation of NADPH p47 Subunit. (A) Densitometry data from the representative western blot shown in 2B with p47 subunit protein in the membrane and cytosolic fractions expressed as a percentage of the total p47 protein densitometry data at that time point. (B) Representative western blot showing the NADPH oxidase p47 subunit expression in membrane and cytosolic fractions of cells treated with 100 µg/ml OxLDL for the times indicated.

baseline control following 18 hours of incubation with OxLDL.

Because a critical step in activation of NADPH oxidase involves phosphorylation and translocation of p47^{phox} from the cytosol to the membrane fraction, we verified OxLDL-induced p47^{phox} translocation in our endothelial cells. Within 30 minutes of treatment, p47^{phox} protein was increased in the membrane fraction with a simultaneous decrease in the cytosol (Figure 2). Translocation of p47^{phox} from the cytosol to the membrane

was highest at 1 hour. Translocation was based on the densitometry data from p47^{phox} western blots of membrane and cytosolic fractions and expressed as a percentage of total p47phox subunit densitometry data for each time point (Figure 2A). The corresponding blot is shown in Figure 2B.

The production of $\cdot O_2^-$ was also assessed at 1 hour using untreated, OxLDL-treated (100 µg/ml), or OxLDL-treated endothelial cells pretreated with either DAHP (an inhibitor of GTP cyclohydrolase I, the rate-controlling enzyme for the *de novo* synthesis of BH4) or sepiapterin (a precursor for BH4 production via the salvage pathway). Figure 3A shows light microscopy photographs of the endothelial cells exposed to medium only (Control -- upper left panel), OxLDL (upper right panel), OxLDL following pretreatment with 10 mM DAHP (OxLDL + DAHP -- lower left panel), or OxLDL following pretreatment with 10 µM sepiapterin (OxLDL + Sepiapterin -- lower right panel). Formation of blue formazan granules in response to reaction of WST-1 and $\cdot O_2^-$ can be seen in response to OxLDL treatment alone. Exacerbation of $\cdot O_2^-$ production, seen as an increase in color generation, was brought about by pretreatment with DAHP, which lowers cellular BH4 levels. In contrast, raising cellular BH4 levels, by pretreatment with sepiapterin, decreased $\cdot O_2^-$ production.

Figure 3B shows a significant increase in $\cdot O_2^-$ production in OxLDL-treated endothelial cells compared to untreated endothelial cells, as assessed by absorbance readings of solubilized formazan. Optical density measurements increased from 0.50 ± 0.03 in untreated endothelial cells to 0.69 ± 0.01 in OxLDL-treated endothelial cells (p < 0.05). Pretreatment with DAHP, 2 hours prior to OxLDL treatment, increased $\cdot O_2^-$ production even more than OxLDL alone (0.75 ± 0.03 vs. 0.69 ± 0.01, respectively, p < 0.05). Pretreatment with sepiapterin 2 hours prior to OxLDL treatment significantly reduced $\cdot O_2^-$ production compared to OxLDL treatment alone (0.61 ± 0.03 vs.0.69 ± 0.03, respectively, p < 0.05), but $\cdot O_2^-$ production still remained significantly greater than in untreated endothelial cells (0.61 ± 0.03 vs. 0.50 ± 0.03, respectively, p < 0.05).

BH4 levels and nitrite production were assessed in untreated endothelial cells and endothelial cells treated with OxLDL for 1-24 hours (Figure 4). Both BH4 (Figure 4A) and nitrite (Figure 4B) were significantly decreased following as little as 1 hour of OxLDL treatment. BH4 levels increased with time but at 24 hours remained significantly below those of control EC. Nitrite accumulation normalized by 18 hours. When eNOS protein expression was assessed in endothelial cell lysates by western blot analysis (Figure 5), there was a substantial decrease in eNOS protein expression following a 1-hour treatment with OxLDL.

