REVIEW

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Oxidative stress as a mediator of cardiovascular disease

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During physiological processes molecules undergo chemical changes involving reducing and oxidizing reactions. A molecule with an unpaired electron can combine with a molecule capable of donating an electron. The donation of an electron is termed as oxidation whereas the gaining of an electron is called reduction. Reduction and oxidation can render the reduced molecule unstable and make it free to react with other molecules to cause damage to cellular and sub-cellular components such as membranes, proteins and DNA. In this paper, we have discussed the formation of reactive oxidant species originating from a variety of sources such as nitric oxide (NO) synthase (NOS), xanthine oxidases (XO), the cyclooxygenases, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase isoforms and metal-catalyzed reactions. In addition, we present a treatise on the physiological defences such as specialized enzymes and antioxidants that maintain reduction-oxidation (redox) balance. We have also given an account of how enzymes and antioxidants can be exhausted by the excessive production of reactive oxidant species (ROS) resulting in oxidative stress/nitrosative stress, a process that is an important mediator of cell damage. Important aspects of redox imbalance that triggers the activity of a number of signaling pathways including transcription factors activity, a process that is ubiquitous in cardiovascular disease related to ischemia/reperfusion injury have also been presented.

Introduction

Free radicals are molecules containing one or more unpaired electrons in atomic or molecular orbitals.[1] Reactive free radicals play a crucial part in different physiological processes ranging from cell signaling, inflammation and the immune defense.[2] There is increasing evidence that abnormal production of free radicals lead to increased stress on cellular structures and causes changes in molecular pathways that underpins the pathogenesis of several important human diseases, including heart disease, neurological disease and cancer and in the process of physiological ageing.[3,4] Understanding the contribution of free radical stress in the pathogenesis of disease will allow us to study the development of oxidative stress; a condition that occurs due to an imbalance between cellular production of oxidant molecules and the availability of appropriate antioxidants species that defend against them. It is hoped that this knowledge will subsequently lead to the development of effective therapeutic interventions against oxidative stress.

One of the major contributors of oxidative stress is the reactive oxygen species (ROS) family of molecules. These include free radicals such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), lipid radicals ($ROO^{\cdot}$) and nitric oxide (NO). Other reactive oxygen species, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl), although are not free radicals but they have oxidizing effects that contribute to oxidative stress. ROS has been implicated in cell damage, necrosis and cell apoptosis due to its direct oxidizing effects on macromolecules such as lipids, proteins and DNA.[5] Production of one free radical can lead to further formation of radicals via sequential chain reactions.[6] Reactions between radicals and polyunsaturated fatty acids within cell membrane can result in fatty acid peroxyl radicals, which accumulate in cell membrane and alter protein function and signal transduction. Under oxidative stress, excessive superoxide also releases free iron from iron-containing molecules, which further generate highly reactive hydroxyl radicals ($HO^{\cdot}$) by reacting with hydrogen peroxide in the Fenton reaction.[7] ROS can also induce the opening of the mitochondria membrane permeability transition pore (PTP) and cause a release in cytochrome *c* and other factors that can lead to apoptosis-mediated cell death.[8,9] $O_2^{\cdot-}$ radicals can further interact with the signaling molecule nitric oxide (NO) resulting in the formation of reactive nitrogen species (RNS), which further reduce NO bioavailability and cause NO toxicity known as "nitrosative stress".[10] Like ROS, excessive production of reactive nitrogen species results in nitrosylation reactions that change the structure of proteins, leading to loss or change of protein function.[11]

In physiological conditions, cells would increase activities of antioxidant enzymes and other antioxidant defences to counteract occurrence of oxidative stress.[12-14] These include manganese dependent superoxide dismutase such as manganese superoxide dismutase (Mn-SOD), Copper/Zinc superoxide dismutase (Cu/Zn SOD), glutathione peroxidase, glutathione reductase and catalase (CAT). MnSOD and Cu/ZnSOD convert $O_2^{\cdot-}$ to hydrogen peroxide, which is then transformed to water by glutathione peroxidase or catalase. Other antioxidant defences

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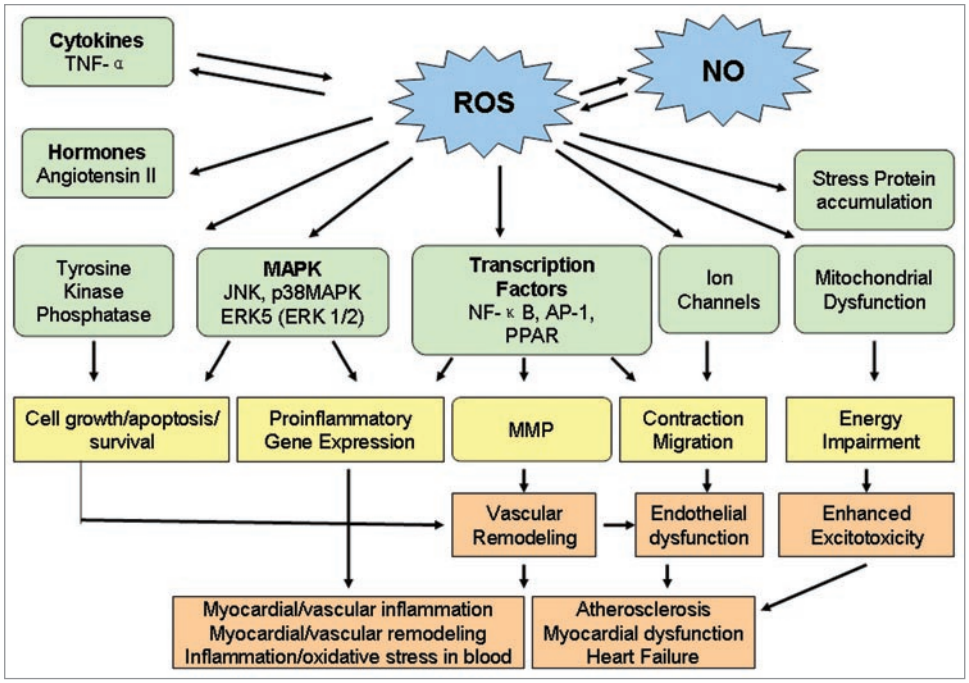

