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Endoplasmic Reticulum Stress, NRF2 Signalling and Cardiovascular Diseases in a Nutshell

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Abstract

Purpose of Review This short review is intended primarily to summarize the understanding of the interrelated roles of endoplasmic reticulum (ER) stress, oxidative stress and inflammation in cardiovascular diseases.

Recent Findings Insults interfering with ER function lead to the accumulation of unfolded and misfolded proteins in the ER. An excess of proteins folding in the ER is known as ER stress. This condition initiates the unfolded protein response (UPR). When the UPR fails to control the level of unfolded and misfolded proteins, ER-initiated apoptotic signalling is induced. Moreover, the role of the protective nuclear erythroid-related factor 2 (Nrf2)/antioxidant-related element (ARE) and the activation of the pro-inflammatory nuclear factor-kappa B (NF-kB) are analysed. Authors summarize evidence that oxidative stress, inflammation and ER stress are closely entwined phenomena. They are involved in the pathogenesis of different cardiovascular diseases. Current literature data are presented, focusing on three topics of related pathologies: atherosclerotic plaque, coronary artery disease and diabetes.

Summary This review will provide a basic platform for study and application to several other conditions in which oxidative stress, ER stress and inflammation are key features. Future studies in this area may identify the most promising molecules to be investigated as common targets for cardiovascular diseases.

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Introduction

Different perturbations in the endoplasmic reticulum (ER) function cause the accumulation of unfolded and misfolded proteins in the ER. The proteins folding excess is the so-called ER stress. This condition induces the unfolded protein response (UPR). When the UPR fails to control the unfolded and misfolded proteins level, ER-initiated apoptotic signalling is activated. The protective nuclear erythroid-related factor 2 (Nrf2)/antioxidant-related element (ARE) and the activation of the pro-inflammatory nuclear factor-kappa B (NF-kB) have a major role in cardiovascular diseases.

Oxidative stress, inflammation and ER stress are narrowly entwined conditions. They are involved in the pathogenesis of different cardiovascular diseases.

Emerging Factors in Cardiovascular Diseases: Endoplasmic Reticulum Stress, Oxidative Stress and NRF2 Signalling

In eukaryotic cells, the majority of secreted and transmembrane proteins fold in the endoplasmic reticulum (ER) lumen [1•]. Proteins usually enter the ER as "unfolded" polypeptides [1•]. The entrance into the ER can vary depending on the conditions of the cell. As a result, cells modulate the protein-folding capability of the ER basing it to their needs. Such homeostatic control is achieved by sensors at the entrance of the ER lumen and effectors that communicate the messages to other cell zones. ER trans-membrane sensors detect the accumulation of the unfolded proteins and activate

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transcriptional and translational pathways that deal with the unfolded and misfolded proteins. This is known as the unfolded protein response (UPR) [1•].

Incompletely folded or misfolded proteins are subjected to ER-associated degradation (ERAD) that occurs in the cytoplasm. However, the failure to relieve prolonged or severe ER stress is the cause of the cell apoptotic death [1•].

When ER stress occurs, three ER transmembrane sensors are activated to initiate adaptive responses [2]. These sensors consist of protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and the transcriptional factor activating transcription factor 6 (ATF6). PERK, IRE1 and ATF6 are maintained in an inactive form through the interaction of their N-terminus with glucose-regulated protein 78 kDa (GRP78/BiP) [3]. When unfolded proteins accumulate, GRP78/BiP releases PERK, IRE1 and ATF6 to allow their oligomerization. Then BiP triggers the UPR. If UPR fails to control the level of unfolded and misfolded proteins, ER-initiated apoptotic signalling is prompted with the activation of the death factor: CCAAT/enhancer binding protein homologous protein (CHOP) [3].

The UPR applies several mechanisms to minimize ER stress. One of these mechanisms involves the activation of chaperones synthesis in order to improve and intensify the intraluminal protein folding.

Another mechanism blocks the transfer of proteins to prevent further proteins loading into the ER. Among ER stress sensors, IRE1 is the most evolutionarily preserved. In normal conditions, IRE1 interacts with GRP78/BiP and prevents IRE1 activation [1•]. IRE1 is then activated by self- phosphorylation. The activated IRE1 specifically splices mRNA, encoding Xbox binding protein (XBP)1, and so inducing the activation of XBP1 [4]. XBP1 increases the transcription of chaperones and other UPR-related proteins and enhances the degradation of the misfolded proteins. Eventually, IkB kinase is activated followed by IkB kinase-mediated suppression of the inhibitor of kB protein and the induction of the nuclear factor (NF)-kB. IRE1 is therefore confirmed as a link between the ER stress and inflammation [5].