The effect of modulating $\cdot O_2^-$ production on the OxLDL-induced decrease in BH4 was assessed after 24 hours. The BH4 content of endothelial cells treated with 100 µg/ml OxLDL was significantly reduced (p < 0.05) from the levels measured in untreated endothelial cells (Figure 6A). Pretreatment with the NADPH oxide inhibitor,
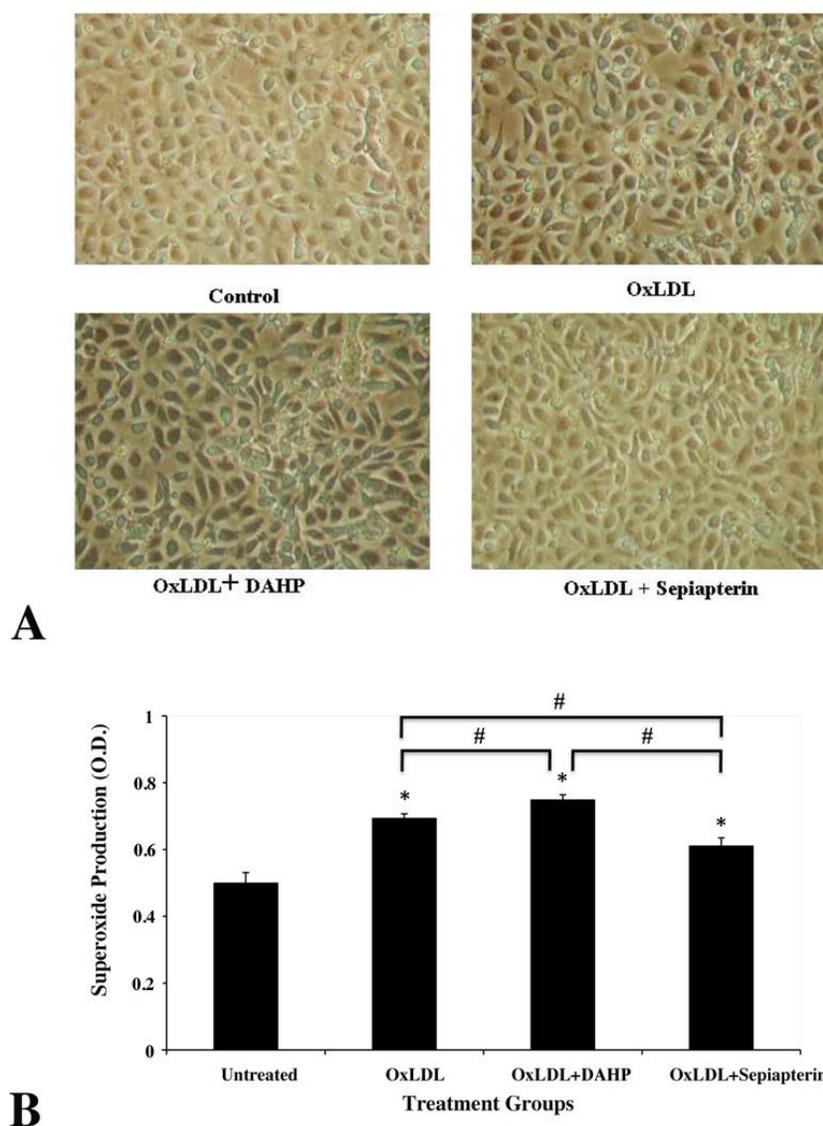
Figure 3. BH4 Modulation of OxLDL-Induced Superoxide Production. (A) Phase contrast microscopy showing formation of blue formazan crystals in response to the addition of WST-1 to untreated endothelial cells (control), OxLDL-treated endothelial cells (OxLDL), OxLDL-treated endothelial cells pretreated with 10 mM DAHP (OxLDL + DAHP), and OxLDL-treated endothelial cells pretreated with 10 μM sepiapterin (OxLDL Sepiapterin). (B) WST-1 reduction in untreated endothelial cells (control), OxLDL-treated endothelial cells (OxLDL), OxLDL-treated endothelial cells pretreated with 10 mM DAHP (OxLDL + DAHP), and OxLDL-treated endothelial cells pretreated with 10 μM sepiapterin (OxLDL + Sepiapterin) measured spectrophotometrically at 450 nm. Data are means ± standard error of the mean, $n = 7$, * $p < 0.05$ vs. untreated control endothelial cells, [#] $p < 0.05$ for indicated pairs of treatments.

apocynin, and the antioxidant, N-acetyl-L-cysteine, returned BH4 levels to untreated levels or above untreated levels, respectively, following addition of OxLDL. The decrease in BH4 levels in endothelial cells treated with OxLDL was attenuated ($p < 0.05$) by pretreatment with apocynin or N-acetyl-L-cysteine.

