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Reactive oxygen species in cardiac signalling – from mitochondria to plasma membrane ion channels

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Summary

1. Reactive oxygen species have been considered deleterious to cell function and there is good evidence to suggest that they play a role in the pathophysiology of a number of cardiac disease states. However reactive oxygen species are also now being recognised as important regulators of cell function by altering the redox state of proteins.

2. Possible sources of production of reactive oxygen species in cardiac myocytes are the mitochondria and NAD(P)H-oxidase. The generation of reactive oxygen species and antioxidant defense mechanisms in the heart are discussed.

3. The evidence for a role for reactive oxygen species in the development of disease states such as atherosclerosis, ischaemia, cardiac hypertrophy and hypertension is presented. It is now recognised that cardiac ion channel function is regulated by reactive oxygen species. Implications with respect to cardiac arrhythmia are discussed.

Introduction

Oxygen is vital to life but as a diatomic molecule it is remarkably unreactive. However, oxygen is the substrate for the generation of a variety of reactive species, some which may be deleterious to cell function. Recent studies have found that although they are involved in pathology, they are also integral to modulating functional responses. Reactive oxygen species (ROS) are generally speaking oxygen molecules in different states of oxidation or reduction, as well as compounds of oxygen with hydrogen and nitrogen. Although superoxide is produced directly from the reduction of oxygen, the biologically active species are hydrogen peroxide, hydroxyl radicals, hypochlorite ion and peroxynitrites (Figure 1).

Superoxide is produced as a result of the donation of an electron to oxygen. It is a weak base at physiological pH, highly soluble in water and therefore does not easily cross lipid membranes, although it can be transported via anion channels.[1,2] Because it is a weak base, it is not a strong oxidant at neutral pH. It is however a one-electron reductant of metals and the reduction of Fe^{3+} to Fe^{2+} by superoxide is the basis of analytical methods used to identify superoxide such as cytochrome *c* reduction.[3] Superoxide can also be protonated to hydroperoxy radical at low pH (pK_a = 4.8), but the principle fate of superoxide at physiological pH lies in its dismutation to hydrogen peroxide by superoxide dismutase. The rate of dismutation is rapid ($k=1.6 \times 10^9$ $M^{-1}s^{-1}$) maintaining the concentration of superoxide extremely low, in the picomolar-nanomolar range.[4]

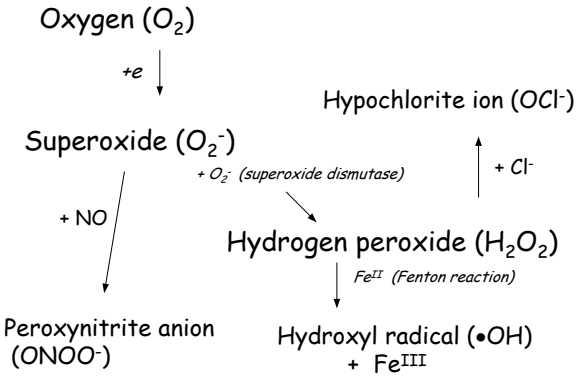

Figure 1. Schematic of ROS arising from oxygen (O_2). *Superoxide (O_2^-) is generated directly from the reduction of oxygen and then dismutated to hydrogen peroxide (H_2O_2). Reactions with chloride ion (Cl^-), nitric oxide (NO) and iron (Fe^{II}) are as indicated.*

In contrast to superoxide, hydrogen peroxide is a relatively strong oxidant and a highly stable small molecule that freely crosses membranes as its biological diffusion properties are similar to water.[5] This makes hydrogen peroxide a biologically important candidate as a ROS and signalling molecule. The naturally occurring enzyme (and antioxidant) catalase metabolises hydrogen peroxide to water and oxygen and is principally responsible for maintaining intracellular hydrogen peroxide in the nanomolar range.[6] Hydrogen peroxide can participate in one-electron reactions with metal ions (Fenton reaction) and generate hydroxyl radicals. Hypochlorite ion is generated when hydrogen peroxide reacts with Cl^-. Both hydroxyl radical and hypochlorite ion are highly reactive.[7,8] Other reactive species are derived from nitric oxide, a well recognised signalling molecule implicated in cardiovascular function as an important vasodilator. Nitric oxide reacts with oxygen to produce nitrogen dioxide that participates in the peroxidation of lipids and with superoxide to produce peroxynitrite, also a stable strong oxidant of lipids.[9,10]