Figure 1. In physiological and disease states, the involvement of the inflammatory state initiated within cellular environment. This entails increased activities of antioxidant enzymes and other antioxidant defences to counteract occurrence of oxidative stress mainly characterised by nitric oxide (NO) and reactive oxygen species (ROS). This molecular fiasco illustrates into cellular pathways that regulate redox status of cells and the consequence of imbalance between free radical production and antioxidant activity during the cardiovascular disease process.

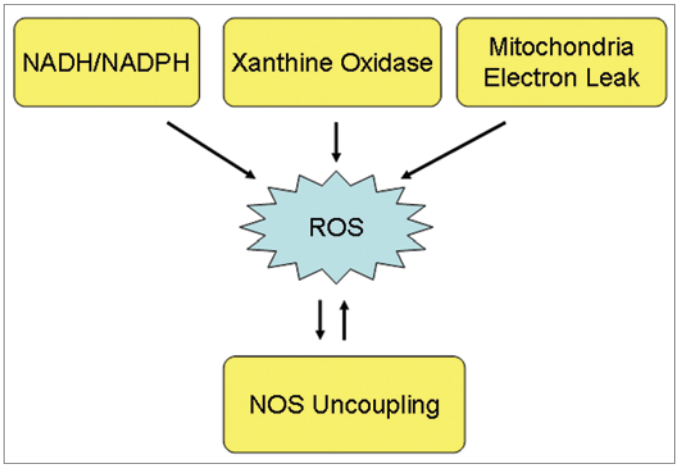

Figure 2. Several interlinked pathways that include mitochondria respiration, NADPH oxidases, xanthine oxidase and uncoupled NO synthases are associated with the production of free radicals within cells under physiological conditions. Mitochondria produce significant amounts of cellular ROS via aberrant O_2 reaction. This rate of mitochondrial respiration and ROS formation is largely influenced by the coupling state of the mitochondria, and in turn by factors such as internal and external Ca^{2+} levels and antioxidant activity. In response to the presence of respiratory burst explained in the text, NADPH oxidase activity get modulated by upregulation of component mRNAs and other inflammatory mediators such as TNFalpha thus dependent on the increase in transcription of p22phox, an important subunit of NAD(P)H oxidase.

include radical scavengers such as vitamin E, beta carotene and vitamin C.

This article aims to illustrate in detail, molecular pathways that regulate redox status of cells and the consequence of imbalance between free radical production and antioxidant activity during the cardiovascular disease process (**Fig. 1**).

Physiological Sources of Reactive Oxidant Species in Cells

Several mechanisms or pathways are associated with the production of free radicals within cells under physiological conditions. These include mitochondria respiration, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase and uncoupled NO synthases (**Fig. 2**).

Mitochondrial respiration as a source of reactive oxidant species in cells. Mitochondrial respiration involves transport of electrons from NADH or flavoprotein-linked dehydrogenases which ultimately result in reduction of oxygen to water, producing ATP in the process. This transport chain involves oxidative phosphorylation (OxPHOS) of complexes that are both nuclear and mitochondrial DNA encoded. Mitochondria produce significant amounts of cellular reactive oxidant species (ROS) via aberrant O_2 reaction.[15,16] During electron transport, approximately 2–5% of electrons escape to react with O_2 resulting in the production of ROS, which primarily occur at complexes I and III.[17] This process in physiological conditions is tightly controlled with majority of ROS produced remaining inside intact mitochondria.[18] In addition, some elements of the mitochondrial outer membrane

