Review Article

Microdialysis technique for in-vivo monitoring of $\cdot\text{OH}$ generation on myocardial injury in the rat

Toshio Obata*

School of Nursing, Faculty of Health Sciences, Osaka Aoyama University, 2-11-1 Niina, Mino City, Japan

***Address for Correspondence:** Toshio Obata, Ph.D. School of Nursing, Faculty of Health Sciences, Osaka Aoyama University, 2-11-1 Niina, Mino City, Japan, Tel & Fax: +81-72-737-6754; Email: t-obata@osaka-aoyama.ac.jp

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Abbreviations: ROS: Reactive Oxygen Species; EPR: Paramagnetic Response; DHBA: Dihydroxybenzoic Acid; HPLC: High-Performance Liquid Chromatography; EPR: Electron Paramagnetic Response; XO: Xanthine Oxidase; MAO: Monoamine Oxidase; NA: Noradrenaline; ACE: Angiotensin-Converting Enzyme; $[\text{K}^+]_o$: Extracellular Potassium-ion Concentration; K_{ATP} : ATP sensitive K^+ ; 5-HD, 5-Hydroxydecanoate; NO: Nitric oxide; NOS: NO synthase; ONOO $^-$: peroxynitrite; L-NAME: NG-nitro-L-arginine Methyl Ester; LDL: Low-density Lipoprotein; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; 1O $_2$: singlet oxygen; ECG, Electrocardiogram; DOPGAL, 3,4-dihydroxyphenylglycol-aldehyde

Abstract

The micro dialysis procedure is a technique that has been established for some years. Although free radical reaction is a part of normal metabolism, sustained elevation of noradrenaline (NA) in the extracellular fluid can be autoxidized, which in turn leads (possibly by an indirect mechanism) to the formation of cytotoxic free radicals. Reactive oxygen causes excessive Na^+ entry through the fast Ca^{2+} channel, leading to intracellular Ca^{2+} overload through the Na^+ - Ca^{2+} exchange system. However, the interaction between intracellular Ca^{2+} overload and oxygen free radicals in myocardium is not clear. Angiotensin converting enzyme (ACE) inhibitor is associated with cardioprotective effect due to suppression of NA-induced hydroxyl radical ($\cdot\text{OH}$) generation in the heart. Low-density lipoprotein (LDL) oxidation may be related to NA induced $\cdot\text{OH}$ generation. Although the neuroprotective effects of nitric oxide (NO) is discussed, NO contributes to the extracellular potassium concentration ($[\text{K}^+]_o$)-induced $\cdot\text{OH}$ generation via NO synthase (NOS) activation. Opening of ATP sensitive K^+ channel (K_{ATP}) channel may cause $\cdot\text{OH}$ generation. These findings may be useful in elucidating the actual mechanism of free radical formation in the pathogenesis of heart disorders.

Introduction

Ischaemia-reperfusion tissue injury is a major pathophysiological process responsible for severe organ damage in human health problems [1-3]. Oxygen-derived free radicals play an important role in several models of experimentally induced reperfusion injury [4]. The deleterious effects of reperfusion of the ischaemic myocardium have been linked to the production of oxygen-derived free radicals [5]. Although free radical reactions are a part of normal metabolism, overproduction or reduced efficiency of defense system towards reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$) may contribute to ischaemia-reperfusion injury [6,7]. An oxygen-derived free radicals such as O_2^- itself is somewhat poorly reactive in aqueous solution, but does it participates in reactions with iron ions, generating the more damaging $\cdot\text{OH}$ [8]. The $\cdot\text{OH}$ radicals are extremely reactive and rapidly react with a number of compounds, including lipids, proteins and nucleic acid [9]. It is important to explain the role played by free radicals in some disease states. We developed the flexibly mounted microdialysis technique [6]. This technique has made it possible not only to collect dialysis samples from interstitial fluid with minimum injury to the cardiac muscles located the probe of the microdialysis system. Therefore, we will demonstrate and discuss the effects of the several drugs of the role of played by radicals in some disease state.

