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Mini-Review

Cardiac Toxicity of Singlet Oxygen: Implication in Reperfusion Injury

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ABSTRACT

Oxygen-derived free radicals ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$) that are produced during postischemic reperfusion are currently suspected to be involved in the pathogenesis of tissue injury. Another reactive oxygen species, the electronically excited molecular oxygen ($^1O_2$), is of increasing interest in the area of experimental research in cardiology. In this review are discussed the main potential sources of singlet oxygen in the organism, particularly in the myocardium, the various cardiovascular cytotoxic effects induced by this reactive oxygen intermediate, and the growing evidence of its involvement in ischemia/reperfusion injury. Antioxid. Redox Signal. 3, 63–69.

INTRODUCTION

MANY EXPERIMENTAL STUDIES have provided evidence that oxygen-derived free radicals are directly involved in the pathophysiological consequences of postischemic reperfusion injury (4, 5, 68). These reactive oxygen species can attack subcellular structures causing functional, metabolic, and structural disturbances and leading ultimately to cell death (37). Most of the studies available in the literature on this topic have focused on superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) as mediators of ischemia/reperfusion injury. However, for several years, a growing interest is given to another nonradical reactive oxygen species, singlet oxygen ($^1O_2$), to its role in pathological situations and particularly in the oxidative stress induced by postischemic reperfusion (67). $^1O_2$ corresponds to an electronically excited state of molecular oxygen. It is a short-lived species with a lifetime of 10^{-6} s. It is produced in cells under normal and pathophysiological conditions, and it likely contributes to the damaging effects of various biological processes (26). No direct evidence of $^1O_2$ formation *in vivo* has been described to date because no specific chemical trap able to measure $^1O_2$ production is available. However, a number of indirect indices, such as the results of studies examining the toxic effects of exogenous generation of $^1O_2$ on the cardiovascular system or testing the potential beneficial action of some $^1O_2$ quenchers, show that this species could play an important role in the oxidative stress resulting from postischemic reperfusion of the myocardium.

POSSIBLE SOURCES OF $^1O_2$

Several mechanisms have been shown to produce reactive oxygen species in biological environment by enzymatic-dependent or non–enzymatic-dependent chemical reactions (3). Some of these mechanisms, known to generate $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$ during oxidative stress in the myocardium, could also be some

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potential sources of $^1O_2$, but their real implication still remains uncertain.

The production of oxygen-derived free radicals via leukocyte activation during ischemia and reperfusion has been proposed by Romson *et al.* (49), who showed that polynuclear neutrophil depletion reduced infarct size to a degree comparable to the reductions seen using the oxygen-radical scavengers superoxide dismutase and catalase. It is now well established that, during phagocytosis, the enzymatic system NADPH oxidase catalyzes the reduction of oxygen to $O_2^{\cdot -}$ and H_2O_2 and the enzyme myeloperoxidase (MPO), which is secreted by neutrophils into the cellular, lysosomal, and extracellular compartments and produces hypochlorite (HOCl) in the presence of H_2O_2 and Cl^-. Moreover, Rosen and Klebanoff (50) have shown that the MPO/H_2O_2/halide from human neutrophils can also produce $^1O_2$ (Eq. 1 and 2):

$$\xrightarrow{\text{MPO}}$$

$$H_2O_2 + H^+ + Cl^- \rightarrow H_2O + HOCl \quad [1]$$

$$H_2O_2 + HOCl \rightarrow H_2O + H^+ + Cl^- + {}^1O_2 \quad [2]$$

Using a chemiluminescence technique, Cutrin *et al.* (14) have recently shown a clear relationship between $^1O_2$ formation, phagocyte activation, and ischemia/reperfusion injury development in the rat liver.

Prostaglandin H_2 (PGH_2) synthesis is potentially stimulated during postischemic reperfusion (28). During arachidonic acid metabolism, the cyclooxygenase activity of prostaglandin synthase (PG synthase) induces the formation of prostaglandin G_2 (PGG_2), which is, in turn, converted into PGH_2 by the hydroperoxidase activity of the enzyme. Prostaglandin hydroperoxidase is known to produce $^1O_2$ in the presence of NADH or NADPH (35); however, this enzyme has also been described to form $^1O_2$ (11) especially by a mechanism involving an oxo-ferryl complex intermediate [$(FeO)^{3+}$] (37) (Eq. 3 and 4):

$$\xrightarrow{\text{PG synthase}}$$

$$PGG_2 + Fe^{3+} \rightarrow PGH_2 + (FeO)^{3+} \quad [3]$$

$$PGG_2 + (FeO)^{3+} \rightarrow PGH_2 + Fe^{3+} + {}^1O_2 \quad [4]$$

Other enzymatic systems such as xanthine oxidase or lipoxygenase have also been reported as potential sources of $^1O_2$ (16, 27).