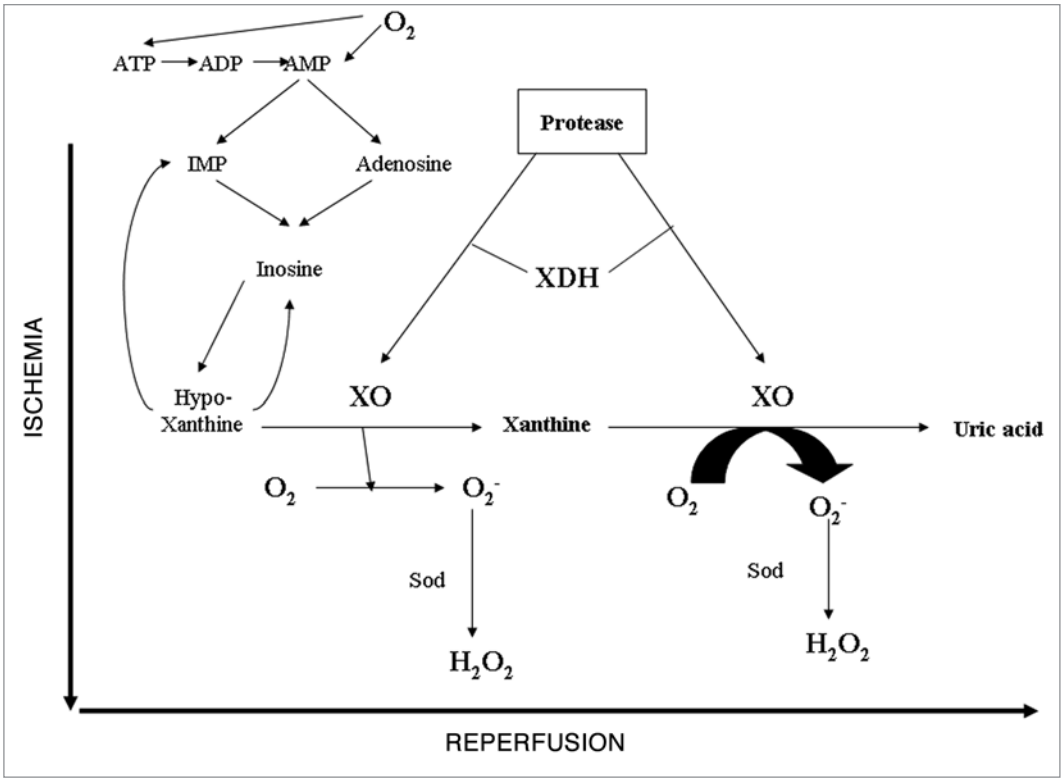

Figure 3. Proposed mechanism of xanthine oxido-reductase pathways. The enzymatic inhibition results in an increased availability of hypoxanthine for purine nucleotide synthesis via 5'-nucleotidase and inosine monophosphate (IMP) and adenosine monophosphate (AMP) dephosphorylation, thereby facilitate dissipating adenosine triphosphate (ATP) synthesis. On one hand, transmembrane ion gradients push cytosolic concentrations of calcium to rise, which in turn, activities protease that irreversibly converts xanthine dehydrogenase (XDH), predominant in vivo, in to xanthine oxidase (XO). At the same time, cellular ATP is catabolised to hypoxanthine, which accumulates in the diseased cell. During the reperfusion phase, XO using readmitted oxygen and hypoxanthine generates superoxide and hydrogen peroxide. Scheme derived from Puig et al., 1989.[140]

such as monoamine oxidases produce NO or H_2O_2 which result in increased free radical stress.[19] The rate of mitochondrial respiration and ROS formation is largely influenced by the coupling state of the mitochondria, and in turn by factors such as internal and external Ca^{2+} levels and antioxidant activity. Mn-SOD located in the mitochondrial matrix is an important antioxidant regulating ROS production.

The balance between oxidants and antioxidants commonly termed "redox state" of mitochondria also influences the opening of mitochondrial permeability transition pore (MPTP), which is associated with energy uncoupling and further ROS production[20] and development of disease process. For instance, overproduction of mitochondrial ROS/NO is associated with early atherosclerosis and hypercholesterolemia. Mitochondrial ROS is also linked to vascular cell pathology from hyperglycaemia induced glycation and protein kinase C activation.[21] Mitochondrial source of H_2O_2 play a key role in flow-mediated dilatation in human coronary resistance arteries.[22]

In endothelial cells hypoxia induces ROS generation and decreases activator protein-1 (AP-1) transcriptional activity, shown to be limited by inhibition of mitochondrial complex III with myothioxol, which suggest mitochondrial ROS are involved in hypoxia-induced signaling.[23]

NADH/NADPH oxidase system as a source of reactive oxidant species in the cell. NADH/NADPH oxidases are membrane-associated enzymes that catalyse the 1-electron reduction of oxygen using NADH or NADPH as the electron donor. NADPH is particularly important in generation of ROS in phagocytic cell systems in response to the presence of foreign organisms in a series of changes known as "respiratory burst".[24] NADH/NADPH oxidases are also important sources of endovascular ROS. NADH/NADPH oxidases are the major oxidases in vascular tissue and in cardiac cells.[25]

NADH/NADPH activity is regulated by a number of factors known to be involved in the pathogenesis of cardiovascular disease including cytokines, hormones, local metabolic changes and haemodynamic forces. Exposure of human umbilical endothelial cells to 5–20 dyne/cm^2 unidirectional lamina shear stress results in a transient elevation in NADH-dependent $O_2^{\cdot-}$ formation whereas oscillatory shear causes sustained increase in oxidase activity.[26] NADH/NADPH-dependent oxidase activity is also increased in vascular smooth muscle cells by stimulation with the vasoactive agonist angiotensin II. Angiotensin II increases NADH and NADPH driven $O_2^{\cdot-}$ production in cultured vascular smooth muscle cells (VSMC) and aortic adventitial fibroblasts.[27] NADH/NADPH-dependent $O_2^{\cdot-}$ formation is increased in experimental rat model of angiotensin II-induced hypertension.[25]

Activation of the oxidase can be mediated by intracellular second messengers.[28] For instance, lipoxygenase metabolites of arachidonic acid mediate angiotensin II stimulation of NAD(P)H oxidase in VSMC.[29] Furthermore oxidase activity can also be modulated by upregulation of component mRNAs. TNFalpha increases NAD(P)H oxidase activity in VSMC over 24 hours, which is dependent on the increase in transcription of p22phox, an important subunit of NAD(P)H oxidase.[30]

Treatment with exogenous antioxidant SOD improves blood pressure and vascular reactivity in rat model of angiotensin II induced hypertension.[31] Re-oxygenation following a period of hypoxia is accompanied by an increase in lactate that stimulates NADH oxidase activity in cardiac myocytes.[32] Various factors including thrombin, platelet-derived growth factor, and cytokines such as tumor necrosis factor-alpha (TNFalpha) also induce NADH/NADPH-dependent oxidase activity.[30,33] Elevated levels of NADH-dependent $O_2^{\cdot-}$ have been found to be associated with diabetes and hypercholesterolemia in human saphenous vein segments obtained from patients undergoing coronary artery bypass surgery[34] and in cardiac remodelling following myocardial infarction.[35]

Xanthine oxido-reductase system as a source of reactive oxidant species in the cell. Xanthine oxido-reductase exists in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase.[36] The first form reduces NAD^+ whereas the latter reacts with molecular oxygen, leading to the production of superoxide anion and hydrogen peroxide.[37] In the purine catabolism, xanthine oxido-reductase catalyses oxidative hydroxylation of hypoxanthine to xanthine, and then from xanthine to uric acid, which is a strong antioxidant and a free radical scavenger (**Fig. 3**). The dual role of xanthine oxidase means that it is an important regulator of cellular redox state.