Detection of $\cdot\text{OH}$ generation

Although improved colorimetric [10] and fluorescence based [11,12] assays for measurement of hydroxylated products have been described, complete determination of hydroxylated product formation can be achieved only by use of gas chromatography [10,13] or high-performance liquid chromatography (HPLC) with or UV-or electrochemical (EC) detection, or both [14].

The $\bullet$ OH react with salicylate and generates 2,3- and 2,5-dihydroxybenzoic acid (DHBA), which can be measured electrochemically in picomoles quantity by an HPLC-EC procedure [15,16]. The *in vivo* generation of $\bullet$ OH free radicals in myocardium can be measured by *in vivo* microdialysis perfusion of salicylate, avoiding many of the pitfalls inherent in the systemic administration of salicylate. After minimizing the contribution of enzyme and/or blood-borne 2,5-DHBA, the present data demonstrate the validity of the use of 2,3-DHBA as an index of $\bullet$ OH formation in the heart. Therefore, we focused on the possible use of salicylate hydroxylation as an *in vivo* trapping procedure for monitoring the time course of 2,3-DHBA generation in the myocardium. However, 2,3-DHBA can be non-enzymatically formed by aromatic hydroxylation [17,18].

Microdialysis technique

Microdialysis technique was recently introduced for *in vivo* heart experiments to measure interstitial biological substances, such as catecholamine, $\bullet$ OH and purine metabolites [19]. We measured $\bullet$ OH in rat hearts by use of a microdialysis technique. With this technique it was feasible to make stable long-term measurements of $\bullet$ OH. The concentration profile of the administered dialysate is unknown; in general given through the probe would never reach the concentration in the dialysis probe [20]. This is an unavoidable limitation of the microdialysis technique that should be kept in mind when interpreting the experimental data.

We designed a system for holding the microdialysis probe [6] which includes loose fixation of the tube and synchronization of the probe with that of the heart (Figure 1). Details of the technique necessary for manipulation of the flexibly mounted microdialysis probe in *in vivo* rat hearts (to measure the 2,3-DHBA) were described previously [6]. We created a suitable microdialysis probe. In brief, the tip of the microdialysis probe (3 mm in length and 220 μ m o.d. with the distal end closed) was made of dialysis membrane (cellulose membrane 10 μ m thick, blocking components with molecular weights >50 kDa). Two fine silica tubes (75 μ m i.d.) were inserted into the tip of the cylinder shape dialysis probe and one of these served as the inlet for the perfusate and the other as the outlet for the dialysate. The inlet tube was connected to a micro-injection pump, and the outlet tube was led to the dialysate reservoir. These tubes of the dialysis probe (~15cm long) were supported loosely at the mid-point on a semi-rotatable stainless-steel wire, so that their movement fully synchronized with the rapid up-and-down motion of the tip caused by the heart beats.

The probe was implanted from the epicardial surface into the left ventricular myocardium and was perfused through the inlet tube. For trapping •OH radicals in the myocardium, sodium salicylate in Ringer's solution was perfused by a micro-injection pump, and the basal level of 2,3-DHBA during a definite time period was determined. In the preparation of ischemic rat heart, after the microdialysis probe implantation in the ischemic zone, the left anterior descending coronary artery (LAD) branch was clamped by a thread through a tube surrounding the coronary artery. The heart was subjected to regional ischemia for 15 min by the occlusion of the LAD coronary artery followed by reperfusion for 60 min. In order to ascertain the protective effect of myocardial infarction and reperfusion damage, the serum creatine phosphokinase (CPK) assay was measured [21].