Medium from the cultured cells was used for the determination of nitrite levels from untreated and OxLDL-treated endothelial cells. Nitrite production, expressed as a percentage of the production by untreated control endothelial cells, was decreased ($p<0.05$) following a 1-hour and 8-hour OxLDL treatment period compared to untreated endothelial cells ($37 \pm 4\%$ and $32 \pm 1\%$ of control, respectively, Figure 6B). There was a significant attenuation of the decrease in nitrite production if OxLDL-treated endothelial cells were pretreated with apocynin ($60 \pm 2\%$) or N-acetyl-L-cysteine ($57 \pm 2.5\%$) prior to the 8-hour OxLDL treatment. However, nitrite production did not completely return to baseline levels following pretreatment with either apocynin or N-acetylcysteine.

A

B
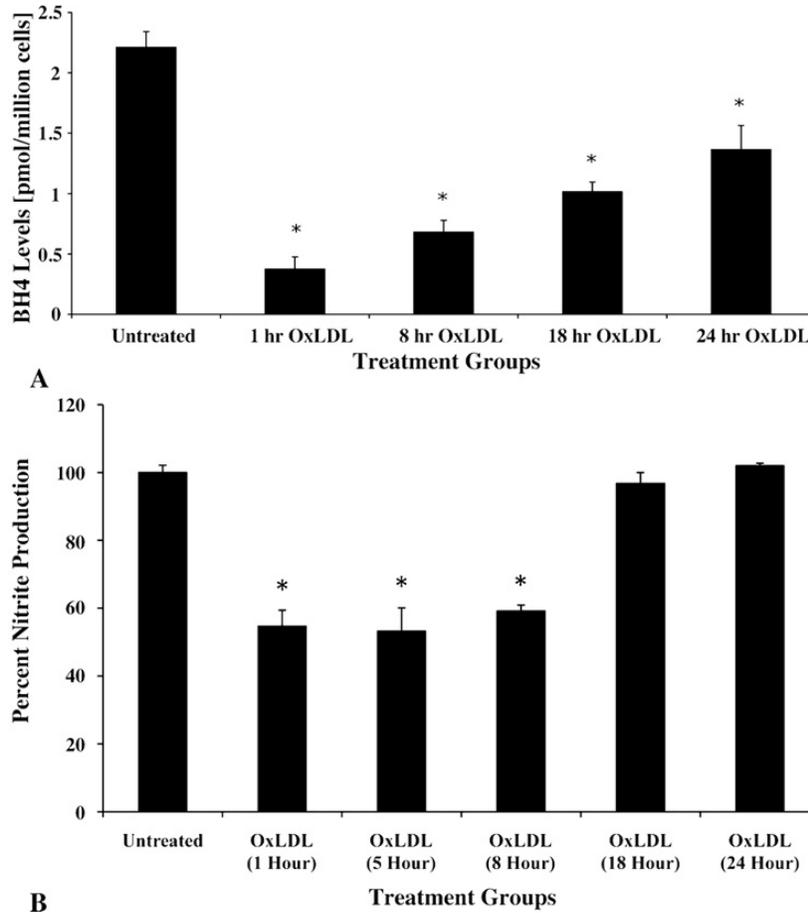
Figure 4. OxLDL-Induced Reduction in BH4 Levels and Nitric Oxide Synthesis. BH4 (A) and nitrite production (B) following incubation with 100 µg/ml OxLDL for the times indicated. Data are means ± standard error of the mean, n = 4, * $p < 0.05$ vs. untreated control endothelial cells.