Similar to IRE1, PERK is activated through dissociation of GRP78/BiP from the luminal binding domain and self-phosphorylation in stress conditions [1•]. PERK downregulates the eukaryotic translation initiation factor 2 (eIF2α) that allows the release of the transcription factor ATF4 permitting it to trigger CHOP [1•].

Phosphorylation of eIF2α by PERK down regulates other protein synthesis, which is a compensatory control to preserve the cell from the protein overproduction stress. ATF6, in presence of ER stress, is cleaved by two proteases associated with the Golgi complex. After the cleavage, the cytosolic N-domain of ATF6 trans-locates into the nucleus and there it activates the expression of many UPR-related genes including GRP78/BiP and XBP1. [1•].

The UPR also generates excess levels of reactive oxygen species (ROS). This process is driven by proteins involving protein disulphide isomerase and ER oxido-reduction [6].

One of the most important cellular defence mechanisms against ROS excess is controlled by nuclear erythroid-related factor 2 (Nrf2), that is a PERK-dependent master transcriptional activator. It regulates many of the antioxidant defence genes [7–9]. Nrf2 is part of a family of transcription factors containing a unique basic-leucine-zipper (bZIP) motif (cap-n-collar (CNC) family [10, 11]). Nrf2 is the main mediator of cellular adaptation to redox stress [11].

Nrf2 activates a number of enzymes with antioxidant and detoxifying activity, with key role in the cell protection against different environmental stresses, such as electrophiles, ROS, and reactive nitrogen species. Moreover, Nrf2 drives the transcription of several drug metabolizing enzymes, transporters, cellular reducing molecules (glutathione, GSH and nicotinamide adenine dinucleotide phosphate, NADPH, oxidase and proteasomes) [12]. Nrf2 is responsible for both constitutive and inducible expression of the antioxidant response element (ARE)-regulated genes [13]. Nrf2-null mice have reduced expression of antioxidant genes, increased oxidative stress, decreased reducing and antioxidant capacity [14]. These data suggest that the Nrf2/ARE pathway is critical for the regulation of intracellular redox status. Under basal conditions, Nrf2-dependent transcription is blocked by its negative regulator Keap1. When cells are exposed to oxidative stress or electrophiles, Nrf2 accumulates in the nucleus and triggers the expression of its target genes [13]. The mechanisms by which Nrf2 is released from Keap1 have been deeply investigated. One proposed mechanism is that cysteine thiol groups of Keap1 function as sensors for oxidative stress [15, 16]. This mechanism causes the formation of disulphide bonds between cysteines of two Keap1 peptides and leads to the conformational changes that make Keap1 unable to bind Nrf2. Alternatively, dissociation of Nrf2 from Keap1 has been described to be caused by protein kinase C-induced phosphorylation at Nrf2 Ser-40. These two mechanisms can work in concert [15]. A feedback self-regulatory loop between Keap1 and Nrf2 controls cellular abundance of them [16]: Nrf2 regulates Keap1 by regulating its transcription, and Keap1 controls Nrf2 by facilitating its degradation. Besides mediating stress-stimulated induction of antioxidant and detoxification genes, Nrf2 contributes to adaptation by tempering intermediary metabolism. In particular, Nrf2 inhibits lipogenesis, activates the oxidation of fatty acids, simplifies the flux through the pentose phosphate pathway and increases NADPH regeneration and purine biosynthesis [17].

Among the different transcription factors that are regulated by UPR, Nrf2 has a major role in modulating the non-antioxidant and antioxidant response triggered by UPR [18]. PERK participates in the regulation of Nrf2 phosphorylation and dissociation from Keap1 [19]. Moreover, recent studies

show that the proteasome-mediated ERAD is in part regulated by Nrf2 [20].

The Authors have recently reviewed [21••] the updated role of Nrf2 in cardiovascular diseases and its protective activity.

To summarize, a series of studies with interventions on ER stress and Nrf2 activation have been shown to reduce myocardial infarct size and cardiac hypertrophy in an animal model of heart failure exposed to ischemia/reperfusion injury and pressure overload respectively [22, 23].

Nrf2 provides also an exciting opportunity in prevention strategies. Its activity can be increased by thiol-reactive foreign compounds, which are called "indirect antioxidants" because they stimulate Nrf2-mediated induction of antioxidant genes by inhibition of Keap1 [24]. Indirect antioxidants are known to protect against oxidative stress and inflammation, though little is known about whether they can prevent ER stress. The benefits of such agents in the setting of cardiovascular diseases are uncertain. Based on these uncertain effects of Nrf2 activation on the cardiovascular system, further research is clearly needed.