Oxygen-derived free radical in post ischaemic tissue injury

It appears likely that free radical production may make a major contribution at certainly stages in the progression of the ischaemic and reperfusion injury [22]. The primary source of superoxide in reperfused reoxygenated tissues appears to be the enzyme xanthine oxidase (XO), released during ischaemia by a calcium-triggered proteolytic attack on xanthine dehydrogenase [4]. Dysfunction induced by free radicals may be a major component of ischaemic disease of the heart. The XO is also thought to be a source of $O_2^{\cdot-}$. The $O_2^{\cdot-}$ itself is somewhat poorly reactive in aqueous solution, but also participate in the reaction in which the iron ions are involved, leading to the generation of more damaging •OH species. Theoretically, •OH may be formed *in vivo* during non-enzymatic oxidation [23,24] and/or enzymatic oxidation. According to the reaction pathway in Figure 2. •OH was generated by the presence of oxygen. The •OH can be also arise from an interaction between H_2O_2 and $O_2^{\cdot-}$ (Haber-Weiss reaction). On the other hand, iron (II) in the presence of H_2O_2 results in further formation of •OH [25,26]. These results suggest that iron (III) may reduce •OH formation by Fenton reaction in the heart. The suppression of •OH formation by iron (III) may play a key role in the cardioprotective effect of iron (III) on the rat heart [27]. There are other sources of •OH generation. The effect of pargyline, a monoamine oxidase (MAO) inhibitor, on the generation of •OH was confirmed in the rat hearts. The accumulation of noradrenaline (NA) in the extracellular fluid by pargyline can be autoxidized, which in turn, leads (possibly by an indirect mechanism) to the formation of cytotoxic •OH [28]. Based on the mass-action principle, an increase of iron (III) should reverse the Fenton reaction and reduce the •OH production.

Figure 2: The reaction pathway in rat heart illustrates the formation of hydroxyl radical in the presence of iron (III) and oxygen. Abbreviations: XO, xanthine oxidase; $O_2^{\cdot-}$, superoxide anion; •OH, hydroxyl radical; MAO, monoamine oxidase, SOD, superoxide dismutase.

Although the mechanism of $\bullet\text{OH}$ generation in the ischaemic heart is obscure, a concomitant increase of NA and $\bullet\text{OH}$ takes place in myocardial injury. In case of ischaemic arrhythmia appeared, the presence of NA and free radicals was observed in ischaemia-reperfused rat heart [6]. The accumulation of NA in the extracellular fluid by ischaemia-reperfusion can be autoxidized, which in turn leads (possibly by an indirect mechanism) to the formation of $\bullet\text{OH}$ radicals [29]. In the presence of iron (III), ischaemia-reperfusion failed to increase in $\bullet\text{OH}$ production [30]. The suppression of $\bullet\text{OH}$ formation by iron (III) may play a key role in the cardioprotective effect of iron (II) in the heart.

Effect of angiotensin-converting enzyme (ACE) inhibitor

Angiotensin-converting enzyme (ACE) inhibitor is known to have a cardio protective effect by reducing reperfusion arrhythmia [31,32]. It is well known that antihypertensive action of the ACE inhibitor is associated with an inhibition of NA release from peripheral sympathetic neuron [33]. ACE inhibitors cardioprotective effects are associated with suppression of $\bullet\text{OH}$ generation [29,34]. It is well known that angiotensin II causes an increase of NA release from peripheral sympathetic neuron [35]. The accumulation of NA may cause cytotoxic $\bullet\text{OH}$ generation. The free radical scavenging action of ACE inhibitor (i.e. captopril) is believed to be due to the presence of an SH-group in its structure [36]. Numerous investigators have reported that myocardial ischaemia also increases catecholamine levels [36-38].

The accumulation of NA in the extracellular fluid elicited by ischaemia/reperfusion can be autoxidized, which in turn, leads (possibly by an indirect mechanism) to the formation of cytotoxic $\bullet\text{OH}$ radicals. NA release by ischaemia/reperfusion of the myocardium may be associated with oxidant damage in $\bullet\text{OH}$ generation. Endogenous angiotensin II may facilitate NA release via presynaptic angiotensin II receptors [39,40] or inhibit NA uptake at synaptic nerve terminals [41]. Rona [42] documented that catecholamines play an important role in reperfusion and ischaemic myocardial injury, and proposed a common pathway in the pathogenesis of catecholamine-induced and reperfusion myocardial injuries. It is possible that ACE inhibitors may reduce catecholamine release and hence ameliorate myocardial injury. ACE inhibitors have been shown to potentiate the effects of endogenous bradykinin in animal models and in humans [43]. However, the role of bradykinin in therapeutic effects of ACE inhibitors has remained controversial, mainly due to the lack of potent and specific bradykinin-blocking agents with which to test this hypothesis experimentally.

