Published in final edited form as:

Curr Atheroscler Rep. 2012 October ; 14(5): . doi:10.1007/s11883-012-0266-8.

Evolving Concepts of Oxidative Stress and Reactive Oxygen Species in Cardiovascular Disease

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Abstract

Cardiovascular disease continues to be a substantial health-care burden, despite recent treatment advances. Oxidative stress has long been regarded as a key pathophysiological mediator that ultimately leads to CVD including atherosclerosis, hypertension and heart failure. Over the past decade, emerging evidence has shifted our understanding of reactive oxygen species (ROS) from its harmful role to being signaling molecules. Here, we reviewed recent advances in our understanding of ROS that mediate the complex process of cardiovascular diseases, with a focus on major ROS signaling and sources such as mitochondria and NADPH oxidases.

Introduction

Our concepts of how oxidative stress contributes to chronic diseases has undergone considerable evolution in the past two decades. Historically, reactive oxygen species (ROS) and their resultant oxidative stress have been examined in the context of damage to biologically important targets such as proteins, lipids, and DNA. However, many clinical studies with antioxidants have failed to materially impact the course of human disease, including atherosclerosis and cardiovascular disease. As a consequence, the focus of how oxidative stress impacts vascular disease has shifted towards an ever increasing appreciation of oxidized targets as biomarkers and the importance of ROS as signaling molecules. With regards to the latter, research in model systems have clearly established that ROS mediate a number of physiologic and pathophysiologic processes. We are only now beginning to appreciate how these concepts are applicable to human clinical disease and this review will focus on recent clinical evidence.

Oxidative Stress: Janus-Faced Implications for Disease

With regard to cardiovascular disease, many of the pathogenic components of the disease are strongly linked to oxidative stress. For instance, LDL oxidation, endothelial dysfunction and inflammation processes are mediated, in part, by increased cellular ROS production. Similarly, other pathologic conditions associated with cardiovascular disease such as insulin resistance, metabolic syndrome, and obesity are characterized by overproduction of ROS and excess oxidative stress (1,2).

In contrast, ROS are also physiologic with one of the best examples being chronic granulomatous disease (CGD) that results from genetic defects in the phagosomal NADPH oxidase limiting ROS production (3). Patients with CGD have defective responses to

pathogens manifest as recurrent infections and the lack of ROS production produces the inability to resolve innate immune responses (4). This involvement of ROS in the control of innate immunity has also been extended to mitochondrial ROS that appear to have a role in regulating Toll-like receptor responses (5). Overall, reparative processes appear to be particularly dependent upon ROS as both angiogenesis (6) and pulmonary remodeling (7).

Thus, ROS can mediate both cellular damage and physiologic events on a contextual basis. One important element in reconciling these conflicting ROS functions involves understanding the chemical nature of ROS and their molecular targets. Molecular oxygen is the parent element for ROS that are derived by sequential reduction as outlined in the scheme below:

Both superoxide and hydroxyl radical are single-electron species that have limited half lives, typically act locally, and can mediate cellular damage. In contrast, H_2O_2 is better suited as a signaling molecule based upon its relatively longer half-life, diffusion capacity, and reaction with protein thiol moieties (8). Thus, the relative likelihood of cell damage versus signaling is dependent, in part, upon the type of ROS produced.

Another important aspect of ROS signaling versus damage is the amount of ROS produced. In the setting of inflammation, the phagocytic respiratory ROS burst produces local H_2O_2 concentrations between 5 and 15 μM (9). In contrast, H_2O_2 levels in neurons at baseline are approximately 0.2 nM, and increase to 2.5 nM with insulin treatment (10). Similarly, epidermal growth factor-induced H_2O_2 production yields cellular levels of 2 nM using advanced fluorescent single-walled carbon nanotube technology (11). Thus, physiological levels of H_2O_2 are typically less than 20 nM and cellular damage involves levels greater than 1 μM (12). This dichotomy is reminiscent of the nitric oxide system where low nM levels of $\text{NO}\bullet$ production by endothelial nitric oxide synthase is regulatory, whereas μM levels of $\text{NO}\bullet$ from inducible nitric oxide synthase contribute to the pathophysiology of septic shock. Thus, the type and amount of ROS produced provide an important basis for distinguishing physiological signaling from pathophysiological cellular damage.