A

B
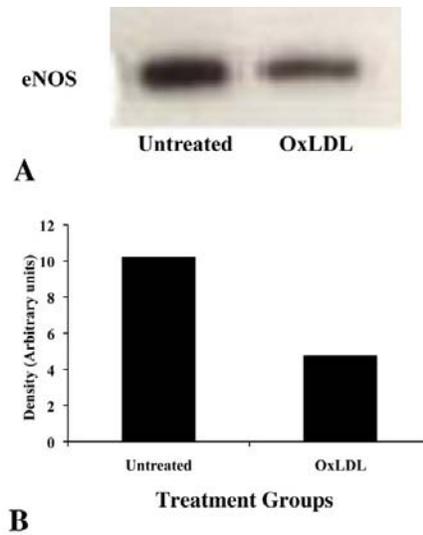
Figure 5. OxLDL-Induced Reduction in eNOS Expression. (A) Western blot showing eNOS expression in endothelial cells incubated for 1 hour in the absence or presence of 100 µg/ml OxLDL. (B) Densitometric measurement of bands shown in (A).
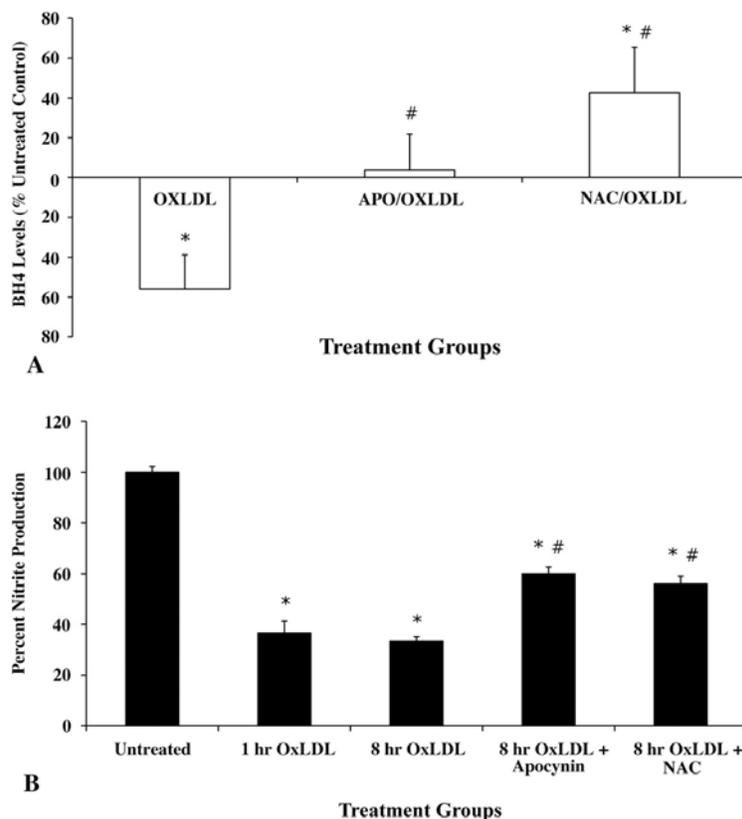
Figure 6. Modulation of OxLDL-Induced Decrease in BH4 Levels and NO Synthesis. BH4 levels in cells (A) and nitrite concentration in medium (B) from OxLDL-treated endothelial cells, OxLDL-treated endothelial cells pretreated with apocynin, or OxLDL-treated endothelial cells pretreated with N-acetyl-L-cysteine compared to untreated (control) endothelial cells. Values are shown as a percentage of the BH4 or nitrite level measured in untreated endothelial cells following incubation with 100 µg/ml OxLDL for the times indicated. Data are means ± standard error of the mean, n = 5, * p < 0.05 vs. untreated control endothelial cells, # p< 0.05 vs. OxLDL treatment alone.

5. DISCUSSION

BH4 is an essential enzymatic cofactor for NO synthesis that maintains and stabilizes eNOS dimers. BH4 is also a potent cellular antioxidant (18,19). Increased NADPH oxidase activity in endothelial cells, in response to oxLDL exposure, leads to the generation of $\cdot O_2^-$, which can react with NO to form peroxynitrite, a highly reactive oxidant that consumes cellular antioxidants, including BH4, reducing the ability of the cell to combat oxidative stress (10-12). Therefore, in order to avert a decrease in NO production and thus maintain endothelial function in the face of increased oxidative stress, cellular pools of BH4 need to be preserved.