Potassium depolarization induce $\bullet\text{OH}$ generation

The interaction between depolarization and oxygen free radicals are not clear. It is well known that in the case of acute myocardial infarction or ischaemia, there is a marked increase in the extracellular potassium-ion concentration, $[\text{K}^+]_o$, and the resulting membrane potential of the ventricular muscle in the infarcted area is remarkably depolarized [44]. In the heart, the release of NA was induced by nerve depolarization [45,46]. Catecholamine release contributes to the formation of cytotoxic free radicals. K^+ depolarization enhances calcium ions (Ca^{2+}) overload by $[\text{K}^+]_o$ -induced depolarization and may generate $\bullet\text{OH}$ radicals in the myocardium. Although the precise mechanism of $\bullet\text{OH}$ generation in the ischaemic heart is obscure, we previously found concomitant increase of NA and $\bullet\text{OH}$ generation in myocardial injury [6].

This data are in agreement with the data that K^+ depolarization evokes $\bullet\text{OH}$ radicals [16]. However, the release of NA was induced by nerve depolarization [45]. NA may also have a deleterious effect on the myocardium by serving as a source of free radicals [47]. It has been suggested that free radical production may mainly contribute to certain stages in the progression of the injury [22]. Oxygen-derived free radicals are thought to be responsible for post ischaemic reperfusion injury [4,48]. Although controversial, a considerable effect on ischaemia/reperfusion exists [49] to support the notion that oxygen radicals play a role in ischaemia/reperfusion injury.

Calcium overload and $\bullet\text{OH}$ generation

Calcium ions (Ca^{2+}) might have been a very important factor in the induction of irreversible ischaemic injury [50]. Intracellular Ca^{2+} overload is then considered to lead to cell death under physiological conditions such as ischaemia and reperfusion injury [51]. Several experimental studies have shown that oxygen-derived free radicals contribute to myocardial damage induced by ischaemic and reperfusion injury [52]. The occurrence of intracellular Ca^{2+} plays a key role in cardiac dysfunction. Intracellular Ca^{2+} level builds and catalyzes the conversion of xanthine dehydrogenase to XO [53], which produces superoxide. Ca^{2+} may be a very important factor in the induction of irreversible ischaemic injury [54]. However, the interaction between intracellular Ca^{2+} overload and oxygen free radicals in myocardium is not clear. Intracellular Ca^{2+} level has been proposed as a source of oxidative toxicity [55].

Elevation of intracellular Ca^{2+} levels causes the conversion of xanthine dehydrogenase to XO [53,56], which produces superoxide. Superoxide, either directly or after conversion to $\bullet\text{OH}$ [57], damages the biological membranes [58] and other cellular components, including DNA [59], resulting in cell death. The ischaemia is attributed to the occurrence of intracellular Ca^{2+} overload [60]. Intracellular Ca^{2+} overload has been reported to be one of the causes of ischaemia reperfusion injury [61]. Although the participation of $\text{Na}^+-\text{Ca}^{2+}$ exchange in the mechanism of intracellular Ca^{2+} elevation has been suggested [62], detailed information about the relationship between the status of $\text{Na}^+-\text{Ca}^{2+}$ exchange and oxygen free radical formation has not been available. Free radical formation may be attributed to the free radical formation by Ca^{2+} [63].

Effect of activation of ATP sensitive K^+ (K_{ATP}) channels on hydroxyl radical production