Under pathophysiological stress conditions, xanthine oxido-reductase is an important source of oxidative stress.[13] In experimental atherosclerosis caused by diet induced hypercholesterolemia, excess superoxide production was inhibited using oxypurinol, a xanthine oxidase inhibitor.[38] Xanthine oxidase generates ROS via purine metabolism pathway and is involved in causing endothelial dysfunction in patients with coronary disease and contractile dysfunction in heart failure.[39]

NOS uncoupling as a source of reactive oxidant species in the cell. Uncoupled NO Synthase (NOS) contribute to ROS generation and result in vascular endothelial dysfunction.[40,41] Endothelial NOS (eNOS) is a cytochrome P450 reductase-like enzyme that catalyses flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. NOS are the major source of endogenous NO. eNOS can produce both NOS via its oxygenase function and superoxide through its reductase function, the later dependent on NADPH. This enzyme requires tetrahydrobiopterin (BH-4) bound near this heme group to transfer electrons to guanidino nitrogen of L-arginine to form nitric oxide (NO).[42] Uncoupling of eNOS contribute to ROS when deficiency of L-arginine or BH-4.[43] In the absence of L-arginine or BH-4, eNOS can produce $O_2^{\cdot-}$ and H_2O_2.[41] The product of reaction between NO and $O_2^{\cdot-}$ can oxidize BH_4 and this may lead to further eNOS uncoupling.[44]

NO is a major cell signaling molecule involved in a large variety of different physiological processes including neurotransmission, regulation of vascular dynamics and immune regulation.[45] It is one of the main mediators of endothelium-dependent relaxation (EDR). NO release is induced by either vascular shear-stress or by eNOS activation in response to cytokine activation[46] and plays a protective role in suppressing abnormal proliferation of vascular smooth muscle cells (VSMCs) following various pathological situations.[47] NO has been shown to react with and cancelled by reaction with excess ROS directly inactivating it.[48] $O_2^{\cdot-}$ reacts with NO to produce peroxynitrite, which reduces bioavailability of NO and produces more damaging secondary species. ROS may also affect NO responses by oxidizing sites on the protein that reacts with NO (direct competition) or which would otherwise influence NO binding (allosteric modulation). There concentration of NOS in blood vessels is therefore dependent on the balance between production of NO on one hand and destruction by ROS on the other. Xanthine oxidase and NADPH oxidases are inhibited by NO, thereby NOS activity also regulate free radical production and maintain ROS/NO homeostasis.[49] Evidence of eNOS contribution to cellular ROS is present in the context of hypercholesterolemia,[50] atherosclerosis,[51] coronary artery disease,[52] aging and diabetes.[53]

Imbalance between endothelial NO and ROS production is one of the major contributor of endothelial dysfunction which plays an important part in atherosclerosis and cardiac disease.[54]

Regulation of Physiological Function and Disease Process by Reactive Oxidant Species Signaling

In addition to its direct damaging effect on macromolecules, ROS can act as biochemical messengers that regulate various intracellular signaling pathways. ROS have been implicated in regulation of calcium (Ca^{2+}) induced signaling in the vasculature which in turn can activate calcium dependent protein kinases activity such as PKC and calcineurin.[55] Intracellular ROS also affect the activity of protein kinase pathways by influencing the redox state of the cell. Alterations in the redox state of protein cysteinyl residues result in changes in protein conformation and function. Influence of Tyrosine kinase activity by ROS has been observed in a variety of cell types.[55] Stimulation of vascular smooth muscle cell (VSMC) by platelet-derived growth factor (PDGF) transiently increases H_2O_2 production that induces tyrosine phosphorylation, mitogen activated protein kinase (MAPK) activation and chemotaxis, effects that were not observed in the higher intracellular concentration of the antioxidant N-acetylcysteine.[43] Stimulation of VSMC with $O_2^{\cdot-}$ generating agent LY83583 also resulted in the increase of MAPK activity in a concentration dependent manner.[56] LacCer, a glycosphingolipid implicated in the proliferation of VSMC and atherosclerotic plaque formation has also been shown to stimulate $O_2^{\cdot-}$ production, activation of NADPH and MAPK pathway induction.[57]

Gene expression pattern is also regulated by ROS via modulation of transcription factor activity particularly nuclear factor kappaB (NFκB), AP-1 and the peroxisome proliferators-

activated receptor (PPAP) family of transcriptional activators[2] where redox cycling of cysteinyl residues plays an important part in this transcription factor regulation process.[58]

ROS signaling via NFκB activation. NFκB is one of the most commonly studied transcriptional factors influenced by cellular redox state.[59] NFκB is a family of inducible transcription factors first described as B-lymphocyte-specific nuclear proteins essential for transcription of immunoglobulin kappa (κ) light chains. It is important in regulation of inflammation, stress responses, expression of cytokines and cell adhesion molecules, regulation of immune response and programmed cell death.[60]