ROS in Vascular Disease and Physiology

ROS and Oxidative Stress in Atherosclerosis

Atherosclerosis involves LDL entrapment in the arterial wall and an inflammatory response to the local LDL. The complexity of this process precludes a thorough discussion here. Nevertheless, common features of atherosclerosis include LDL oxidation, endothelial dysfunction, and inflammation (13). Importantly, these three features of atherosclerosis all involve ROS in their pathophysiology.

The oxidation of LDL is a well-described phenomenon in atherosclerosis. A number of enzyme systems have been proposed to contribute to LDL oxidation *in vivo* and there are a number of reviews available on this topic (13–15). The most complete data concerning ROS-mediated LDL oxidation *in vivo* involve the contributions of NADPH oxidases and mitochondria. Human atherosclerotic coronary arteries contain increased immunostaining of p22phox (16), an NADPH oxidase subunit. This protein is principally associated with Nox2 in lesional macrophages, and the p22phox expression level is positively associated with atherosclerosis severity (17). Animal studies using the ApoE-null atherosclerosis model indicate that mice lacking the Nox2 isoform of NADPH oxidase exhibit a 50% reduction in lesions, along with a marked decrease in aortic ROS production, suggesting that inhibition

of Nox2 NADPH oxidase could limit atherosclerosis (18). Humans express an NADPH oxidase isoform (Nox5) that is not found in rodents, and this oxidase may also contribute to blood vessel ROS as coronary arteries with atherosclerosis exhibit an increased expression level and activity of Nox5 (19).

Mitochondrial ROS have been implicated in many chronic diseases, including atherosclerosis (20). Emerging data now link mitochondrial ROS production to the control of inflammation. For example, mitochondrial ROS are important for signaling events critical to innate immunity (5) and activation of the NLRP3 inflammasome (21) that is known to contribute to both animal and human atherosclerosis (22).

As atherosclerotic lesions mature, they develop a fibrous cap overlying a lipid core. Acute vascular events are often caused by weakening of this fibrous cap and plaque necrosis is a key mechanism for fibrous cap weakening and rupture (23). The apoptosis of macrophages, and the inability to clear these apoptotic cells, are key contributors to plaque necrosis. When macrophages become apoptotic, they also stimulate a process known as autophagy in which the cells consume their own cellular components in an organized manner. It turns out that autophagy is an important adaptive mechanism for oxidative stress and without autophagy, ROS production (via Nox2 NADPH oxidase) is increased and plaques become more prone to rupture (24). Thus, oxidative stress is dependent upon autophagy and recent data suggests that stimulating autophagy could have beneficial effects for atherosclerosis.

ROS in Hypertension

Hypertension is an important risk factor for atherosclerosis and it also contributes to left ventricular hypertrophy and heart failure. Clinical studies have clearly indicated that inhibition of the renin-angiotensin-aldosterone system (RAS) is of major benefit for patients with hypertension (25). One potential reason for the benefit of inhibiting the RAS system is the reduction of oxidative stress and dysfunctional ROS signaling. For example, activation of the RAS system in model systems upregulates several NADPH oxidase isoforms such as Nox1 and Nox2, and subunits p22phox, p47phox, and p67phox (26–28). Genetic mouse models have dissected the specific consequences of the NADPH oxidase isoforms. Animals without the Nox1 protein develop less hypertension with angiotensin II infusion (29), but exhibit the same pathologic arterial medial hypertrophy as wild-type animals. In contrast, Nox2 appears to have no impact on the blood pressure response to angiotensin II, but prevents pathologic events such as RAS-induced medial hypertrophy (30). The RAS also has implications for vascular inflammation as angiotensin infusion recruits inflammatory cells, particularly T cells, to the vascular adventitia (31). Adventitial T cells, and their ROS production, is linked to the pathophysiology of hypertension as adoptive transfer of T cells lacking functional NADPH oxidase prevents angiotensin II-induced hypertension (31). Thus, in model systems, oxidative stress in the form of NADPH oxidases plays a critical role in the pathophysiology of hypertension. These data have not yet reached full appreciation in human studies as early reports from human studies using genome-wide association strategies have yet to identify any NADPH oxidase gene loci with hypertension.