The toxicity associated with high levels of ROS depends on the antioxidant defense mechanisms of the cell. In certain circumstances ROS toxicity is beneficial to the host when defending against microorganisms or pathogens

for example in plants and eukaryotes.[11,12] In low concentrations, ROS may be necessary in normal cellular function. Hydrogen peroxide precisely regulates the catalytic activity of enzymes by redox modification of cysteine residues.[13] These include tyrosine phosphatase activity,[14,15] the translocation and activation of serine/threonine kinases such as protein kinase C[16] and the induction of gene expression.[13] For example, a hypoxia-induced decrease (40%) in hydrogen peroxide concentration in PC12 cells (measured with the fluorescent indicator 2',7'-dichlorofluorescein) results in the induction of tyrosine hydroxylase mRNA, the rate-limiting enzyme in catecholamine biosynthesis. This can be mimicked when cells are treated with catalase, that specifically converts hydrogen peroxide to water and oxygen.[17]

Where excessive production leads to oxidative stress ROS have been implicated in a variety of pathophysiological conditions. With respect to the cardiovascular disorders these include atherosclerosis, ischaemic heart disease, hypertension, cardiac hypertrophy and cardiac failure. However the detection of ROS is difficult due to the short life and reactive state of many species leading to discrepancies in the literature. This review aims to give an overview of the available evidence for a role for ROS both in physiological signalling in the heart and the development of disease states. Limitations associated with techniques used to detect ROS will be discussed. Some recent insights into the role of ROS in regulating cardiac ion channel function and implications with respect to cardiac arrhythmia will also be discussed.

Where do reactive oxygen species originate?

In mammalian cells including cardiac myocytes, the mitochondria are a major source of generation of ROS.[18,19] Electron transport within the mitochondria is generally very efficient. However a small leakage of single electrons can participate in the reduction of oxygen to superoxide. Most of the generation of superoxide and some hydroperoxy radical occurs at the ubisemiquinone site in complex III, but complex I may also be a source of ROS when electrons flow backwards from complex II.[20-22] In addition, superoxide production has been shown to be dependent on mitochondrial membrane potential.[23]

A number of other sites within the cell participate in the production of superoxide. These include xanthine/xanthine oxidase reactions, autoxidation of catecholamines and arachidonic acid metabolism.[24] An important source of superoxide in vascular smooth muscles and endothelial cells is the plasma membrane-bound NAD(P)H-oxidase. Since Griendling *et al.*[25] first identified that physiologically relevant concentrations of angiotensin II increase NAD(P)H-oxidase activity in rat vascular smooth muscle, a number of studies have confirmed the importance of the oxidase and ROS generated by the oxidase in vascular pathology. Other intracellular organelles contribute to the production of hydrogen peroxide and superoxide, such as the endoplasmic reticulum and peroxisomes in the liver and kidney, but since most of the reactive species do not cross the organelle membranes their roles are limited to local oxidation of proteins.[26]

To counteract the effects of oxidants on cellular function, a number of antioxidant defense mechanisms exist. These include superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione. Cardiac myocytes express a cytosolic copper-zinc form of superoxide dismutase and a mitochondrial manganese form of superoxide dismutase (MnSOD).[27] The presence of these forms of superoxide dismutase ensure that intracellular superoxide concentrations are maintained at picomolar concentrations.[7] Hydrogen peroxide generated from the dismutation of superoxide can then be converted to water and oxygen by catalase or glutathione peroxidase. The ratio of reduced to oxidised glutathione is often used as a biochemical marker of cellular oxidative stress. Less than 5-10% of total cellular glutathione should normally be in the oxidised form. Reduced and oxidised glutathione can be assayed spectrophotometrically using the fluorimetric probe o-phthalaldehyde.[28] Alternatively protein bound thiols in the reduced form can be measured using Ellman's reagent.[29]

Problems with detection of ROS – why the discrepancies?

The high reactivity, variable diffusion rate across cell membranes and the instability of ROS make them extremely difficult to detect in cellular systems. In addition, the methods of detection carry limitations. Typically the assessment of ROS has been made using indirect measurements relying on the oxidation of probes or detector molecules to elicit fluorescent signals. Detection of superoxide has been made feasible using chemiluminescence substrates such as lucigenin and coelenterazine that can access intracellular sites of superoxide production and are relatively non-toxic to the cell. However, as with other methods of detection of superoxide such as paraquat and nitroblue tetrazolium (see Tarpey & Fridovich[3] for review), lucigenin is capable of increasing superoxide in the xanthine oxidase/xanthine as well as the glucose oxidase/glucose reactions.[30] Therefore these detectors are non-specific and rates of superoxide production may be underestimated.

