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Endothelial cell oxidative stress and signal transduction

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ABSTRACT

Endothelial dysfunction (ED) is an early event in atherosclerotic disease, preceding clinical manifestations and complications. Increased reactive oxygen species (ROS) have been implicated as important mechanisms that contribute to ED, and ROS□s may function as intracellular messengers that modulate signaling pathways. Several intracellular signal events stimulated by ROS have been defined, including the identification of two members of the mitogen activated protein kinase family (ERK1/2 and big MAP kinase, BMK1), tyrosine kinases (Src and Syk) and different isoenzymes of PKC as redox-sensitive kinases. ROS regulation of signal transduction components include the modification in the activity of transcriptional factors such as NFkB and others that result in changes in gene expression and modifications in cellular responses.

In order to understand the intracellular mechanisms induced by ROS in endothelial cells (EC), we are studying the response of human umbilical cord vein endothelial cells to increased ROS generation by different pro-atherogenic stimuli. Our results show that Homocysteine (Hcy) and oxidized LDL (oxLDL) enhance the activity and expression of oxidative stress markers, such as NFkB and heme oxygenase 1. These results suggest that these pro-atherogenic stimuli increase oxidative stress in EC, and thus explain the loss of endothelial function associated with the atherogenic process.

Key words: oxidative stress, signal transduction, gene expression, endothelial cell, atherosclerosis, antioxidants.

INTRODUCTION

The endothelial cell (EC) is situated at the interface between the circulating blood and the vessel wall. It serves as a sensor and transducer of signals within the circulatory microenvironment and is integral in maintaining the homeostatic balance of the vessel through the production of factors that regulate vessel tone, coagulation state, lipid transport, cellular proliferative response and leukocyte trafficking (13,36). Although the role of the endothelium in the vascular diseases *in vivo* remains unproven, accumulating evidence indicates that endothelial dysfunction is pivotal in the development of atherosclerosis and its complications (13,37).

Atherosclerosis is a complex, multifunctional disease with numerous predisposing factors, including hyperlipidemia, hypertension, diabetes, mechanical stress, hyperhomocysteinemia, smoking and inflammation, in which the cellular and molecular processes involved in the pathogenesis are unknown (13,38). Evidence increasingly suggests that several proatherogenic stimuli lead to increased production of reactive oxygen species (ROS) within the endothelial microenvironment and the resultant oxidative stress plays a key role in mediating the pathologic manifestations of EC dysfunction associated with atherosclerosis (37,38).

Cells respond to extracellular stimuli through activation of intracellular messengers. Various forms of cellular stress apparently constitute primary events that are transduced into the cytoplasm and alter the expression of genes. Recently, the production of ROS has been recognized as a key chemical process that regulates signal transduction pathways, which ultimately control gene expression and post-translational modification of proteins (1). These functions add to the known capacity of ROS to oxidize biological macromolecules such as DNA, protein and lipids (50) that contribute to the pathogenesis of a variety of diseases.

We review here the notion that ROS□s play a role in the EC function as physiological regulators of signal transduction and gene expression through the modulation of specific redox-sensitive pathways and explore the pathophysiological implications for vascular diseases such as atherosclerosis.

Oxidative stress and atherogenic risk factors

Oxidative stress occurs when redox homeostasis within the cell is altered. This imbalance may be due to either an overproduction of ROS or a deficiency in antioxidant defense mechanisms. The intracellular sources for free radicals include, but are not limited to, normal products of mitochondrial respiration, NADPH oxidase, nitric oxide (NO) synthases, cycloxygenases, lipoxygenases, cytochrome P-450 monooxygenases and xanthine oxidase (1,19). ROS□s include both free radicals, which typically have an oxygen- or nitrogen-based unpaired electron, and other species, such as H_2O_2. Classic examples of free radicals are superoxide anion (O_2^{-}), hydroxyl radicals ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$) (1,19). The relative contribution of any of these sources of ROS and the role of individual species in the pathogenesis of diseases of the vasculature is not well established, and their relative contribution will likely vary with cell type and physiological state of the cell.

A variety of well-established risk factors for the development of atherosclerosis such as hyperlipidemia, hyperglycemia, hypertension, hyperhomocysteinemia and local hemodynamic stresses are known to mediate elevated levels of ROS in the vasculature (38). The molecular and cellular mechanisms linking these diverse risk factors to a common mechanism are unclear. However, a current hypothesis suggests that modulation of the expression of selected vascular genes by intracellular oxidative signals may provide a molecular mechanism to link these risk factors with the early stages in the pathogenesis of atherosclerosis (3,10,13). These ROS□s and their modified target biomolecules (for example, oxidized LDL) then serve as second messenger molecules that transmit these extracellular signals to induce the expression of atherogenic gene products in EC, such as adhesion molecules (27,39). Thus, ROS□s may act as specific regulators in the signal transduction network to relay environmental and physical signals generated at the cell membrane to nuclear regulatory signals resulting in modulation of gene expression in EC.