NFκB forms functional dimerized structure composed of members of the Rel family, which include p65 (RelA), NFκB-1(p50), NFκB-2(p52), c-Rel and RelB.[61] Members of the Rel family carry a Rel homology domain which contains a nuclear localization signal (NLS). The homodimers and hetero-dimers are kept inactive by structurally association with IκB family of inhibitory proteins in the cytoplasm including IκB-a, IκB-b, IκB-e, as well as p105 and p100 precursors of p50 and p52.[62]

Dissociation of NFκB from its IκB inhibitory protein is the initial step of NFκB activity. Upon activation by stimulatory signals phosphorylation of IκB by ubiquitin-dependent protein kinase results in its ubiquitination and proteolytic degradation. Amino acid residues Ser-32 and Ser-36 of IκB are essential for phosphorylation and Lys-21 and Lys-22 are important for ubiquitination process. The activated NFκB rapidly translocate to the nucleus to regulate NFκB responsive genes.[63] Members of IκB are also nuclear transcription factors that interact with NFκB family members directly in the nucleus, influencing its function. Many of the stimuli related to atherosclerosis are upstream regulators of NFκB such as oxidized LDL, cytokines (TNFα, IL-1), UV light, ionizing radiation and infectious agents.

ROS is an important intermediate second messenger of NFκB activation by upstream stimuli such as TNF and IL-1.[64] Antioxidants including vitamin E and N-acetylcysteine have been shown to reverse activation of NFκB by stimuli,[65] however it is thought that a non-antioxidant action on NFκB activity may also be responsible.[65]

NFκB cooperates with other transcription factors in orchestrating gene expression. The interaction between NFκB and AP-1 is particularly important as many genes involved in the regulation of inflammation require both transcription factors working together.[66] The modulation of NFκB signaling is affected by post-translational modifications, including reversal acetylation of the RelA/p65 subunit. Full transcriptional activity of RelA/p65 requires the acetylation of locus Lys-310, which can be deaceytylated by sirtuin-1 (SIRT1), a class II histone deacetyl transferase.[67] The small molecular agonist of SIRT1, Resveratrol, has been shown to inhibit NFκB signaling by promoting the deacetylation of RelA/p65.[68]

NFκB induce the transcription of more than 200 genes, of which many are involved in regulation of inflammation, the production of cytokines, and upregulation of prothrombotic markers, processes associated with pathogenesis of atherosclerosis.[69] NFκB is found to be upregulated in atherosclerotic vessels[70] and its nuclear translocation has been detected in the intima and media of atherosclerotic lesions and in smooth muscle cells, endothelial cells, macrophages and T cells of atherosclerotic plaques.[71] It has also been reported that NFκB also plays a role in mediating T-cell signaling in atheromatous plaques.[72,73]

Cardiac specific blockade of NFκB in rodents has been shown to reduce myocardial infarct size following ischaemia/reperfusion injury. NFκB knockout mice have also been shown to have a decreased infarct size and improved haemodynamics after ischaemia/reperfusion injury, although the effects have not been seen in the long term.[74]

NFκB activation in atherosclerosis can be due to several factors. Local vascular injury has been shown to decrease inhibitory protein IκB and result in increased macrophage infiltration.[75] Localized adventitial hypoxia has been implicated in NFκB activation.[76] Oxidized low density lipoprotein (LDL) which is implicated to plays a major role in atherosclerosis formation also regulate NFκB activity. In an in vivo study, injected LDL particles localised to arterial walls and underwent oxidative modification and subsequent activation of endothelial NFκB activity and expression of NFκB-dependent genes.[77] Advanced glycation end products (AGEs) formed through non-enzymatic reactions of sugars with amino groups of proteins, nucleotides and lipids have also been shown to accumulate in atherosclerotic plaques and are thought to activate monocytes infiltration through NFκB.[78] Furthermore the hormone angiotensin II, in addition to its effects on blood pressure also participates in the formation of atherosclerosis through expression of adhesion molecules and IL-6 in smooth muscle cells, this process is partly regulated through NFκB.[79]

Involvement of infectious agents has been implicated in the pathogenesis of atherosclerosis. Expression of Toll Like Receptors (TLR), found to activate NFκB, are involved in the initial recognition of pathogenic antigens such as bacterial lipopolysaccharides have been found to be upregulated in human atherosclerotic arteries compared to low levels in normal arteries.[80]

ROS signaling via AP-1 activation. AP-1 is a family of basic domain/leucine zipper (bZIP) transcription factors characterized by their specific trans-activation through the cis-acting transcriptional control DNA element, the 12-O-tetradecanoyl phorbol-13-acetate (TPA) response element (TRE). AP-1 is a heterodimer of members of FOS and JUN families, or a homodimer of JUN proteins.[81]

AP-1 activity is controlled by both transcriptional and post-translational mechanisms in response to variety of extracellular stimuli.[82] The expression of c-JUN and c-FOS is induced by various mitogens[83] such as hydrogen peroxide,[84] UV,[85] and ionizing radiation.[17] Arachidonic acid metabolite concentration and Mitogen kinase pathway activity are involved in mediating activation of AP-1.[86] AP-1 is also important in the regulation of gene expression of various target genes involved in cell proliferation and transformation.[87]

In smooth muscle cells AP-1 expression or DNA-binding activity have been shown to be upregulated by H_2O_2, oxidized LDL and lipid peroxidation.[88] It is suggested that AP-1 activation under oxidative conditions may be mediated by phosphorylation

of Jun proteins.[89] However a number of antioxidants such as cysteine based redox regulators of glutathione and thioredoxin pathways have also been shown to stimulate DNA binding and transcriptional activity of AP-1.[90] Therefore the regulation of AP-1 by oxidative free radicals is complex and requires further elucidation.