Other ROS sources have been investigated with regards to hypertension. In an animal model of salt-induced hypertension, inhibitors of the mitochondrial respiratory chain complexes I and III, as well as a mitochondrial SOD2 mimetic all demonstrated an antihypertensive effect (32), consistent with a role for mitochondria in hypertension. Similarly, antioxidants either targeted to the mitochondria (*e.g.* mitoTEMPO and MitoQ) or endogenously expressed in the mitochondria (SOD2 and thioredoxin 2) have all been shown to attenuate angiotensin II-induced hypertension (33,34).

One recent concept relating oxidative stress to hypertension involves cross-talk between the NADPH oxidase system and mitochondria. Endothelial NADPH oxidase-derived ROS are involved in the pathogenesis of hypertension through induction of mitochondrial dysfunction and ROS production (35). However, it also appears that mitochondria impact the NADPH oxidase system. Angiotensin treatment in animal model systems demonstrate increased mitochondrial superoxide production that results in NADPH oxidase activation as treatment with mitochondria-targeted antioxidants or excess mitochondrial SOD both limit cellular NADPH oxidase activity and angiotensin-induced hypertension (33). Thus, emerging evidence indicate both mitochondria and NADPH oxidases are important ROS sources in hypertension and may represent pathologic feed-forward mechanism. These data prompt speculation that interrupting such a vicious cycle of ROS catalyzed ROS production may hold therapeutic promise in the future.

ROS in angiogenesis

One critical vascular function is the maintenance of oxygen homeostasis and the recruitment and development of new blood vessels in the setting of tissue ischemia. This process is dependent upon hypoxia inducible factor (HIF), a transcription factor controlling gene expression critical for angiogenesis. Mitochondria have proven critical for upregulation of HIF-1 α via an ROS-dependent mechanism that involves the inhibition of prolyl hydroxylases that control HIF-1 α degradation (36). Inhibition of mitochondrial ROS or mitochondria-targeted antioxidants prevent HIF-1 α upregulation and angiogenesis in model systems.

Another source of ROS implicated in controlling angiogenesis include the NADPH oxidase isoforms. Mice lacking the Nox2 NADPH oxidase have defective angiogenesis responses manifest as reduced VEGF responses (37) and impaired blood flow recovery to hindlimb ischemia (38). This latter effect appears to be due to the Nox2 NADPH oxidase present in inflammatory cells rather than the vascular wall (6).

In the vascular wall, the Nox4 NADPH oxidase appears particularly important in regulating angiogenesis responses. This oxidase primarily produces H₂O₂ that is known to promote angiogenesis and Nox4 is upregulated in both hemangiomas (39) and ischemic hindlimb (40). Endothelial upregulation of Nox4 promotes endothelial proliferation and ROS-dependent angiogenesis (40). Conversely, mice lacking Nox4 have impaired blood flow recovery after hindlimb ischemia (41). The mechanism whereby Nox4 contributes to angiogenesis is believed to be the production of H₂O₂ that activates endothelial nitric oxide synthase (42) that is known to be important for angiogenesis.

Thus, from the preceding paragraphs it is clear that considerable preclinical data indicate that ROS are important modulators of reparative responses and play an important role in both the physiology and pathophysiology of vascular disease. One consequence of pathophysiologic ROS production is the modification of cellular constituents such as lipids. As a consequence, we will now turn our attention to oxidative biomarkers and their role in providing insight into the pathophysiology of vascular disease.