Apart from lowering oxidative stress, there are other different approaches that could potentially reduce either the ER stress itself or manipulate the UPR. Chemical chaperone-like molecules, such as 4-phenylbutyric acid and tauroursodeoxycholic acid (TUDCA) have been used to stimulate the correct protein folding [25].

Moreover, natural products and plant-derived phytochemicals such as sulforaphane, curcumin, resveratrol, allicin and garlic organosulfur compounds are Nrf2 activators. Curcumin is known to moderate acute doxorubicin-induced cardiomyopathy in animal models [26, 27].

Up to now, safety and clinical daily usefulness data are not definitive. The most intriguing points about this topic have been carefully reviewed [28•].

Focus on: Human Atherosclerotic Plaque

Atherosclerotic plaque provides conditions that can trigger ER stress and activate UPR [29•]. In this context, reduced apoptosis and plaque necrosis have been shown in mice lacking CHOP [30], providing direct evidence for a causal link between the ER-stress effector CHOP and plaque progression.

Moreover, markers of oxidative stress have been identified in human atherosclerotic plaques. In fact oxidized derivatives of PUFA such as arachidonic and linoleic acids have already been identified and postulated to function as second hits to trigger apoptosis in macrophages exposed to low levels of ER-stress [29•, 31]. Some of these oxidized derivatives of arachidonic and linoleic acids are 8-iso-prostaglandin-F2α (8-iso), the prototype of F2-isoprostanes, 9-hydroxyoctadecadienoic acid (9-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE). They have also been shown to be mediators of

important biological effects through their binding to human thromboxane A2 (TP) receptor and to G2A receptor [31, 32].

Our research has demonstrated [33] that some oxidized derivatives of polyunsaturated fatty acids (PUFAs, in particular hydroxyoctadecadienoic acids and hydroxytetraenoic acids) contained in the tissue around the necrotic core (TANC) of human carotid plaques may have a role in the expansion of necrotic core. They induce defective efferocytosis [33].

Newer studies have evaluated the relationship between macrophage apoptosis and the ER-stress in TANC and in the periphery of human carotid plaques [34] and the role of oxidized derivatives of PUFAs in promoting ER-induced macrophage apoptosis.

TANC of carotid plaques was characterized by abnormal amount of apoptotic cells, which was attributed at least in part related to sustained ER-stress. In fact, CHOP and apoptosis-related genes expression prevailed in TANC, while PERK and survival genes prevailed in the periphery. Different concentrations of oxidized derivatives of PUFAs contributed to drive this specular expression. As a result, ER-stress may promote macrophage apoptosis in TANC and favour the expansion of necrotic core, which is an important contributor to plaque disruption and luminal thrombosis. To understand if the oxidative derivatives of PUFAs in the extracts were able to stimulate the expression of PERK and CHOP and of Nrf2/ARE related genes and apoptosis, monocytoid cells were incubated with increasing amounts of the periphery and TANC extracts. The periphery extract determined a dose-dependent increase of Nrf2 and related genes. These preliminary, these results suggest that the concentrations of these oxidative derivatives may play a role in determining the direction toward apoptosis or survival.

The postulated roles of the oxidative and ER stress in triggering and orchestrating the atherosclerotic plaque vulnerability haves been recently described [35••].

Macrophage apoptosis is an important characteristic of atherosclerotic plaque development, and many studies have demonstrated that apoptotic macrophages accumulate in advanced atherosclerotic plaques [36].

If phagocytes cannot sufficiently eliminate the apoptotic macrophages, these apoptotic cells become necrotic and coalesce over time into a key feature of the vulnerable plaque, the necrotic core [37, 38]. This process is called "defective efferocytosis". Efferocytosis is an important mechanism in which there is the phagocytes attraction to the apoptotic, necrotic, or damaged cells with the precise engulfment and endosomal/lysosomal destruction of the apoptotic cells [36]. Apoptotic cells and phagocytes that engulfed them produce anti-inflammatory cytokines, (TGF-alpha, IL-10 and others). Although macrophages can inhibit atherosclerosis development, the overloaded phagocytes, such as foam cells in atherosclerotic plaques, likely have compromised engulfment mechanisms. This fact causes the defective efferocytosis and

the conversion of apoptotic into necrotic cells. The final step is the release of pro-atherogenic factors and accumulation of cell debris, which enhance atherosclerosis progression [38]. The early phagocyte attraction occurs by a host of "find me" signals [39]. Then the phagocytes must encounter appropriate "eat me" signals displayed by the apoptotic cells such as phosphatidylserine (PS) and different opsonin-like proteins that link to various receptors on the phagocytes. This topic has been reviewed by Van Vrè et al. [38]. PS exposure by itself does not suffice for adequate efferocytosis. Several bridging molecules facilitate the interaction of PS on the cells with receptors on phagocytes. The consequence of accelerated macrophages apoptosis coupled with defective efferocytosis is the expansion of necrotic core [39].