ROS signaling via peroxisome proliferators activated receptors (PPAR). The dimerisation of PPAR with retinoid X receptorbeta (RXRbeta) occurs in response to various metabolic stimuli. PPAR/RXRbeta transcription factor is responsible for inducing acetyl coenzyme A oxidase, an enzyme that transfers electrons to oxygen to produce H_2O_2.[91] PPAR transcription factor has been implicated in the inflammatory processes involved in pathogenesis of atherosclerosis.[92] Oxidized LDL has been shown to increase expression of PPAR in foam cells of atherosclerotic lesions.[93] However PPAR does not have a sole role in mediation of inflammation. Activation of PPARalpha and PPARgamma isoforms results in anti-inflammatory responses in blood vessel wall. Specific agonists of PPARgamma has been shown to suppress pro-inflammatory gene expression in monocytes.[94] Activators of PPAR alpha has also been shown to block inflammatory responses in aortic smooth muscle cells and PPARgamma activation was recently shown to mitigate the inflammation associated with chronic and acute neurological insults.[93]

Reactive Oxidant Species Formation and Cardiovascular Disease

Oxidative/nitrosative and endothelial dysfunction in aterosclerosis. One of the key concepts of free radical mediated pathogenesis of cardiovascular disease is endothelial dysfunction, whereby the regulation of vascular wall microenvironment is disrupted. An important element in this concept is that vascular endothelium is an active component of the vasculature, which plays a part in the regulation of vascular tone, platelet activity, thrombosis, inflammation and atherosclerosis. Endothelium vasoactive tone is maintained by the release of substances like prostacyclins, endothelins and the endothelium-derived relaxation factor nitric oxide (NO) or a related compound.[95]

Reduction in endothelium-dependent vasorelaxation caused by the reduction in NO bioavailability plays a significant role in endothelial dysfunction. Decreased NO bioavailability disrupts the non-thrombogenic intimal surface and promotes platelet adhesion and aggregation as well as deposition of platelets on the abnormal endothelial surface.

Thrombus formation during acute coronary syndromes results in the release of various vasoactive substances such as thrombin and serotonin.[96-98] In normal endothelium these substances mediate vasodilatation.[99] In the setting of endothelial vasodilator dysfunction vasoconstriction occurs.[100] This is potentiated by the presence of endothelins, whose concentrations are elevated concentration in plaques of patients with acute coronary syndrome.[101]

The impairment of vasodilatation in response to vasodilator acetylcholine is a measurement of endothelial dysfunction and it correlates with increased local ROS production and reduced superoxide dismutase activity.[102] The lack of myeloperoxidase activity suggests that elevated leukocyte/macrophage production of ROS is not involved in the process.

Endothelial vasodilator dysfunction can lead to paradoxical vasoconstriction effects and occurs in situations with sympathetic activation such as exercise. In the cardiac vasculature this can result in angina pectoris.[96] During increased metabolic demand, vasodilator dysfunction in coronary vessels has been shown to result in ischemia, even in the absence of pathological stenosis.[99]

Endothelium dysfunction has been observed in patients with established coronary artery disease or risk factors for both coronary and peripheral vascular disease.[100] Impairment in endothelium-dependent vasorelaxation predicts adverse cardiovascular events and long-term outcomes.[101]

Atherosclerosis in coronary arteries even at early stages is associated with evidence of endothelium dysfunction.[101] Long-term cigarette smoking is an independent risk factor for impaired endothelium-dependent coronary vasodilation regardless of the presence or absence of coronary atherosclerotic lesions.[102] Studies in the human forearm have demonstrated decreased flow-dependent dilation in chronic smokers.[103] The blunted endothelium dependent relaxation (EDR) was improved with antioxidant vitamin C in chronic smokers, indicating the involvement of ROS in the pathogenesis.[104] Hypertension is also linked to increased vascular oxidative stress in a number of animal models of hypertension.[105] Hypertension leads to blunting of EDR through effect of oxidative stress.[106]

Endothelium-dependent coronary blood flow regulation is blunted in the presence of elevated cholesterol levels. Furthermore the type of cholesterol appears to produce different response. Reduced EDR is closely related to LDL cholesterol levels but ameliorated with high HDL-levels.[106]

At sites of plaque growth, NO release is increased in response to increased shear-stress acting on the vessel wall. This may lead to vasodilation and structural vessel wall remodelling.[107] Baseline vasomotor tone is also decreased in atherosclerotic vessels by a functional mechanism.[108]

Reduction in NO bioavailability can be decrease by several mechanisms including reduction in eNOS expression, lack of substrate or cofactors for eNOS activity, alterations of eNOS cellular signaling and increase in NO degradation.[109] Mice with eNOS knockout are more prone to develop typical atherosclerotic lesions in response to adventitial vessel wall injury compared to wild-type mice.[110]

The bioavailability of NOS is more important for coronary artery dilatation than the activity of eNOS and generation of NOS. The bioavailability of NOS is influenced by the amount of ROS present that can transform NO to $ONOO^-$ and oxidized tetrahydrobiopterin to dihydrobiopterin which lead to eNOS uncoupling and further ROS production.[95] Upregulation of tetrahydrobiopterin which improves bioavailability of NOS has been shown to improve endothelial function and reduce superoxide production.[111] Supplementation of antioxidant superoxide dismutase has also been shown to improve endothelium dependent vasodilatation of coronary arteries.[112] Treatment with L-arginine, precursor of intracellular NOS, has been found to improve

endothelium dependent vasodilation in patients with cardiac risk factors.[113,114] In addition, calorie restrictions for 3–12 months lead to enhanced eNOS expression and cGMP formation in various tissues in mice.[112] This was accompanied by mitochondrial biogenesis, increased oxygen consumption and ATP production and enhanced expression of sirtuin-1.[112] These effects were abrogated in eNOS null mutant mice.[112]

Furthermore NO generated by vascular endothelium has traditionally been thought to have a purely paracrine role. Interestingly, recent evidence show that NO display hormonal function by reacting with haemoglobin to form stable metabolites, which can be transported through the blood stream and subsequently release NO at sites distant to the site of production.[115] During oxygenation in the lung some NO transfers to the highly conserved beta chain Cys93 residue of haemoglobin to produce S-nitrosyl haemoglobin (SNO-Hb). Following deoxygenation in peripheral circulation, SNO-Hb is able to then release NO bioactivity. The significance of this mode of NO activity is still under investigation.