The two electron oxidation of dihydroethidium to the fluorophore ethidium bromide is relatively specific for superoxide but can also be oxidised by cytochrome *c*. Electron spin resonance has been used with the stable spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) offering greater specificity for detection of superoxide. Hydrogen peroxide is typically assayed using horseradish peroxidase catalysed assays or the fluorescence indicator 2'-7'-dichlorofluorescein (DCF). However under certain conditions these assays also lack specificity in that lipid peroxides and peroxynitrite can generate DCF fluorescence in the absence of hydrogen peroxide. Therefore the use of more than one detection system may be necessary in order to yield the most reliable results. A

number of excellent reviews discussing the difficulties associated with detection of ROS and advice with regard to interpretation of data are available.[3,31,32]

Role of reactive oxygen species in cardiac physiology and pathophysiology

ROS play an important role in modulating cell signalling pathways, and when produced at high levels inducing cell death. They are believed to interact with cell signalling pathways by way of modification of key thiol groups on proteins that possess regulatory functions. These proteins may be second messengers such as serine/threonine, tyrosine and MAP kinases, growth factors and transcription factors such as NF-κB (for a review of this area see Wolin[7] and Thannickal & Fanburg[26]). As mentioned previously, the activity of some intracellular second messengers is under the tonic regulation of cellular ROS and this appears to be necessary for normal cellular function. Epidermal growth factor and insulin-induced intracellular hydrogen peroxide formation are dependent upon the inhibition of protein tyrosine phosphatase activity by hydrogen peroxide. Tyrosine phosphatase activity is inhibited via reversible oxidation of its catalytic cysteine. Formation of a sulphenyl-amide intermediate (upon reaction with hydrogen peroxide) causes unusual protein modifications to the active site of tyrosine phosphatase 1B that both protects it from irreversible oxidation and promotes reversible reduction by thiols .[14,15] The regulation of catalytic activity of the enzyme by hydrogen peroxide is both precise and rapid. The reversibility of an oxidised cysteine residue by thiols appears to be dependent upon the intermediate state formed (sulfinic acid versus sulfonic acid).[13]

ROS can also play a central role in the genesis of abnormalities. For example, an initial step in the development of atherosclerosis is the uptake of oxidised low density lipoprotein that then further stimulates the production of ROS and apoptosis.[33] Under these circumstances, oxidative stress plays a part in the formation of nitric oxide-containing species preventing vascular relaxation and increasing the risk of thrombosis as a result of inactivation of prostaglandins.

Reperfusion of the ischaemic heart during coronary occlusion remains the primary objective to prevent further cardiac damage in the clinical setting. However, the reperfusion period imposes deleterious effects due to the generation of ROS. Contractile dysfunction or “myocardial stunning” has been linked to increases in cellular generation of ROS and includes impaired sarcoplasmic reticulum calcium handling and contractile responses.[34,35] In addition, the development of cardiac hypertrophy is associated with activation of hypertrophic signalling pathways such as ERK and MAP-kinase by ROS.[36,37] The source of ROS has been linked to chronic activation of NAD(P)H-oxidase.[38,39] Hydrogen peroxide can regulate the activity of specific isoforms of protein kinase C in the heart that are implicated in the development of cardiac hypertrophy,[16,40,41] and the transition from hypertrophy to failure.[42] Animal studies indicate that treatment with antioxidants reduce morbidity and mortality after myocardial infarction.[43,44] Mice with targeted disruption of superoxide dismutase are vulnerable to ischaemic damage,[45] while over-expression of superoxide dismutase protects from postischaemic injury.[46]

In vascular smooth muscle it is well recognised that ROS produced as a result of activation of NAD(P)H-oxidase by angiotensin II are involved in the development of vascular smooth muscle hypertrophy and hypertension.[47,48] Angiotensin II-induced superoxide production in the brain is also involved in the central regulation of blood pressure.[49] Cardiomyopathy and vascular disease associated with chronic diabetes is linked to reactive oxygen and nitrogen species. Antioxidants preserve cardiomyocyte morphology and contractile function.[50,51]

