Endoplasmic Reticulum Stress and Atherosclerosis

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Oxidized LDL and Atherosclerosis

Oxidized LDL (Ox-LDL) are known to promote atherogenesis through foam cell formation and inflammatory responses. The process involves receptor-mediated endocytosis of Ox-LDL which leads to lipid accumulation and vascular cell dysfunction including induction of apoptosis which represents major cause of plaque growth and rupture.

Plaque formation is induced by the accumulation of Ox-LDL, at the subendothelial level. Small oxidized lipids that are components of Ox-LDL, such as oxysterols, oxidized fatty acids, and aldehydes, are potent inducers of ROS production. They have been shown to increase mitochondria ROS generation and to trigger cytochrome c release leading to caspase activation and apoptosis. Thus, the death of vascular cells and monocyte-derived foam cells has been shown to modulate the cellularity of the plaque, and are thought to play important roles in plaque growth, as well as promoting procoagulation and plaque rupture [1].

Increased production of reactive oxygen species (ROS) in the vascular wall is a characteristic of inflammatory diseases including atherosclerosis, diabetes, and hypertension. ROS causes oxidation of lipids and target proteins and mediates a wide range of pathological processes in the endothelium, smooth muscle cells, and inflammatory cells. ROS are generated by enzymes expressed in cells of the vascular wall such as NAD(P)H oxidase, xanthine oxidase, and nitric oxide synthase. The increased activities and levels of these enzymes represent major vascular disease risk factors and are involved in vascular disease states in which oxidative stress is prominent.

Non-phagocytic NAD(P)H Oxidases and ROS Production

Several lines of evidence indicate that non-phagocytic NAD(P)H oxidases termed Nox are a major source of vascular ROS production and that they control the fine tuning of oxidative events, which in turn regulate multiple signaling pathways.

A new family of Nox has been defined on the basis of their homology with the catalytic subunit of phagocyte NAD(P)H oxidase gp91phox. Four homologues Nox-1, Nox-3, Nox-4, and Nox-5 proteins have been discovered in non-phagocytic cells [2,3]. Recent studies have demonstrated that the Nox-1, Nox-4, and Nox-5 homologues are mainly expressed in cultured vascular smooth muscle cells (SMC) [4]. Their activity is modulated by a variety of mediators such as angiotensin II, thrombin, platelet-derived growth factor, and tumor necrosis factor-α (TNF-α) known to play a deleterious role in vascular diseases. Coronary artery restenosis, a frequent complication of angioplasty, is accompanied by an increase in Nox-generated ROS production [5]. Likewise balloon injury of the carotid artery is known to result in an increase in ROS production throughout the vessel wall and this is associated with an up-regulation of Nox proteins. This increased in ROS appears to be derived from SMCs in the media and neointima of
the arterial wall [6]. However, the implications of oxysterols in the regulation of Nox and their cytotoxic effects in human vascular SMCs have not yet been investigated.

Our recent studies indicated that the cytotoxicity of 7-ketocholesterol (7-Kchol), a major oxysterol found in Ox-LDL, is tightly mediated through the upregulation of Nox-4 in SMCs. We show that 7-Kchol upregulates Nox-4 protein, triggers overproduction of ROS, and induces an endoplasmic reticulum (ER) stress and activates the unfolded protein response (UPR) leading to apoptosis and SMC death [7].

7-Kchol Stimulates Nox-4 Expression and ROS Production in SMC

To date, a number of studies have shown that oxysterols constitute an important family of oxygenated derivatives of cholesterol that exert potent biological effect in the pathogenesis of atherosclerosis [8]. Among the oxysterols that have been identified, those oxidized at the C7 position, such as 7-Kchol, are the ones most frequently detected at high levels in atherosclerotic plaques and in the plasma of patients with high cardiovascular risk factors. 7-Kchol exerts deleterious effects on SMCs including the stimulation of ROS production [9] and the induction of apoptosis, two major events involved in atherogenesis.

We found that 7-Kchol specifically induces the over-expression of Nox-4 at both the mRNA and protein levels and enhances ROS production in SMCs. Using a RNA interference strategy, we show that these two effects were reduced in SMC transfected with Nox-4 siRNA, whereas no effect was observed with scrambled siRNA demonstrating the crucial role of Nox-4 in 7-Kchol-induced ROS generation [7].