Increases in ROS may affect four fundamental mechanisms that contribute to atherosclerosis: endothelial cell dysfunction, vascular smooth muscle cells (VSMC) growth, monocyte migration, and oxidation of LDL (oxLDL) (3,10). ROS and oxLDL induce the expression of different molecules in the EC surface, which stimulate monocyte binding and subsequent macrophages differentiation, such as vascular cell adhesion molecule 1 (VCAM-1), monocyte chemotactic protein 1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (33,51). Thus, antioxidant therapy appears to be an important alternative approach to the control of decrease atherogenesis. It has been shown that the endothelial dysfunction and oxidation of LDL in animals and patients with hypercholesterolemia can be controlled with antioxidants (4,29,53). These and other findings strongly suggest that the vascular redox state plays an important role in the pathogenesis of atherosclerosis.

Hypertension is a well-established risk factor of vascular disease, and both clinical and experimental evidence support a role of the renin-angiotensin system in the pathogenesis of hypertension-associated atherosclerosis (3). Angiotensin II (Ang II) activates NADPH oxidase to produce superoxide in EC (20,30) and induce the expression of VCAM-1, ICAM-1 and MCP-1, in processes blocked by NADPH oxidase inhibitors and catalase (16,34), suggesting that these enzymes can contribute to oxidative stress and the regulation of vascular genes. Thus, through the induction of oxidative stress and increased gene expression in endothelium, Ang II may serve as a molecular link between hypertension and the pathogenesis of atherosclerosis.

Diabetes-associated **hyperglycemia** causes intracellular oxidative stress, which contributes to vascular dysfunction (9). The effects of hyperglycemia on EC function can be mediated by several pathways: (a) production of ROS; (b) accumulation of sorbitol; (c) nonenzymatic glycoxidation of macromolecules; and (d) direct activation of protein kinase C (13,41). Because glycoxidation of proteins and lipids occurs ubiquitously in patients with diabetes and is irreversible, its consequences are especially relevant to long-term vascular dysfunction. The initial glycoxidation of proteins results in the formation of early glycation products. Further molecular rearrangement occurs, due in part to oxidation, and results in irreversible AGE (advanced glycation end products)-modified proteins. The interaction of AGEs with cell surface receptors (RAGEs) has been shown to generate ROS, decrease the levels of reduced gluthatione and activate the redox-sensitive transcription factor NFkB (12,54). Chronic AGE accumulation, observed in diabetic patients, promotes VCAM-1 expression and monocyte-endothelial interactions with the formation of atherosclerotic lesions, an effect that can be blocked by antioxidants (42). These observations indicate that pathologic processes associated with diabetes may cause intracellular oxidative stress and activate gene expression in endothelium.

Experimental and clinical studies have shown that **hyperlipidemia** impairs endothelial function and promotes atherosclerosis. In general, the degree of endothelial dysfunction of the coronary microvasculature correlates with total serum cholesterol levels (18), however the basic mechanisms that induce this process remain to be elucidated and cannot be deduced from clinical studies. One of the earliest events in atherosclerosis is the oxidative modification of lipoproteins, in particular LDL, in the vessel wall (13,18,36). Oxidative modification of LDL is probably one of the most important and critical events in the atherogenic process (11). OxLDL alters the intracellular redox status of the cell, in part through the generation of superoxide, and has been implicated as an important pro-oxidant signal in the EC (10,11,52). Although controversial, several studies suggest that oxLDL act as a pro-oxidant signal to regulate monocyte adhesion to EC, vascular gene expression such as ICAM-1 and VCAM-1 (11,14), and redox-sensitive

transcriptional factors such as activator protein-1 (AP-1) and NFkB, which are inhibited by antioxidants (28). Non-modified (native) LDL has also been shown to induce adhesion molecule expression on EC (44). In contrast, high density lipoprotein (HDL) inhibits the cytokine-induced expression of adhesion molecules (E-selectin, VCAM-1 and ICAM-1) in EC (6). Therefore the role of oxidation of the LDL and the exact nature of the cellular signals that mediate endothelial/leukocyte adhesion is an area for additional investigation.