The role of ROS in cardiovascular disease has been extensively reviewed and the reader is referred to a number of excellent reviews.[52,53]

Reactive oxygen species and the regulation of ion channel function

The one electron reduction of oxygen to superoxide occurs rapidly. Therefore in addition to the longer-term effects of altering cellular transcription, ROS are also capable of triggering rapid changes in protein function as a result of altering the redox state of the proteins. In cardiac myocytes rapid changes in cellular oxygen can contribute to electrophysiological instability in the myocardium and the development of arrhythmias.[54-56] Understanding how ion channels respond to changes in oxygen tension may help with developing strategies to prevent the trigger of arrhythmias.

A number of cardiac ion channels respond to changes in oxygen partial pressure.[57-65] Channel function can be modified by thiol reducing or oxidising agents[19,58,66,60,67,68] and thiol reducing agents mimic the effect of hypoxia.[19,60] This would suggest that ion channel function is modified during hypoxia as a result of redox modification of the channel protein or a nearby protein. We examined the role of ROS in regulating the function of the L-type Ca^{2+} channel and the sensitivity of the channel to β-adrenergic receptor stimulation in adult guinea-pig ventricular myocytes. Cells were perfused intracellularly with catalase, a naturally occurring antioxidant that specifically converts hydrogen peroxide to water and oxygen, and the sensitivity of the channel to β-adrenergic receptor stimulation was measured. We hypothesised that if hypoxia is associated with a decrease in cellular production of ROS then perfusing myocytes intracellularly with catalase should mimic the effect of hypoxia. In the presence of active catalase, the $K_{0.5}$ for activation of the channel by isoproterenol (a β-adrenergic receptor agonist) was significantly less than the $K_{0.5}$ recorded when cells were perfused intracellularly with heat-inactivated catalase (0.4 vs 2.7 nM; Figure 2), and similar to the $K_{0.5}$ recorded during hypoxia.[19,60] Pre-exposing cells to hydrogen peroxide attenuated the effect of catalase and the effect of