Evidence also points to a critical role of Nrf2 in stimulating the antioxidant response through the interaction with the UPR-genes regulation [40]. In this context, Nrf2 signalling has been shown to upregulate the expression of the proteasome catalytic subunits in different cell types and to contribute to the ER stress response by enhancing proteasome-mediated ERAD [41]. So far, however, there is no precise data showing that this mechanism is active also in macrophages of vulnerable plaques.

Focus on: Coronary Artery Disease

Coronary artery disease is a common complex atherosclerotic pathology associated with substantial morbidity and mortality [42]. ER stress and UPR markers have been detected in both human and animal atherosclerotic lesions. An important advance in this area was achieved by examination of histological sections of human atherosclerotic coronary artery lesions obtained by autopsy or after coronary atherectomy by Myoishi et al. [43••]. Both smooth muscle cells and macrophages revealed a prominently increased expression of the ER chaperones GRP78/BiP and GRP94 and CHOP in thin-cap atheroma and ruptured plaques compared to fibrous plaques and thick-cap atheroma. Advanced atherosclerotic plaques have a pathophysiological environment that causes ER stress and activates the UPR due to the existence of oxidized lipids, inflammation, and metabolic stress [44]. Several studies have demonstrated a progressive increase in oxidative-inflammatory markers and monocyte activation from control subjects to stable and to unstable angina patients [45–47]. More recent studies [48] have shown for the first time that the expression of GRP78/BiP, as a representative of UPR, and of CHOP, as a representative of ER-initiated apoptotic signalling, were significantly higher in circulating cells of stable coronary artery disease (CAD) patients compared to healthy controls (C).

Activation of UPR and CHOP in circulating cells of CAD patients indicated also that the circulating environment may be somehow altered in CAD [48]. This study also showed that inflammation, altered redox state and abnormal levels of ox-

LDL were chronically present in CAD, during the acute event despite the achievement of the desired targets for glucose, lipid and blood pressure values. CAD patients, in fact, presented higher plasma levels of high sensitivity C reactive protein and ox-LDL and lower circulating GSH than healthy C. In this context, ox-LDL has been shown to trigger ER stress in vascular cells in vitro, and oxidation products of phospholipid 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC) lead to ER stress and activate the UPR in human aortic endothelial cells [49].

Growing evidence suggests that signalling pathways in the UPR and inflammation are interconnected through various mechanisms and in particular through the activation of NF-κB [50].

In this context, ox-LDL induced an inflammatory response via specific receptors in both stable and unstable CAD patients [45–47]. The phenomenon, however, was much more evident in unstable patients. The slight increase in hs-CRP found in this study in stable CAD patients could be related to a mechanism other than ER stress, and the results cannot exclude that inflammation may have, at least partially, played a role in the induction of UPR. Further studies are needed to explore how ER stress can be affected by the extent of inflammatory response. Authors' results also showed that oxPAPC-induced UPR and ER-initiated apoptotic signalling observed in circulating cells of CAD patients were not associated with an adequate expression of the Nrf2/ARE-related genes. The same inadequacy was reported in other chronic pathologies such as chronic renal failure [51] and chronic obstructive pulmonary disease [52]. Although the precise mechanism of decreased Nrf2/ARE expression in chronic pathologies remains to be fully determined, a partial explanation of these results may derive from a previous study [53], showing that monocytes from heavy smokers with the highest production of ROS did not appropriately react in terms of Nrf2/ARE activation [53]. It is possible that it is an excess of oxidative stress coordinates the switch from the protective UPR and Nrf2/ARE gene expression to ER initiated apoptotic signalling in different pathologies, including coronary artery disease.

Focus on: Diabetes

An emergent body of evidence has linked ER stress and the pathogenesis of diabetes mellitus. ER stress contributes to loss of pancreatic β cells and resistance to insulin [54•]. Insulin synthesis requires a complex series of molecular events that are initiated in the ER. Like many other proteins targeted for secretion on the cell surface, pro-insulin and its converting enzymes require special maturation steps in the ER. Pro-insulin synthesis may vary several-fold under normal and pathological conditions. β cells utilize the UPR homeostatic

mechanism to balance the load of the new formed insulin and the ER capacity to properly fold it [54•].