Oxidative Biomarker and Antioxidative Therapy

The role of ROS in cardiovascular biology and their propensity to produce oxidation byproducts has prompted considerable enthusiasm for the discovery of novel biomarkers for predicting both cardiovascular risk and therapeutic responses. Many excellent studies have characterized novel oxidation byproducts in select circumstances, however, oxidized lipoproteins, including LDL (OxLDL) have a long track record as biomarkers and appear to

be among the most promising oxidation markers to potentially impact clinical practice in the near future.

Oxidized Lipids

Oxidative modification of LDL is known to be a feature of the atherosclerotic process including the initiation, progression, and destabilization of atherosclerotic plaques. Oxidized phospholipids (OxPL) are key components of OxLDL that are believed to contribute to both early and late CVD events (43). A seminal study by Tsimikas et al has demonstrated that plasma levels of OxPL present on apo B-100-containing lipoproteins and predominantly on Lp(a) lipoprotein reflect the presence and extent of angiographically documented coronary artery disease (44). Recent clinical studies have shown that elevated OxPL/apoB levels predict the risk of cardiac death, myocardial infarction and stroke, and provide cumulative predictive value when added to traditional cardiovascular risk factors (43,45). In these studies, the assay utilized a murine monoclonal antibody, E06, that specifically binds to the phosphorylcholine moiety of oxidized, but not native phospholipids. A similar strategy utilizing oxidation-specific antibody-labeled gadolinium micelles and iron oxide nanoparticles has been applied *in vivo* for MRI detection of atherosclerotic lesions in animals (46). This latter finding could provide a clinically useful noninvasive means of atherosclerosis imaging. Thus, oxidized phospholipids. Collectively, these studies highlight the potential role of modified phospholipids as markers and/or contributors to CVD. These findings complement a recent increased interest in phospholipids in atherosclerosis, particularly since gut metabolism appears key to the mechanism of how phospholipids may contribute to CVD (47).

Myeloperoxidase

Myeloperoxidase (MPO) is a leukocyte-derived heme-containing enzyme that functions to catalyze the conversion of hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) to hypochlorous acid (HOCl) as its major reaction. MPO principally is important for anti-microbial action, but is also involved in oxidation of proteins and lipids including LDL and phospholipids. Elevated circulating MPO levels are indicative of endothelial dysfunction as determined by brachial artery flow-mediated dilation, characteristic of atherosclerosis (48). In patients with acute coronary syndromes, serum MPO levels are associated with death and nonfatal MI, and this relation was independent of other biomarkers for CVD such as cardiac troponin, CRP, and soluble CD40 ligand (49). It also appears that serial MPO monitoring is able to detect a subset of at risk patients who have troponin concentrations persistently within the reference range. The results suggested that the MPO concentrations are predictive of outcome up to 16h after presentation with chest pain and predict events missed by troponin testing (50). The biologic basis for MPO participation in atherosclerosis is that MPO-modified proteins are present in human atherosclerotic lesions (51) and it is thought MPO, together with metalloproteinases, may degrade collagen within atherosclerotic plaque and result in plaque erosion or rupture (52–54). Indeed, inflammatory cells producing MPO are found more frequently in ruptured plaques of patients with acute coronary syndromes than in patients with stable coronary artery disease (50,51,53,54). Thus, compelling data support the role of MPO in CAD, but it should be noted that MPO levels are influenced by numerous factors such as unfractionated heparin dosing (55) and the method of collection (56). As a consequence, more work is needed to standardize MPO as a CVD biomarker.

Other Biomarkers

There are a number of biomarkers that have showed promising results related to oxidative events in CVD. Most other markers, however, are not as mature in their development and have not been validated as extensively in multiple clinical populations compared to the

oxidation markers discussed above. Thus, for the sake of brevity we have not entertained an exhaustive description of these potentially promising biomarkers.

Antioxidant Therapy

Clinical trials such as HOPE (57) and HPS (58) have not shown a benefit of Vitamin C and Vitamin E in the treatment of cardiovascular disease. The most likely explanation is that redox processes relevant to atherosclerosis and plaque rupture are not uniform and amenable to simple attenuation with antioxidants. Particularly since these antioxidants (Vitamins C and E) are already present *in vivo* and their effect is likely sufficient in the absence of supplementation.