In the present study, we demonstrate that OxLDL dramatically increases the production of $\cdot O_2^-$ and concomitantly decreases the bioavailability of NO and its cofactor BH4 in human microvascular endothelial cells. Our data support the role of NADPH oxidase in this process based on: a) the increased formation of an active membrane subunit complex in response to OxLDL exposure and b) attenuation of the decrease in NO and BH4

by blockage of the formation of this membrane complex with apocynin. Inhibiting $\cdot O_2^-$ accumulation, with the antioxidant N-acetyl-L-cysteine (a precursor for the synthesis of reduced glutathione), also attenuated the drop in BH4 and NO levels in response to OxLDL exposure, suggesting that $\cdot O_2^-$ may decrease levels of BH4 by oxidizing it. These data support a link between NADPH oxidase-induced $\cdot O_2^-$ production, decreased BH4 levels, and endothelial dysfunction.

NO production from L-arginine is important for vascular homeostasis, keeping the vasculature dilated, protecting the intima from platelet aggregation or leukocyte adhesion, and preventing smooth muscle cell proliferation to retard atherosclerosis. Loss of the NO-mediated modulatory role of the endothelium may be a critical initiating factor in the development of vascular disease. NO is synthesized in endothelial cells by the enzyme eNOS when levels of BH4 are adequate. A BH4 deficiency leads to uncoupling of eNOS from its L-arginine substrate. As a result, eNOS transfers electrons to molecular oxygen, forming $\cdot O_2^-$ instead of NO and citrulline (18). Increased production of reactive oxygen species, coupled with

decreased synthesis of NO, alters the homeostatic environment of the blood vessel.

Through its strategic role in the enzymatic action of eNOS, BH4 plays a critical role not only in increasing the rate of NO synthesis but also in controlling the formation of $\cdot O_2^-$ by this enzyme in endothelial cells (18). When BH4 levels in endothelial cells are inadequate and eNOS is uncoupled from NO production, production of $\cdot O_2^-$ is favored. However, when BH4 levels are adequate, $\cdot O_2^-$ levels are decreased, since normal eNOS activity generates NO. NO can react with $\cdot O_2^-$ produced by multiple sources to form peroxynitrite. BH4 can directly scavenge peroxynitrite and $\cdot O_2^-$ independent of its role in the production of NO (19,20). Unfortunately, when oxidative stress levels increase, oxidation of BH4 to BH2 also increases. BH2 can block production of NO, acting as a competitive inhibitor of BH4 for binding to eNOS (21). In addition, as BH2 levels rise in the cell, the concomitant decrease in BH4 levels leaves the cell more susceptible to oxidative injury. Indeed, *in vitro* studies utilizing purified eNOS have shown that BH4 acts as a redox switch in the catalytic mechanism of eNOS and that it is the balance between reduced and oxidized BH4 (i.e., the balance between BH4 and BH2) that controls $\cdot O_2^-$ formation by this enzyme (18,21). Thus, BH4 protects endothelial cells by increasing their antioxidant capacity and providing an additional pathway for metabolism of reactive oxygen species.

Our findings demonstrate that OxLDL increases $\cdot O_2^-$ production while decreasing NO synthesis. Other investigators have demonstrated a similar increase in $\cdot O_2^-$ production by endothelial cells following incubation with 100 μg/ml OxLDL (22,23,24). Mehta *et al.* (25) demonstrated a 2-fold sustained increase in $\cdot O_2^-$ production following a 24-hour incubation with lower concentrations of OxLDL (10-80 μg/ml). Increased $\cdot O_2^-$ production has also been shown to affect eNOS expression and activity. Napoli *et al.* (26) demonstrated that both glycosylated and oxidized LDL reduce eNOS expression in human coronary endothelial cells, while Motoshima *et al.* (23) reported a 45-47% reduction in the activity of eNOS following a 16-hour incubation of bovine aortic endothelial cells with OxLDL (10 μg/ml).