In addition to these enzyme-dependent mechanisms, several nonenzymatic processes could contribute to $^1O_2$ formation. For example, interaction of $O_2^{\cdot -}$ with H_2O_2 during Haber–Weiss reaction leads to $^1O_2$ formation (29) (Eq. 5 and 6):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [5]$$

$$H_2O_2 + O_2^{\cdot -} \rightarrow {}^{\cdot}OH + OH^- + {}^1O_2 \quad [6]$$

Haber–Weiss reaction does not occur frequently under normal biological conditions, but should be stimulated under pathophysiological conditions such as ischemia, which is characterized by a reduction of intracellular pH.

Generation of $^1O_2$ may also occur during spontaneous disproportionation of H_2O_2 (Eq. 7) as evidenced by Smith and Kulig (52) using the detection of 5α-hydroperoxide of cholesterol, a specific trap of $^1O_2$ as a marker.

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + {}^1O_2 \quad [7]$$

Lipid peroxidation occurs under pathological conditions of oxidative stress such as ischemia and reperfusion (1), during which an apparent accumulation of lipid peroxides in the tissue is measured (47). Reactive oxygen species produced upon postischemic reperfusion may initiate this chain reaction leading to membrane injury and resulting in the development of severe arrhythmias (43, 62). A number of studies have shown that lipid peroxidation in microsomes is associated with production of $^1O_2$ (15, 31, 45): lipid hydroperoxides (ROOH) are very unstable, and their decomposition yields peroxy ($ROO^{\cdot}$) and alkoxy ($RO^{\cdot}$) radicals. The self reaction of lipid peroxy radicals leads to the formation of $^1O_2$ and excited carbonyl compounds that on decay to the ground state emit chemiluminescence (8, 49).

CELLULAR TARGETS OF $^1O_2$

$^1O_2$ is highly reactive and can oxidize many cellular components. As far as DNA and its constituents are concerned, it is known that $^1O_2$

reacts with guanine, initiating very complex reaction pathways as described by Cadet and Teoule (12), which lead to DNA damage (61). Like $^{\cdot}OH$ radicals, $^1O_2$ could be a direct initiator of membrane lipid peroxidation (25, 54) leading to major alterations of biological membrane components. Moreover, several amino acids are subjected to $^1O_2$-induced damage, and histidine, tryptophan, and methionine are particularly sensitive to $^1O_2$ attack (18, 50). These kinds of interactions can ultimately result in protein alteration (46) and/or inactivation of many enzymes (20, 61).

In cardiovascular studies, cytotoxic effects of $^1O_2$ have been investigated mainly by using an exogenous generating system: photoactivated Rose Bengal (RB). This photosensitive dye is a fluorescein derivative and is very soluble in aqueous solutions. RB strongly absorbs light in the range of 530–590 nm and reaches an excited state. The transfer of excitation energy to molecular oxygen (type II pathway) leads to the formation of $^1O_2$. Charge transfer from excited RB to oxygen can also produce $O_2^{\cdot -}$ but in relatively small proportion (42, 64). Thus, photoactivated RB is considered as an efficient $^1O_2$ generating system. Borgers *et al.* (6) and Ver Donck *et al.* (60) were the first to exploit the photodynamic properties of RB as an investigative tool for transient bursts of reactive oxygen intermediates in experimental cardiology. Ver Donck *et al.* (60) have shown that exposition of isolated cardiomyocytes to illuminated RB induced a rapid morphologic transformation into hypercontracted rounded cells, suggesting that $^1O_2$ induces cellular damage probably via Ca^{2+} homeostasis disturbances. Subsequently, Hearse *et al.* (21) and Kusama *et al.* (41) have reported the effects of photoactivated RB on isolated perfused rat hearts. Low concentrations of RB induced a very rapid occurrence of electrophysiological changes that resulted in a variety of arrhythmias, providing evidence of severe membrane dysfunction. The photoactivation of RB also results in dose- and light-dependent changes in coronary flow, characterized by an initial transient vasodilation followed by a progressive time-dependent vasoconstriction (21, 41). Moreover, Tarr and Valenzeno (56) have shown that RB illumination induces changes in cardiac electric activity with a prolongation, followed by a reduction, of action potential in atrial myocytes. The electrophysiological, cytochemical, and ultrastructural signs of $^1O_2$ toxicity on the heart were confirmed by Vandeplassche *et al.* (59), and their homologies with postischemic reperfusion injury suggest that $^1O_2$ could be involved, as well as other reactive forms of oxygen, in oxidative stress of reperfusion.