Oxidative/nitrosative stress and hypertension. Clinical studies have demonstrated that there is increased ROS production in patients with essential hypertension, renovascular hypertension, malignant hypertension and pre-eclampsia.[116-119] Vascular ROS are produced in endothelial, adventitial and vascular smooth muscle cells (VSMCs) and derived primarily from NAD(P)H oxidase, a multi-subunit enzyme catalyzing $O_2^{\cdot-}$ production by the 1 electron reduction of oxygen using NAD(P)H as the electron donor:[120]

$$2O_2 + NAD(P)H \rightarrow 2O_2^{\cdot-} + NAD(P) + H^+$$

Interestingly, all major trials on antioxidant supplementation have failed to show significant cardiovascular benefits and antioxidants are not recommended for the prevention or treatment of hypertension. In contrast, dietary approaches are highly recommended, supported by evidence from a trial which demonstrated that subjects consuming high fruit and vegetable diets had significantly reduced blood pressure.[121] On the other hand, direct cardiovascular effects of some pharmaceutical agents have been attributed to direct inhibition of NAD(P)H oxidase activity, as shown for angiotensin 1 (AT_1) receptor blockers, and to intrinsic antioxidant properties of the agents. Classical antihypertensive agents such as β-adrenergic blockers (carvedilol), ACE inhibitors, AT_1 receptor antagonists, and Ca^{2+} channel blockers may be mediated, in part, by decreasing vascular oxidative stress.[122-124]

Oxidative/nitrosative stress and cardiovascular disease outcomes. Oxidative stress is linked with negative outcomes in cardiovascular disease.[88] As discussed above, free radical stress can lead to cardiovascular disease by influencing the endothelial function.[125] ROS can cause direct cardiac injury by oxidizing cellular constituents, disruption of proteins critical for excitation-contraction (E-C) coupling and by diminishing NO bioactivity.[126] Blood sample from patients with ischaemic heart disease has been shown to contain evidence of oxidative/nitrosative stress.[127]

Oxidative/nitrosative stress and cardiovascular ischemia. In myocardial ischaemia, hypoxia and reoxygenation induces an increase in free radical production in cardiac tissues and are principal causes of reperfusion injury. ROS produced through reoxygenation lead to direct oxidative damage to cellular components and also through indirect injury via activation of localized inflammation.[1] ROS can also act as signaling messenger in activating biochemical pathways responsible for altering cellular function.[128] For instance, Akt activation in VSMC induced by exogenous angiotensin II, which leads to VSMC hypertrophy, has also been shown to be mediated by H_2O_2 and can be abrogated by overexpression of catalase.[54] Hypoxia/reoxygenation in cardiac myocytes also leads to induction of p38 MAPK and JNK pathways and that the activity of these pathways can be attenuated by pre-incubation with antioxidants and tyrosine kinase inhibitors.[57]

ROS mediated effect in cardiovascular disease is also reflected in nuclear transcription factor activity. The nuclear transcription factor NFκB activity has been found in myocardial biopsies of patients with unstable angina.[129] Nuclear translocation of RelA has also been found to be increased in human coronary artery plaques.[130]

The pathogenesis of atherosclerosis has been thought of as an inflammation-mediated process.[131] Atherosclerosis is associated with increased levels of inflammatory markers including CRP, IL-6, ESR, TNF, homocysteine.[132] Hormones and cytokines such as angiotensin II, PDGF and TNFalpha may increase ROS in atherosclerotic lesions by stimulating local vascular myocytes to produce ROS.[133] Mitochondrial dysfunction and increase ROS production has also been shown to associate with early atherosclerotic lesion formation.[134] Multiple cell populations in the vascular wall have been shown to both produce and be regulated by ROS signaling.[135] Free oxygen radicals lead to increase vascular oxidizes LDL and increase adhesion molecule expression in endothelial cells, which result in inflammatory cell infiltration and activate matrix metalloproteinases and vascular remodelling.[136] Reactive oxygen species ($O_2^{\cdot-}$ and H_2O_2) regulate growth and migration of vascular smooth muscle cells in the plaque structure.[42] ROS also trigger extracellular matrix remodelling through regulation of collagen resorption resulting in compromised plaque stability.[136,137]

Oxidative/nitrosative stress and heart failure. Xanthine oxygenase is an important cardiovascular source of ROS. Increase in xanthine oxygenase level and activity has been shown in heart failure.[138] Upregulation of xanthine oxygenase in patients with heart failure is thought to contribute to mechano-energetic uncoupling.[139]

NAD(P)H activity is also increased in cardiovascular disease with increased levels found in myocardial cells from humans with heart failure and in ischaemia-reperfusion models.[140] This increase is due in part by elevated levels of angiotensin II which causes neurohormonal dysregulation of oxidative/nitrosative disequilibrium.[141] In rabbit model of early atherosclerosis, where hypercholesterolemia was induced by defect in LDL-receptor, it was shown that NAD(P)H-induced ROS production increased by 2 fold in the disease group compared with controls.[142]