OxLDL-induced $\cdot O_2^-$ production in our endothelial cells correlated with decreased NO and BH4 bioavailability. At one hour, when $\cdot O_2^-$ production was highest, BH4 and NO levels were also at their lowest. This supports a direct effect of $\cdot O_2^-$ on BH4, via oxidation of BH4 to BH2 (27). Similar results have been reported for the vasculature of rats with high salt-induced hypertension (28). The expression of eNOS protein was also decreased after only one hour of exposure to OxLDL (100 μg/ml). This decrease in eNOS expression could contribute to the decrease in NO production in our endothelial cells.

Inhibiting GTP-cyclohydrolase 1 (GTPCH1), the rate-limiting enzyme for the *de novo* synthesis of BH4, with DAHP decreases the level of BH4 in endothelial cells (29). This same treatment exacerbated OxLDL-induced $\cdot O_2^-$

production. Conversely, pretreatment with sepiapterin, which raises BH4 levels in endothelial cells, decreases OxLDL-induced $\cdot O_2^-$ production (Figure 3). Guzik *et al.* (30) reported a similar normalization of $\cdot O_2^-$ production following sepiapterin treatment of internal mammary artery and saphenous veins from diabetic humans. Thus, the relationship between $\cdot O_2^-$ production and NO and its cofactor, BH4, appears to play an important biological role in vascular homeostasis. If increased $\cdot O_2^-$ production leads to the consumption of BH4 in its role as an antioxidant, less BH4 will be available to support NO synthesis. The result is endothelial dysfunction. Restoring the BH4 levels and increasing the antioxidant status of the endothelium may shift the imbalance between $\cdot O_2^-$ and NO and/or enhance eNOS function, thus preserving an anti-atherogenic environment.

NADPH oxidase is activated by OxLDL in human endothelial cells and this activation could contribute to the observed $\cdot O_2^-$ production in our cells. The correlation of the translocation of the p47^{phox} NADPH oxidase subunit to the membrane and the increase in $\cdot O_2^-$ production supports a critical role for NADPH oxidase in the OxLDL-induced increase in O_2^- production. Apocynin has been used by many investigators as an inhibitor of NADPH oxidase. In our study, apocynin was able to block the OxLDL-mediated decrease in BH4 and attenuate the reduction in NO synthesis (i.e, nitrite accumulation). However, apocynin requires myeloperoxidase to become activated. Since endothelial cells do not have myeloperoxidase, apocynin has recently been proposed to work as an antioxidant in these cells (31). This would still allow apocynin to reduce OxLDL-mediated $\cdot O_2^-$ production, potentially preserving BH4 in its reduced state for NO synthesis, but underscores the lack of any selective NADPH oxidase inhibitor.

N-acetyl-L-cysteine is able to increase the antioxidant defenses of the cell by increasing the intracellular glutathione concentration and has been proposed as an antioxidant therapy (32). N-acetyl-L-cysteine reduced $\cdot O_2^-$ levels to a greater degree than apocynin. Although N-acetylcysteine could completely inhibit the reduction in BH4 levels and actually raised BH4 in OxLDL-treated cells, neither N-acetyl-L-cysteine nor apocynin could completely restore nitrite levels to their baseline values (i.e., values in untreated control endothelial cells) in the time period studied. A longer treatment with N-acetylcysteine possibly at a higher dose may be necessary to result in a greater effect on NO synthesis in endothelial cells under conditions of oxidative stress.

Nitrite represents a stable metabolite of NO and its levels are used to assess NO synthesis (17). However, following reaction with $\cdot O_2^-$, NO may be rapidly converted to peroxynitrite, rather than being metabolized to nitrite. Peroxynitrite has also been suggested to uncouple eNOS (27,30,31). Thus, alternative sources of reactive oxygen and reactive nitrogen species may be involved in the reduction of NO bioavailability in endothelial cells. Additionally, oxidation of BH4 by peroxynitrite or $\cdot O_2^-$ leads to eNOS dysfunction with the formation of even more

reactive oxygen species (18, 21,33). The initiation of OxLDL-induced $\cdot O_2^-$ production may deplete the endothelial cell of its intracellular antioxidants, including BH4, and ultimately change the redox status of the endothelium leading to further increases in reactive species. Therapies designed to increase BH4 levels in endothelial cells should increase the endogenous antioxidant pool to protect the cell from oxidative injury and potentially prevent the initiation of endothelial dysfunction.