Hyperhomocysteinemia (>100 μM) is associated with premature thrombosis and atherosclerosis, and even moderate hyperhomocysteinemia (>10 μM) is associated with increased risk of atherosclerosis and hypertension (31). Clinical studies have shown that patients with hyperhomocysteinemia exhibit endothelial dysfunction and produce increases on oxidative stress, both *in vitro* and *in vivo* (23,25), however the mechanisms by which homocysteine affects EC function are unclear. It is important to consider that most studies have used concentrations of homocysteine that exceed that observed *in vivo*. The experimental increase of plasma homocysteine concentration by methionine loading rapidly impairs endothelial function and ROS in healthy humans. Increased oxidant stress appears to play a key role in the deleterious endothelial effects of homocysteine because the administration of an antioxidant completely prevents these processes (23).

Working with human umbilical vein endothelial cells (HUVEC), we recently demonstrated in our laboratory that at pathophysiological concentrations (100μM), homocysteine increases intracellular ROS (Fig 1). Intracellular levels of ROS were determined using the non-fluorescent probe 2□-7□-dichlorofluorescein (DCF) diacetate. Removal of the acetate groups by intracellular esterases results in the release of dichlorofluorescin, which upon exposure to ROS is oxidized to the fluorescent probe dichlorofluorescein, which allows the quantitation of ROS in living cells.

FIGURE 1. Homocysteine increases intracellular levels of H_2O_2. HUVEC' s were pre-incubated with 10 mM DCF-diacetate, then treated for 1 h in absence (control) or presence of 100 mM DL-homocysteine (Hcy). DCF fluorescence was determined with excitation at 503 nm and emission at 529 nm. Results are presented as relative increase respect the control, and are the means ± SD of three separate experiments. * $p < 0.05$.

In brief, cells were preincubated with 10μM DCF-diacetate for 60 min, then treated with DL-homocysteine (100μM) for 60 min and solubilized in NaOH 0.1N. The fluorescence was determined to be 503/529 nm. Evidence suggests a role of ROS as a common and critical intermediate for various NFkB-activating signals, based on the inhibition by a variety of antioxidants (24). Exposing EC to homocysteine induces NFkB activation (Fig. 2). We used the electrophoretic mobility shift assay (EMSA) to detect NFkB in nuclear extracts of HUVECs obtained after 2 h of incubation with homocysteine (100 μM). To assess whether ROS induction by homocysteine plays a role in the NFkB activation we preincubated the cells with quercetin (20 μM), a natural antioxidant. As shown in Figure 2, this antioxidant prevents NFkB activation. Heme oxygenase (HO) is a widely distributed enzyme associated with the degradation of heme to iron, CO and biliverdin. Two distinct isoforms of HO have been extensively studied: HO-2 is the constitutive isoenzyme and HO-1 (heat shock protein 32), the inducible form (26). HO-1 is a stress response protein, up-regulated by a variety of factors including oxidative stress. The induction of HO-1 may have physiological importance because the end products of heme catabolism, biliverdin and bilirubin, possess antioxidant properties (5,43). We have shown that homocysteine (100 μM) induces HO-1 expression in HUVEC (Fig. 3). Exposure of cells to homocysteine for 6 h result in a significant increase in HO-1 expression, detected through the western immunoblot technique, with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Canada). In additional experiments we observed a direct effect of antioxidants in preventing the homocysteine-upregulation of HO-1, supporting the contribution of ROS to HO-1 induction in EC treated with homocysteine.

FIGURE 2. The effect of Quercetin, a natural antioxidant, on NFkB activation induced by Homocysteine. HUVEC were pretreated for 2 h with or without 20 mM Quercetin (Q) followed by 100 mM DL-homocysteine (Hcys) incubation for 1 h. Nuclear extracts from these cells were analyzed by EMSA with a labeled probe. The arrow indicates specific NFkB binding. A representative autoradiograph from three experiments is shown.

FIGURE 3. The effect of Homocysteine on HO-1 expression in HUVEC. HO-1 protein expression was analyzed by the Western immunoblot technique, using a polyclonal antibody anti-HO-1, in the soluble fraction of HUVEC treated with 100 µM DL-homocysteine (Hcys) for 8 h. A representative result from three experiments is shown.

Oxidative stress and signaling pathways

Oxidative stress can modulate a wide variety of biological processes by coupling signals at the cell surface with changes in gene expression, suggesting that multiple signaling pathways are involved. Indeed, ROS□s may be defined as true second-messenger molecules that regulate various signal transduction cascades upstream of nuclear transcription factors, including modulation of Ca^{2+} signaling, protein kinase and protein phosphatase pathways (32,47).