More recently, Mizukawa and Okabe (45) have reported that $^1O_2$ produced by photoactivated RB inhibits vascular reactivity in rabbit mesenteric artery with a strong dysfunction of endothelium-dependent relaxation.

The primary targets of the postischemic process in the myocardium could be the excitation–contraction coupling system that regulates Ca^{2+} distribution in the cellular compartments (2, 30, 33). Kukreja *et al.* (35, 36) have shown that exposure of cardiac sarcoplasmic reticulum (SR) to irradiated RB produced a significant inhibition of Ca^{2+} uptake and Ca^{2+}-ATPase activity. Moreover, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of SR demonstrated complete loss of the Ca^{2+}-ATPase monomer band. The protective effects of the $^1O_2$-quencher histidine on both the structure and the activity of the protein confirm that SR damage is most likely due to $^1O_2$. On skeletal SR preparations, photoactivation of RB causes a stimulation of Ca^{2+} release via Ca^{2+} channels, probably by interaction of $^1O_2$ with the ryanodine binding site (52, 63). Moreover, Holmberg *et al.* (23) and Holmberg and Williams (22) have described a progressive destruction of structure and function of the cardiac SR Ca^{2+} channels by reactive oxygen species produced by photoactivated RB.

Sarcolemmal Na^+,K^+-ATPase regulates intracellular concentration of Na^+ and plays an important role in membrane potential regulation. Inhibition of this enzyme by ischemia and reperfusion of the myocardium leads to the rise of intracellular Na^+ (30). As a consequence of this Na^+ overload, a Ca^{2+} influx through sarcolemmal Na^+/Ca^{2+} exchange will contribute to the intracellular Ca^{2+} overload, which is one of the characteristic deleterious causes of ischemia/reperfusion injury. Vinnikova *et al.* (61) have observed the action of exogenously generated $^1O_2$ on Na^+,K^+-ATPase activity. The exposure of isolated cardiac sarcolemmal vesicles to irradiated RB resulted in a marked inhibition of the activity of this ATPase. Oxygen

intermediate scavengers superoxide dismutase, catalase, and mannitol were without any beneficial effect on the preparations, whereas histidine exhibited a dose-dependent protection. Again, these observations suggest the involvement of $^1O_2$ in the mechanism of oxidative stress reperfusion-induced arrhythmias.

The enzyme 5′-nucleotidase is thought to be the major pathway of adenosine formation in the ischemic myocardium and is determinant for maintaining myocardial function and regulating microvascular competence. The membrane form of the enzyme (ecto-5′-nucleotidase) was found to be the target of oxygen free radicals during ischemia/reperfusion (32). Zhai *et al.* (68) have shown that perfusion of isolated rat hearts with photoactivated RB causes inhibition of ecto-5′-nucleotidase. Thus, $^1O_2$ might be directly responsible for the attenuation of adenosine release, which could possibly be one of the important mechanisms of oxygen radical-mediated injury during ischemia/reperfusion.

EVIDENCE FOR INVOLVEMENT OF $^1O_2$ CYTOTOXICITY IN ISCHEMIA/REPERFUSION INJURY

Although exogenous generating systems have allowed the study of the cytotoxic effects of $^1O_2$ on subcellular, cellular, or entire organ models, direct evidence of $^1O_2$ formation in biological tissue is very difficult to give. Indeed, up to now, there is no specific marker available for *in vivo* $^1O_2$ detection.

Measurement of chemiluminescence arising from the radiative transition of $^1O_2$ to the ground state constitutes a direct noninvasive method for $^1O_2$ detection. The monitoring of bimolecular transition (634 and 703 nm) allows the measurement of the light emission arising from solutions with enzymatic reactions (50), from suspensions of subcellular fractions (9, 11) and cells (10), or even from the surface of entire organs (7, 57).

However, *in vivo* light emission measurement, *per se*, is not sufficient to identify $^1O_2$. Indeed, spontaneous chemiluminescence also involves photon emission from excited carbonyl groups generated during lipid peroxidation initiated by oxidant stress. Moreover, spontaneous chemiluminescence is very low, and efforts are usually made to enhance it by increasing peroxidative conditions (9). Experimental evidence of $^1O_2$ formation is further provided by associating chemiluminescence detection with spectral analysis (7, 10).