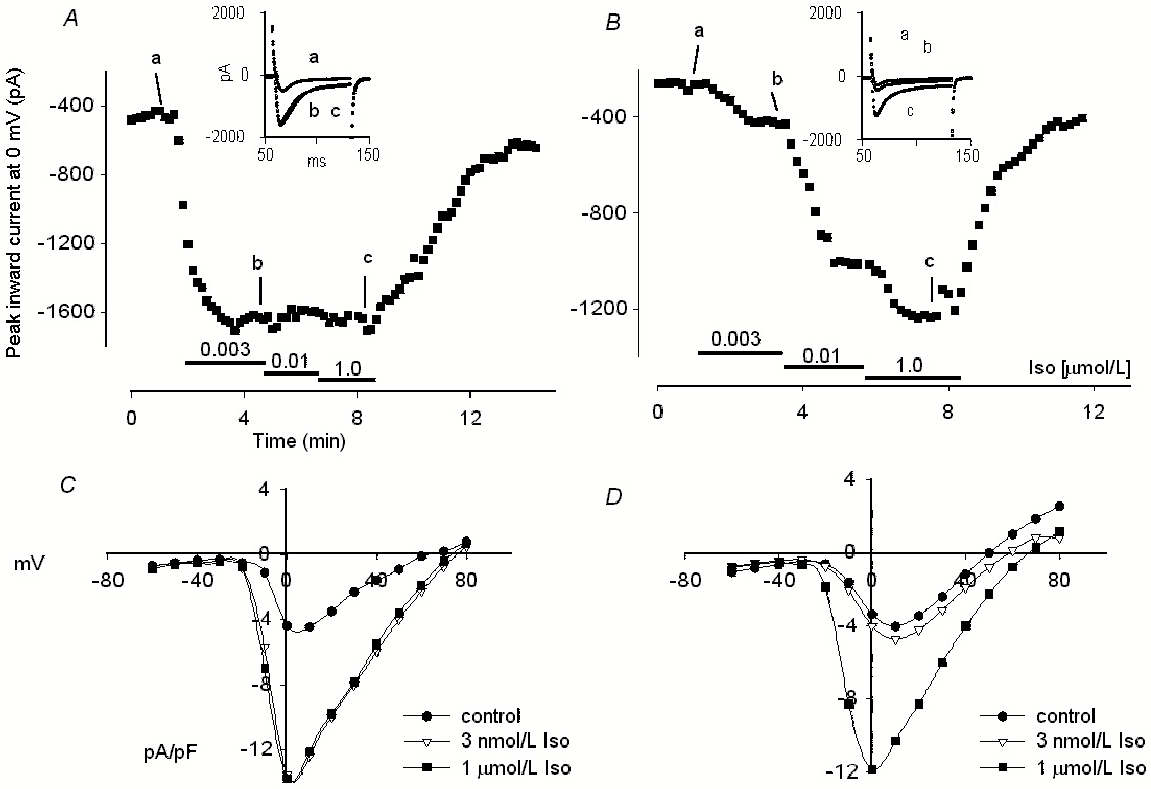

Figure 2. Catalase increases the sensitivity of the L-type Ca^{2+} channel to isoproterenol. *(A) and (B) Time course of changes in membrane current recorded in a cell during exposure to increasing concentrations of isoproterenol (Iso) while being dialysed with active catalase (A) and inactivated catalase in a separate cell (B) including membrane currents recorded at time points indicated (inset). Current-voltage relationship normalised to cell membrane capacitance in the presence of catalase is shown in (C) and in the presence of inactivated catalase is shown in (D). Adapted from Hool & Arthur[19] with permission.*

hypoxia. In addition, responses recorded in the presence of hypoxia and catalase could be attenuated when cells were perfused intracellularly with βPKC peptide inhibitor but not εPKC peptide inhibitor implicating a role for the C2 containing PKC isoforms in the regulation of channel function during hypoxia. The C2 (classical) isoforms are activated by Ca^{2+} and diacylglycerol. This group are the most abundant and comprise the α, β_I, β_{II} and γ isoforms. The novel PKC's comprise ε, η, δ and θPKC and are activated by diacylglycerol but not Ca^{2+}.[69]

We measured cellular hydrogen peroxide using the fluorescent indicator DCF and cellular superoxide using dihydroethidium in the myocytes and confirmed that hypoxia was associated with a decrease in production of reactive species. These data support the idea that cellular ROS can regulate channel function. Hypoxia at a level that does not cause metabolic inhibition (pO_2 17 mmHg) [70] causes an increase in the sensitivity of the channel to β-adrenergic receptor stimulation that is mediated via a decrease in cellular hydrogen peroxide. In addition, when mitochondrial electron transport was partially disrupted with carbonyl cyanide p-(trifluromethoxy)phenyl-hydrazone (FCCP) or myxothiazol, a similar increase in sensitivity of the L-type Ca^{2+} channel to β-adrenergic receptor stimulation to that recorded during hypoxia was measured. These data suggest that the main source of production of ROS in cardiac myocytes is the mitochondria. During hypoxia, cellular production of ROS is less and the channel function is altered either as a result of direct redox modification of the channel protein or an intermediate such as protein kinase C or protein kinase A (Figure 3).

Enhancement of the L-type Ca^{2+} channel promotes arrhythmogenic afterdepolarisations.[71,72] Therefore these data help to explain the mechanisms associated with Ca^{2+}-dependent arrhythmias in the heart. Further understanding the site of redox modification may help to determine specific treatment strategies as antiarrhythmic therapy.

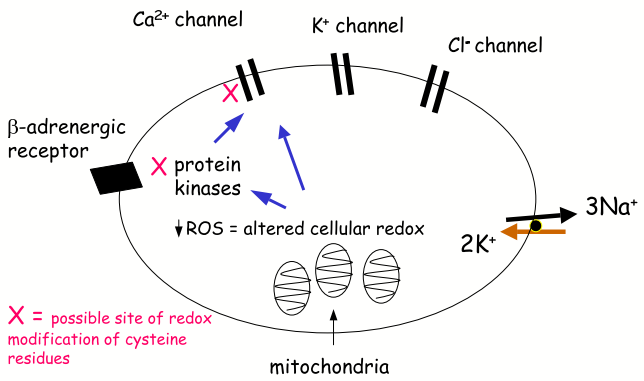

Figure 3. Schematic of proposed role of ROS in the regulation of ion channel function during hypoxia. *A decrease in ROS generated from the mitochondria results in a reduced cytosolic environment that either directly alters the redox state of the channel protein or alters the function of the channel via redox modification of serine/threonine kinases.*

Acknowledgments

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