Some oxidation processes are reversible and can play a role in dynamic regulatory events as a result of variations in the redox conditions within the cell. Such variations may cause changes in signaling proteins and modify transductional pathways. ROS□s in general, and H_2O_2 in particular, are second messengers for various physiological and pathological stimulus, such as inflammatory cytokines, angiotensin, growth factors, ionizing radiation and others (1). For example, platelet-derived growth factor (PDGF) has been reported to increase intracellular H_2O_2 levels in VSMC and induce tyrosine phosphorylation and serine/threonine kinase stimulation (46). More recently, it was proposed that G-protein Ras acts as a mediator of ROS signaling, activating a cascade of kinases, including diverse members of the mitogen activated protein kinase (MAPK) family (22). In the case of ERK5 or BMK1 (big MAP kinase 1), H2O2 appears to be an exclusive activator (2). Dalton et al. have shown that homocysteine, an independent risk factor for atherosclerosis that induces oxidative stress (25), activates a specific receptor/transporter that is coupled to diacylglycerol production and protein kinase C (PKC) activation in VSMC (17).

These observations suggest that ROS may mediate specific signaling pathways within the cell as the proteins may be differently sensitive to oxidation according to their content of critical cysteine residues, to their conformation, or to the intensity of the oxidative stress (47). Hence possible signal specificity may be mediated by oxidative stress.

Different agents that induce oxidative stress have been demonstrated to stimulate **tyrosine kinase** activity, induce tyrosine phosphorylation events and activate downstream kinases such as protein kinase C, c-Src, raf-1 and MAPK (2,32,45-47). However, it is not yet clear whether ROS□s cause direct activation of tyrosine kinase or inhibition of tyrosine phosphatase activity. Because all tyrosine phosphatases have reactive cysteine residues in their active site, it has been proposed that inhibition of these enzymes by oxidants may produce increases in tyrosine phosphorylation. In this regard, PKC-mediated phosphorylation in response to oxidative stress may be the result

of inhibition of protein phosphatese 1 and 2A activity (47). Therefore, oxidants may regulate protein tyrosine phosphorylation by modifying the phosphorylation-dephosphorylation cascade and play a role in modulating several biochemical events that control cell growth and differentiation, smooth muscle proliferation and atherosclerosis.

Mitogen-activated protein kinases (MAPK) relay the mitogenic signaling pathways from the extracellular compartment to the cell nucleus through sequential kinase reactions that target transcription factor modification. The MAPK family consists of the extracellular-signal-regulated protein kinase (ERK) subgroup, the stress-activated protein kinase, or c-Jun N-terminal kinase (SAPK/JNK) subgroup, and the p38 mitogen-activated protein kinase subgroup (1,22).

One of the most characterized functional targets of the MAPK family is the transient phosphorylation of the transcription factor complex that regulates the c-fos promoter. Both c-fos and c-jun are components of redox-sensitive transcription factor AP-1. Considerable experimental evidence supports the notion that the induction of oxidative stress activates signaling pathways involving various members of the MAPK family (15,32,47). Initially, Baas and Berk demonstrated that superoxide increases the MAPK activity in VSMCs (8). Various physiological agents that participate in vascular dysfunction, including oxLDL, Ang II and linoleic acid and its metabolites, have been shown to elicit an increase in intracellular ROS and a rapid phosphorylation of different members of the MAPK family in VSMC□s. Treatment with antioxidants inhibited superoxide generation and blocked MAPK activation (7,35,49). Also, in ECs, H_2O_2 activates p38 MAPK activity with an associated reorganization of endothelial actin microfilaments (21). In cardiac myocytes, several groups have demonstrated that oxidative stress induced by ischemia-reperfusion stimulates MAPK activity (15). MAPK□s phosphorylate and enhance the transcriptional activity of the protooncogenes c-fos and c-jun, and play a role in the activation of nuclear transcription factors such as NFkB and AP-1 (1,32).

Cumulatively, these results imply that ROS initiates MAPK signaling pathways in the vasculature and provides a link to physiological stimuli that alter intracellular ROS levels and change gene expression.

CONCLUSIONS

The chronic disturbance of the cellular redox status leading to oxidative stress is associated with several pathological processes and may alter physiological functions. These disturbances modify different signaling pathways and induce transcriptional activators, which ultimately change cell function. In the pathogenesis of atherosclerosis several risk factors generate oxidative stress, and the resulting ROS could modulate the signal transduction processes leading to changes in vascular gene expression. VCAM-1, E-selectin and MCP-1 represent an important group of genes implicated in the pathogenesis of atherosclerosis, for which regulation is associated with oxidative stress through redox-sensitive signals and transcriptional factors. Therefore, the clarification of precise oxidative stress-regulated signaling pathways and their relationship with the regulation of vascular gene expression and endothelial dysfunction represent an important paradigm for understanding the pathogenesis of atherosclerosis and the development of future therapeutic treatments, which are primarily associated with the benefits of antioxidants(48).

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