In conclusion, our findings demonstrate that BH4 deficiency is a result of OxLDL-induced $\cdot O_2^-$ production, likely via the NADPH oxidase pathway, which ultimately decreases the bioavailability of endothelium-derived NO. This may result from decreased biosynthesis of NO as a result of an $\cdot O_2^-$-induced oxidation of BH4 and eNOS uncoupling as well as inactivation of NO by $\cdot O_2^-$, and formation of other reactive oxygen/nitrogen species. Increasing the BH4 levels in OxLDL-treated cells, via BH4 synthesis or antioxidant therapy, helps to restore BH4-mediated NO synthesis and vascular homeostasis.

6. ACKNOWLEDGEMENT

7. REFERENCES

1. J. Davignon and P. Ganz: Role of endothelial dysfunction in atherosclerosis. *Circulation* 109[suppl III], III-27-III-32 (2004)

2. K.Y. Stokes, E.C. Clanton, J.M. Russell, C.R. Ross, and D.N. Granger: NAD(P)H oxidase-derived superoxide mediates hypercholesterolemia-induced leukocyte-endothelial cell adhesion. *Circ Res* 88, 499-505 (2001)

3. A.J. Berliner, M. Navab, A.M. Fogelman, J.S. Frank, L.L. Demer, P.A. Edwards, A.D. Watson, and A.J. Lusis: Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* 91, 2488-2496 (1995)

4. D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witztum: Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320, 915-924 (1989)

5. B.S. Dhaliwal and U.P. Steinbrecher: Scavenger receptors and oxidized low density lipoproteins. *Clin Chim Acta* 286, 191-205 (1999)

6. M. Chen, T. Masaki, and T. Sawamura: LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther* 95, 89-100 (2002)

7. J. Galle, A. Heinloth, C. Wanner, and K. Heermeier: Dual effect of oxidized LDL on cell cycle in human endothelial cells through oxidative stress. *Kidney Int Suppl* 78, S120-123 (2001)

8. J.L. Mehta, J. Chen, R.L. Hermonat, F. Romeo, and G. Novelli: Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): A critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res* 69, 36-45 (2006)

9. K.K. Griendling, D. Sorescu, and M. Ushio-Fukai: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86, 494-501 (2000)

10. R. Ray and A.M. Shah: NADPH oxidase and endothelial cell function. *Clin Sci* 109, 217-226 (2005)

11. A. Mizrahi, T. Berdichevsky, Y. Ugolev, S. Molshanski-Mor, T. Nakash, I. Dahan, N Alloul, Y. Gorzalczany, R. Sarfstein, M Hirshberg, and E. Pick: Assembly of the phagocyte NADPH oxidase complex: chimeric constructs derived from the cytosolic components as tools for exploring structure-function relationships. *J Leukocyte Biol* 279, 881-895 (2006)

12. U. Forstermann and T Munzel: Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 113, 1708-1714 (2006)

13. D.Y. Li, Y.C. Zhang, M.I. Philips, T. Sawamura, and J.L. Mehta: Upregulation of endothelial receptor for oxidized low-density lipoprotein (LOX-1) in cultured human coronary artery endothelial cells by angiotensin II type 1 receptor activation. *Circ Res* 84, 1043-1049 (1999)

14. J.K. Liao, W.S. Shin, W.Y. Lee, and S.L. Clark: Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* 270, 319-324 (1995)

15. J.M. Li, N.P. Gall, D.J. Grieve, M. Chen, and A.M. Shah: Activation of NADPH oxidase during progression of cardiac hypertrophy to failure. *Hypertension* 40, 477-484 (2002)

16. C.J. Meininger, R.S. Marinos, K. Hatakeyama, R. Martinez-Zaguilan, J.D. Rojas, K.A. Kelly, and G. Wu: Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency. *Biochem J* 349, 353-356 (2000)