Nitrosative stress caused by nitrogen free radicals also plays a role in cardiovascular disease. In acute ischaemia, sepsis or

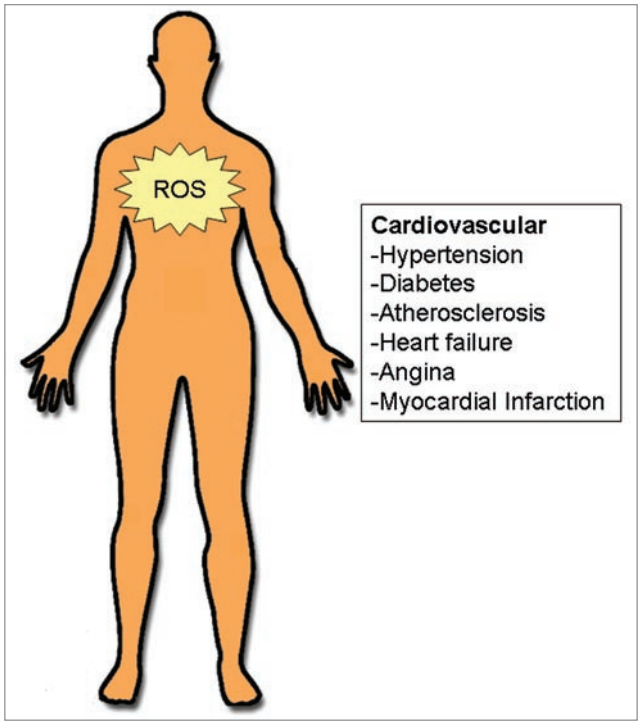

Figure 4. Recent emerging work supports with more overt evidence thus showing the strong relation of human disease mechanisms to the production of reactive oxidant species and the dysregulation of oxidant-antioxidants pathways. These pathways as discussed in this review demonstrate the modulation of signal transduction processes and energy metabolism in response to conditions of oxidative/nitrosative stress.

heart failure, nitrosative stress is increased due to an increase in the amount of iNOS (NOS2), leading to increased levels of S-nitrosylated proteins.[143] The accumulation of heme NO in heart failure is correlated with venous desaturation.[144] Oxidative stress leads to partial uncoupling of eNOS, resulting in the increased production of superoxide and peroxynitrite species.[145] However, the role of NOS activity in cardiovascular disease is not fully understood in that, although NOS gene therapy in carotid arteries of a rabbit model of hypercholesterolemia has been shown to rapidly reduce adhesion molecule expression and inflammatory cell infiltrations,[146] in contrast, eNOS-deficient mice are known to develop smaller aortic lesions than corresponding wild-type control animals fed on an atherogenic diet. It is suggested that superoxide production by eNOS is important in oxidation of LDL during the formation of atherosclerosis in the setting of hyperlipidemia.[147]

The "Vascular Interaction With Age in Myocardial Infarction" (VINTAGE MI) Trial showed that supplementation of L-arginine (precursor of NO) in patients with first ST-segment elevation myocardial infarction did not improve vascular dynamics or cardiac function and may be associated with higher post-infarction mortality.[144]

In addition to direct injury, NO/redox disequilibrium may also impair ion channels within the heart by S-nitrosylation, resulting in functional cardiac impairment.[143] Different NOS isoforms exert varying effects on cardiac physiology. For instance, NOS3 exert its effect on signal transduction at plasmalemmal membrane, inhibiting L-type calcium channel and thus attenuating the beta-adrenergic mediated myocardial contractility.[148] On the other hand, NOS1 isoform exerts its effect on sarcoplasmic reticulum, which facilitates calcium cycling and enhancing myocardial contractility stimulated by catecholamines.[149]

Oxidative/nitrosative stress and postoperative arrhythmias. Atrial fibrillation (AF) is a frequent complication of most types of coronary artery surgery.[150] The incidence of post-operative AF (PAF) in patients undergoing cardiac surgery is between 20–50%, and has been reported up to 65%.[150] It usually occurs within 5 days especially on the second or third day.[151,152] The incidence may in fact be higher in patients with combined coronary artery bypass graft (CABG) and valve surgery than CABG alone.[152,153] There is now a new focus on cardiac specific ROS production, rather than supplementing a systemic antioxidant response with evidence suggesting that myocardial specific-oxidative stress may be the main trigger for PAF.[154] Recent evidence suggests that preoperative angiotensin converting enzyme inhibitor or angiotensin receptor blocker use has a significant impact on the frequency of atrial fibrillation after cardiac surgery,[155] most likely by targeting cardiovascular specific ROS production.

Concluding Remarks

In recent times, important milestones have been reached with the availability of more overt evidence that shows that cardiovascular disease mechanisms are strongly linked to the production of reactive oxidant species and the dysregulation of oxidant-antioxidants pathways. In this regard, the oxidation and nitration of cellular proteins, lipids and nucleic acids, and formation of aggregates of oxidised molecules underlie the loss of cellular function, cellular ageing and the inability of cells to withstand physiological stresses. In addition, reactive oxidants species modulate signal transduction processes and energy metabolism in response to conditions of oxidative/nitrosative stress. Evidence shows that sources of reactive oxidant species, physiological and pathophysiological conditions, and cellular oxidant targets determine the characteristic nature of a disease process and resultant outcomes. Although much information on the relationship between oxidative stress and the disease process is now available (**Fig. 4**), further research with a clearer focus on the development of disease-specific therapeutic targets is required.

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