Nox-4 Mediates 7-Kchol Cytotoxicity

We provided evidence that 7-Kchol induces a loss of the mitochondrial potential a major event in the onset of apoptosis. SMCs treated with 7-Kchol exhibit substantial amounts of apoptotic and necrotic cells (40% and 15% of the total cell population, respectively). Silencing of Nox-4 prevents 7-Kchol-induced cell death and results in inhibition of mitochondrial-caspase dependent apoptosis (the number of apoptotic cells was 75% lower in Nox-4 siRNA transfected cells than in control cells).

Nox-4 Modulates Endoplasmic Reticulum Stress Induced by 7-Kchol

Immunostaining of Nox-4 indicated that Nox-4 is associated with ER where it produces low amounts of ROS required for intracellular signaling. Upregulation of its expression by 7-Kchol suggests that 7-Kchol elicits an ER stress by depleting ER calcium stores and by altering ER oxidizing environment, two factors which favor protein folding and assembly.

7-Kchol overload as well as ROS overproduction may alter ER homeostasis and may lead to the accumulation of unfolded proteins which are prone to aggregation and could interfere with the normal functioning of the ER. To cope with this stress, cells activate a signal transduction system called the unfolded protein response (UPR). The activation of UPR may lead to cell survival (by triggering the synthesis of ER chaperone proteins such as glucose regulated protein of 78 kD (GRP78/Bip) and protein disulfide isomerase (PDI) or alternatively to cell demise via the activation of programmed cell death signals [10-12].

We investigated the expression patterns of several molecular indicators of ER stress that were elicited by 7-Kchol in SMCs transfected with Nox-4 siRNA or in control cells. In all cases,
7-Kchol induced very rapid ER stress as assessed by the occurrence of transient cytosolic Ca$^{2+}$ oscillations. Furthermore, the activity of c-jun-N-terminal kinase (JNK) was increased, as indicated by JNK and c-jun phosphorylation. GRP78/Bip chaperone and CHOP/GADD153 expressions were upregulated by 7-Kchol.

![Signaling pathways of the Unfolded Protein Response (UPR)](image)

Interestingly, we found that the amount of Bax proteins, shown to be potent pro-apoptotic factors, significantly increased, while the level of the anti-apoptotic protein Bcl-2 concomitantly decreased in 7-Kchol-treated cells. Furthermore, all these effects were reversed in Nox-4 siRNA-transfected cells, indicating that Nox-4 is involved in an ER signaling pathway, which is very sensitive to 7-Kchol and probably to other factors known to induce oxidative stress.

**Nox-4 Controls Endoplasmic Reticulum Stress Sensors**

7-Kchol may act directly or indirectly on a stressor of ER, thus resulting in the induction of UPR and subsequent cell apoptosis. Our findings show that 7-Kchol activates one of the transducers of the UPR, IRE-1, and the downstream signaling pathways, which partially regulate both adaptative survival as well as apoptotic pathways [13]. Multiple overlapping pathways, both pro-apoptotic and anti-apoptotic, induced by ER stress and mediated by IRE-1, may be implicated in the cytotoxicity of 7-Kchol. We provide for the first time evidence that 7-Kchol promotes Nox-4 expression by stimulating the IRE-1/JNK/AP-1 signaling pathway. Silencing of IRE-1 by siRNA strategy or the inhibition of JNK activity, both suppressed Nox-4 expression and protected SMCs from 7-Kchol-induced cell death. This demonstrates that Nox-4 expression, which is essential for the ER-stress-induced cell death triggered by 7-Kchol, is controlled by the IRE-1 signaling pathway. Although we still do not know how Nox-4 acts on downstream targets to induce cell death, the Nox-4 silencing experiments clearly show that among the UPR-inducible pro-apoptotic effectors identified, the expression of CHOP is regulated by Nox-4.
Conclusion and Perspectives

Our findings point to a fundamental mechanism underlying the molecular sensing of atherosclerosis. We postulate that Nox-4 may play a pivotal role in maintaining ER homeostasis (at least in vascular smooth muscle cells), its upregulation induces the phosphorylation and dimerisation of the two ER stress sensors, PERK and IRE-1, and stimulates downstream signaling events leading to cell survival or cell death depending of the nature of activators.

Therefore, control of Nox expression (down- or up-regulation) by specific drugs may represent new opportunities for preventing and treating atherosclerosis. It is not known whether Nox is involved to relieve or to sustain ER stress reported in many other chronic diseases, such as obesity, diabetes, hypertension, and neurological disorders. All share disturbed lipoprotein metabolism and enhanced oxidative stress. In the future, studies combining pharmacological and nutritional interventions in appropriate experimental animal models will certainly provide valuable information to better understand how to modulate the ER stress response in vascular smooth muscle cells.

References