17. H. Li, C.J. Meininger, and G. Wu: Rapid determination of nitrite by reversed-phase high-performance liquid chromatography with fluorescence detection. *J Chromatogr B* 746, 199-207 (2000)

18. J. Vasquez-Vivar, B. Kalyanaraman, P. Martasek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo, and K.A. Pritchard Jr: Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 95, 9220-9225 (1998)

19. B. Thony, G. Auerbach, and N. Blau: Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J* 347, 1-16 (2000)

20. K. Nakamura, V.P. Bindokas, D. Kowlessur, M. Elas, S. Milstien, J.R. Marks, H.J. Halpern, and U.J. Kang: Tetrahydrobiopterin scavenges superoxide in dopaminergic neurons. *J Biol Chem* 27, 34402-34407 (2001)

21. J. Vasquez-Vivar, P. Martaek, J. Whitsett, J. Joseph, and B. Kalyanaraman: The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J* 362, 733-739 (2002)

22. J.K. Liao, W.S. Shin, W.Y. Lee, and S.L. Clark: Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem* 270, 319-324 (1995)

23. H. Motoshima, X. Wu, K. Mahadev, and B.J. Goldstein: Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL. *Biochem Biophys Res Commun* 315, 264-271 (2004)

24. U. Rueckschloss, J. Galle, J. Holze, H.R. Zerkowski, and H. Morawietz: Induction of NAD(P)H oxidase by oxidized low-density lipoprotein in human endothelial cells. antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy. *Circulation* 104, 1767-1772 (2001)

25. J.L. Mehta and D.Y. Li: Identification and autoregulation of receptor for ox-LDL in cultured human coronary artery endothelial cells. *Biochem Biophys Res Commun* 248, 511-514, 1998.

26. C. Napoli, L.O. Lerman, F. de Nigris, J. Loscalzo, and L.J. Ignarro: Glycoxidized low-density lipoprotein downregulates endothelial nitric oxide synthesis in human coronary cells. *J Am Coll Cardiol* 40, 1515-1522 (2002)

27. M. Kirsch, H.G. Korth, V. Stenert, R. Sustmann, and H. De Groot: The autooxidation of tetrahydrobiopterin revisited. proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. *J Biol Chem* 278, 24481-24490 (2003)

28. T.R. Nurkiewicz, G. Wu, P. Li, and M.A. Boegehold: Decreased arteriolar tetrahydrobiopterin is linked to superoxide generation from nitric oxide synthase in mice fed high salt. *Microcirculation* 17:147-157 (2010)

29. W. Shi, C.J. Meininger, T.E. Haynes, K. Hatakeyama, and G. Wu: Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells. *Cell Biochem Biophys* 41:415-433 (2004)

30. T.J. Guzik, S. Mussa, D. Gastaldi, J. Sadowski, C. Ratnatunga, R. Pillai, and K. Channon: Mechanisms of increased vascular superoxide production in human diabetes mellitus. role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 105, 1656-1662 (2002)

31. S. Heumuller, S. Wind, E. Barbosa-Sicard, H.H.H.W. Schmidt, R. Busse, K. Schroder, and R.P. Brandes: Apocynin is not an inhibitor of vascular NADPH but an antioxidant. *Hypertension* 51, 211-217 (2008)

32. G. Wu, Y.Z. Fang, S. Yang, J.R. Lupton, and N.D. Turner: Glutathione metabolism and its implications for health. *J Nutr* 134:489-492 (2004)

33. U. Landmesser, S. Dikalov, S.R. Price, L. McCann, T. Fukai, S.M. Holland, W.E. Mitch, and D.G. Harrison: Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111, 1201-1209 (2003)

Abbreviations: BH4, *6R*-L-5,6,7,8-tetrahydrobiopterin; BH2, dihydrobiopterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; NADPH, nicotinamide adenine dinucleotide phosphate; eNOS, endothelial nitric oxide synthase; MDA, malondialdehyde; NO, nitric oxide; $\cdot O_2^-$, superoxide anion; OxLDL, oxidized low-density lipoprotein; TCA, trichloroacetic acid; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.

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