Numerous mechanisms have been proposed to explain the cardioprotective action of the activation of K_{ATP} channels, including vasodilatation of coronary arteries and collateral vessels, inhibition of platelet aggregation, and inhibition of the release of oxygen free radicals. Glibenclamide has been used as a pharmacological tool to block selectively the K_{ATP} channels [64]. Qian et al. [65], demonstrated that glibenclamide blocked cardioprotection conferred by ischaemic preconditioning in the heart. However, Thornton et al. [66], found that glibenclamide, given intravenously to rabbits did not abolish myocardial preconditioning. Thus it appears that controversy exists as to the role of K_{ATP} channels in mediating cardioprotection. Tokube et al. reported [67], that opening of cardiac K_{ATP} channels is induced by oxygen free radicals produced via xanthine oxidase reaction. However, the relationship between the opening of cardiac K_{ATP} channels and oxygen free radicals in myocardium is not clear. It is well known that myocardial ischaemia increases interstitial K^+ concentrations of compromised ventricular muscles and decreases the resting membrane potential, leading to the slow conduction and ventricular tachyarrhythmias [68]. Although interaction between K_{ATP} channels and oxygen free radicals in the myocardium is discussed, increased intracellular Ca^{2+} concentrations cause superoxide production [69]. It is known that Ca^{2+} overload by K^+ induced depolarization may generate $\bullet\text{OH}$ radicals. Although the mechanism of the action of K^+ channel activators in subsequent $\bullet\text{OH}$ production is obscure, K^+ channel activators were assessed by recording $\bullet\text{OH}$ formation in in vivo hearts. Rat hearts, cromakalim and nicorandil both increased ROS, measured with microdialysis probe perfused with sodium salicylate, which reacts with $\bullet\text{OH}$ radicals produced in the muscle to yield a production of 2,3-DHBA [70]. EC_{50} of cromakalim and nicorandil were approximately 10 μM and 1 mM, respectively [70]. Therefore, cromakalim may be a more potent K^+ channel activator. Opening K_{ATP} channels significantly increased the level of 2,3-DHBA.

K_{ATP} channel openers can cause release of catecholamine, irrespective of their ability to open K_{ATP} [71,72]. The sustained elevation of NE in the extracellular fluid by K_{ATP} channel opener can be auto-oxidized, which in turn, leads (possibly by an indirect

mechanism) to the formation of cytotoxic $\bullet\text{OH}$ radicals. Activating K_{ATP} channel on nerve terminal may facilitate NA release. Activation of K_{ATP} channels was proposed to offer a protective effect on the ischaemia reperfused myocardium [73]. It is likely that K_{ATP} channels play, at least, in part a role to protect the heart during episodes of ischemia. Although the relationship between K_{ATP} channels and cardioprotective effect was obscure, glibenclamide decreased the production of $\bullet\text{OH}$ by preventing the opening of K_{ATP} channels. The $\bullet\text{OH}$ generation by cromakalim was suppressed by subsequent treatment with glibenclamide. Glibenclamide decreases the level of 2,3-DHBA [70]. Cardioprotection mediated by attenuated free radical generation may be mitigated or abolished in the presence of K_{ATP} channel blockers. 5-Hydroxydecanoate (5-HD), a selective type of K_{ATP} channel blocker [74] also decreased cromakalim-induced 2,3-DHBA formation. Moreover, another K_{ATP} channel activator, nicorandil, also enhanced $\bullet\text{OH}$ formation. Both glibenclamide and 5-HD also decreased nicorandil-induced 2,3-DHBA formation [70].

Several experimental studies have shown that oxygen radicals contribute to myocardial damage induced by the ischemia-reperfusion [6,75]. When the ischaemic arrhythmia appeared, the presence of free radicals was observed in the ischaemic-reperfused rat heart [6]. Activation of K_{ATP} channel on ischemia-reperfusion of myocardium may be cause release of NA. However, in the presence of glibenclamide or 5-HD, the elevation of 2,3-DHBA was not observed in ischaemic-reperfused rat heart. Glibenclamide is associated with a cardioprotective effect due to suppression of ischemia-reperfusion-induced $\bullet\text{OH}$ generation. It is possible that opening of K_{ATP} in the cardiac nerve terminals facilitates the release of catecholamines, which the increase ROS production to trigger the protection [76]. Cardioprotection mediated by these mechanisms may be mitigated or abolished in the presence of glibenclamide or 5-HD. Although it is well established that high levels of ROS are detrimental [77], moderate levels of H_2O_2 and O_2^- have been shown to elicit a cardioprotective effect similar to that observed with ischaemic preconditioning [78,79]. Forbes et al. [80], proposed that cardioprotective actions of diazoxide, a selective opener of the mitochondrial K_{ATP} channel, are mediated by generation of pro-oxidant environment. Pain et al. [81], also reported that the dioxide-induced reduction infarct size is attenuated by addition of antioxidants. These findings are agreement with that the assumption that both mitochondrial and sarcolemmal channels induced $\bullet\text{OH}$ formation [70].