Chemiluminescence has been applied in ischemia/reperfusion experiments. Ferreira *et al.* (17) have detected a significantly higher chemiluminescence in postischemic myocardial biopsies as compared with preischemic samples. Moreover, in myocardial rat and dog tissue homogenates, Torok *et al.* (57) have shown some differences in *tert*-butyl hydroperoxide-induced photoemission between normally perfused and ischemic samples. Therefore, these authors have suggested that intensification of the light bursts might be considered as a marker of infarcted versus intact tissue.

Finally, the increase in reactive oxygen intermediate production detected by luminol-enhanced chemiluminescence on isolated rat hearts during ischemia and reperfusion could include $^1O_2$ (39).

Direct detection of $^1O_2$ is very difficult; therefore, numerous studies are based on the measurement of more or less specific oxidative products of $^1O_2$ (49, 51, 53). Zhai and Ashraf (67) have provided evidence of $^1O_2$ involvement in ischemia/reperfusion damage by HPLC measurement of the specific product of β-carotene oxidation by $^1O_2$, 5,8-endoperoxide. These authors have shown that $^1O_2$ production during reperfusion is dependent on the duration of initial ischemia. Moreover, the protective effects of histidine on postischemic function were associated with a significant reduction of 5,8-endoperoxide. Thus, $^1O_2$ seems to be one of the major factors in postischemic reperfusion injury.

To confirm the toxicity of exogenously generated $^1O_2$ in biological environments, the specific $^1O_2$ quencher, histidine, has been widely used. Most of the studies have demonstrated a protective effect of histidine against photoactivated RB-induced damage to the Ca^{2+}-ATPase of the cardiac (35, 36) or skeletal (24) SR, the sarcolemmal Na^+,K^+-ATPase (60), the myocardial ecto-5′-nucleotidase (67), or the reactivity of the vasculature (44). Moreover, the use of $^1O_2$ quenchers on ischemia/reperfusion models has provided further evidence of the involvement of $^1O_2$ in reperfusion injury. In this view, Gauduel

and Duvelleroy (19) have shown that histidine limits the severity of the "oxygen-paradox" phenomenon by reducing the level of unsaturated lipid peroxidation during the ischemia/reperfusion sequence. Kukreja and Hess (38) and Cai *et al.* (13) have confirmed the beneficial effects of histidine on isolated perfused rat hearts, showing an improvement of postischemic function, a decrease in the incidence and severity of reperfusion arrhythmias, and a preservation of cellular ultrastructure. Similarly, the reduction of ecto-5′-nucleotidase activity induced by ischemia/reperfusion was significantly limited by addition of histidine (67). Finally, more recently, Lee *et al.* (42) have shown that carnosine, a histidine-derived dipeptide, is potentially more effective in quenching $^1O_2$ *in vitro* and therefore provides cardioprotective effects against ischemia/reperfusion injury. In this study, the lack of protection of the $^{\cdot}OH$ scavenger mannitol further suggests that $^1O_2$ might contribute to reperfusion injury.

Other natural or artificial compounds are able to scavenge $^1O_2$ with more or less specificity, like 1,4-diazabicyclo[2.2.2]octane (DABCO), diphenylisobenzofuran, sodium azide, tryptophan, or β-carotene. Unfortunately, few of them can be used in biological systems because of their potential toxicity and/or limited solubility in aqueous solutions.

CONCLUSION

Oxygen-derived free radicals ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$) have been clearly implicated in postischemic reperfusion injury. A growing interest is attributed to $^1O_2$ in biological systems and especially in the pathophysiology of ischemia/reperfusion. This very highly reactive chemical species exerts marked deleterious effects on the cardiovascular system as shown by the studies using photoactivated RB as exogenous generator of $^1O_2$. Although there is currently no satisfying way to provide direct evidence of $^1O_2$ formation *in vivo*, the detection of specific oxidative products and/or the evaluation of the effects of specific quenchers like histidine strongly suggests that $^1O_2$ might play an important role in myocardial ischemia/reperfusion injury.

ABBREVIATIONS

$(FeO)^{3+}$, oxo-ferryl complex; H_2O_2, hydrogen peroxide; HOCl, hypochlorite; MPO, myeloperoxidase; $^1O_2$, singlet oxygen; $O_2^{\cdot -}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PG synthase, prostaglandin synthase; RB, Rose Bengal; SR, sarcoplasmic reticulum.

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