Statins, although not considered antioxidants based upon their chemical structure, are a well-established treatment for atherosclerosis (59) and one of their consequences is reduced indices of oxidative stress. For example, patients treated with atorvastatin exhibit a dose-dependent decrease in circulating levels of Ox-LDL (60). Studies to investigate the mechanisms of these observations in animals demonstrated that atherosclerosis increases endothelial superoxide production and decreases NO bioavailability due to “uncoupling” of endothelial nitric oxide synthase (eNOS) whereby electrons intended for NO• production instead leak to oxygen producing superoxide. Statins have an indirect antioxidant effect by preventing eNOS uncoupling (61). An important recent study by Antoniadou et al in humans indicated that atorvastatin treatment reduces vascular superoxide generation and improves vascular NO• bioavailability in human internal mammary artery of patients with atherosclerosis. The mechanism involved upregulation of GTP-cyclohydrolase I expression and activity, thereby providing complete tetrahydrobiopterin cofactor levels to improve eNOS coupling (62). Thus, statin therapy has profound implications for endothelial function.

Given that ROS production can be either physiologic or pathologic, it would be important to preferentially target antioxidants to pathologic sources of ROS production. One attempt using this strategy involves MitoQ, an ubiquinol-based compound that is complexed with triphenylphosphonium (TPP) cation that concentrates the molecule several hundred fold in the mitochondrion. Preclinical animal studies demonstrate that MitoQ significantly decreases heart dysfunction, cell death, and mitochondrial damage after ischemia/reperfusion (63), indicating that mitochondrial ROS are important in this condition. Similarly, previous studies had shown that mice heterozygous for the null allele of the *ataxia telangiectasia mutated* (ATM) gene on the ApoE-null background had significant adiposity, metabolic syndrome, and atherosclerosis. Treating this model with MitoQ prevented this phenotype and also reduced the macrophage content within atherosclerotic plaques in concert with preventing oxidative DNA damage (64). Collectively, these findings suggest a potential therapeutic role for mitochondria-targeted antioxidants. Human studies to evaluate MitoQ thus far are limited, but data indicate it can be safely delivered to patients for up to a year and may be effective in decreasing liver damage in Hepatitis C patients (65). Another clinical trial with MitoQ in Parkinsons disease failed to show any effect, perhaps due to the stage of disease or limited penetration of the blood brain barrier (55,66). Nevertheless, early results suggest targeted antioxidants are a safe strategy that may hold promise in certain disease states. More work will be needed to definitively establish the role of targeted antioxidants in treating chronic disease, including atherosclerosis and CVD.

With regard to antioxidant therapy, some other factors that warrant consideration. Genetic factors are clearly involved in the etiology of CVD, thus it is plausible that the extent to which oxidative events contribute to CVD may be individualized. One must consider, therefore, that antioxidant treatment will most benefit the subset of patients who suffer from an excess of vascular oxidative stress. Thus, to properly design trials of targeted antioxidant therapy we will need to develop facile techniques to accurately assess local and systemic

oxidative stress. An example of new evolving technology is the work of Albrecht et al., in which genetically encoded redox probes were used to quantitatively map both the glutathione redox potential and peroxide in defined subcellular compartments (67). Emerging technologies have expanded the scope of antioxidants to include small molecule inhibitors, nucleic acid aptamers (68), and monoclonal antibodies (69), that have the potential to target ROS in a selective manner.

Conclusions

There is a large body of evidence linking ROS to cardiovascular physiology and pathophysiology. It is now clear that the consequences of ROS are highly contextual based upon the sources and targets of these reactive molecules. As our understanding has matured, we have moved beyond simple strategies of treating patients with non-specific antioxidants to the notion that we need to target ROS modulation to specific enzyme systems and cellular compartment will inform us of the biology of ROS and potentially help risk stratification in CVD patients. With continued investigation, it is likely we will be able to target antioxidant therapy more effectively to modify the phenotypic features of cardiovascular disease.

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