The most convincing emphasis for the role of ER stress in β cell failure was initially found by studying rare genetic disorders. The model of the Akita mouse is the most convincing example that links ER stress and β cell failure. The Akita mouse expresses a pro-insulin variant gene and the misfolded insulin accumulates in the ER. Akita mice develop diabetes due to β -cells loss caused by ER stress [55].

Another genetic example of ER stress-induced diabetes derives from the Wolfram syndrome [56]. Mutations in Wolfram Syndrome gene 1, which encodes an ER calcium channel, lead to young-onset diabetes associated with selective β cell loss. At last, a mutation in PERK causes the Wolcott-Rallison syndrome, a rare disorder characterized by early-infancy insulin-dependent diabetes [57]. Evidence for ER stress in animal and human pancreatic islets has been confirmed in conditions where β cells were subjected to high concentrations of glucose. This in vitro condition has been seen to overwhelm the ER folding capacity, causing an imbalance in homeostasis and leading to UPR activation and to the ER apoptosis [58, 59]. Also glucose deprivation can cause ER stress, with established stimulation of the UPR and the ER apoptosis [60]. Moreover, in human studies, ER stress and UPR activation markers were increased in β cells from pancreatic sections of type 2 diabetes mellitus (T2DM) patients compared with non-diabetic pancreatic tissue [61].

Our group has investigated the UPR and the ER initiated apoptotic signalling in circulating cells of T2DM patients without the recommended glycemic goals [62].

The study examined effects of the prolonged glycemic, inflammatory and oxidative stress as possible UPR and ER apoptosis inductors in activating and modulating the ER stress response and the Nrf2/ARE pathway activation.

Major findings were that there was an activation of the UPR and of the ER apoptosis in circulating cells of T2DM without glycemic target achievement. This may be associated to the chronic hyperglycemia, to the augmented inflammation and oxidative stress, without a corresponding Nrf2/ARE defence activation.

To date, precise data about how and why circulating cells are susceptible in T2DM is lacking. However, the finding that the UPR and the ER apoptosis were stimulated in circulating cells indicates that the circulating environment may also be somehow altered, with similarities with their previous results in CAD [48]. Glucose dose-dependently increased ROS at different times, and this increase was inhibited by diphenyliodonium (DPI), a specific NADPH oxidase inhibitor. The glucose-induced ROS generation was linked, after prolonged incubation and with the highest concentration of glucose, to a considerable increase of CHOP expression. The results of this study also indicated that malondialdehyde (MDA) concentration was higher in T2DM patients than in controls. The results

concerning increased MDA levels in T2DM were consistent with previous data [63]. Increased production of ROS in T2DM patients may therefore affect oxidation of fatty acids. In fact, MDA is a collection of end-products of different types of oxidized fatty acids, and it is used to estimate in vivo lipid peroxidation. Inflammation and ER stress are linked through several mechanisms and in particular through the activation of NF-kB [50], with proven involvement in T2DM. In fact, insulin itself has been shown to have a strong acute anti-inflammatory effect, by reducing NF-kB activity [64].

Authors' data confirm that ER is a critical site where metabolic and inflammatory pathways touch [65]. Activation of NF-kB may also be a consequence of oxidative stress. Moreover, NF-kB regulates many inflammatory responses in β cells, including the expression of several cytokines and chemokines, and its activation contributes to development of diabetes in mice [54•].

Also in presence of ER stress and of oxidative stress and inflammation augmented levels, there is an inadequate response of Nrf2/ARE response in circulating cells of T2DM patients [62], in agreement with the previous study where the same inadequacy was reported in patients with CAD [48]. The in vitro experiments suggest that an excess of oxidative or metabolic stress that causes the switch from the protective UPR and Nrf2/ARE gene expression to ER-initiated apoptotic signalling.

On the basis of these data, it can be argued that oxidative stress, ER stress and inflammation are integral interconnected pathological features in T2DM, as recently reviewed [65].

Conclusions

This short review is intended primarily to summarize the understanding of the roles of ER stress, oxidative stress and inflammation in cardiovascular diseases. The current field of research is wide. Nevertheless, this review provides a basic platform for study other different diseases in which oxidative stress, ER stress and inflammation are key features.

While considering these elements, the reader has to appreciate the reiteration of such pathways and elements in the different settings. This "combined" approach will be useful to fully understand this area and to facilitate an informed therapeutic solution.

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