Prazosin attenuate $\bullet\text{OH}$ generation

Prazosin has been demonstrated to exhibit a protective effect on myocardial ischaemic injury [82]. It is known that prazosin has a Na^+ channel blocking action as well as an α_1 -adrenoceptor blocking action [83]. Reactive oxygen causes excessive Na^+ entry through the fast Ca^{2+} channel, leading to intracellular Ca^{2+} overload through the Na^+ - Ca^{2+} exchange system, and hence myocardial damage [84]. Ca^{2+} overload is then mediated via activation of Na^+ - Ca^{2+} exchange due to increased cytosolic Na^+ content. Na^+ channel blocking drugs have been demonstrated to provide protection in ischaemia/reperfusion model [85]. Although the role of catecholamines in the myocardial cellular injury is unclear, NA was induced by ischemia/reperfusion [6,86]. NA released is thought to play a significant role in the etiology of various cardiac pathophysiological disorders [87]. Accumulation of endogenous NA can leads to the formation of cytotoxic $\bullet\text{OH}$ radicals. Radical scavengers or anti-oxidant substances have been shown to attenuate myocardial dysfunction [88,89]. Theoretically, $\bullet\text{OH}$ may be formed *in vivo* during non-enzymatic oxidation [23] and/or enzymatic oxidation of NA [90]. We demonstrated that tyramine (a catecholamine releaser)-induced $\bullet\text{OH}$ formation was attenuated by prazosin [91]. It is possible that Na^+ accumulation triggers Na^+ - Ca^{2+} exchange leading to Ca^{2+} overload. It is well known that Ca^{2+} overload may involve the formation of free radicals [26,92].

According to recent studies [83], prazosin has a Na^+ channel blocking action as well as the α_1 -adrenoceptor blocking action. Su et al. [93], have found that prazosin inhibits inward Na^+ current in the rat, guinea-pig and human myocardium and that this action of prazosin is unrelated to the blockade of α_1 -adrenoceptors. On the other hand, the reactive oxygen causes excessive Na^+ entry through the fast Na^+ channel, leading to intracellular Ca^{2+} overload through the Na^+ - Ca^{2+} exchange system, and hence myocardial damage [94]. The beneficial effect of prazosin on the H_2O_2 -induced derangements, therefore, may be due to its blocking effect on the Na^+ channel of the cardiac cells. This notion is in agreement with the data from a recent study, in which α_1 -adrenoceptor antagonistic action of prazosin may not contribute to its action to attenuate the free radical induced damage, although the possibility of contribution of α_1 -adrenoceptor blockade cannot be completely excluded [95].

Intracellular extracellular Ca^{2+} overload is considered to be the pathway leading to cell death in several pathological condition. Increases in intracellular Na^+ levels could be caused by modified Na^+ channel function [94,96]. Contribution of the Na^+ channel blocking activity of prazosin to attenuate the ischaemia/reperfusion induced damage remain to be studied. During myocardial ischaemia, reduced ATP production generates disturbances in intracellular ion homeostasis, which ultimately lead to cellular Ca^{2+} overload and contractile failure. Ca^{2+} overload is then considered to be maintained via activation of Na^+ - Ca^{2+} exchange due to increased cytosolic Na^+ content under ischaemic conditions. Intracellular Ca^{2+} overload is then considered to contribute to cell death under pathological conditions such as ischaemia/reperfusion injury [97]. These results suggest that Na^+ -mediated Ca^{2+} overload may be relevant to ischaemia/reperfusion Ca^{2+} overload [94]. This is a new cardioprotective principle.

Nitric oxide induces $\bullet\text{OH}$ generation

Nitric oxide (NO) is responsible for tissue damage during ischaemia. NO is a free radical which regulates a variety of biological function and pathogenesis of cellular injury. NO is synthesized from L-arginine by NO synthase (NOS) [98]. It is known that O_2^- and NO rapidly to form the stable peroxynitrite (ONOO^-) and then its decomposition generates $\bullet\text{OH}$ [99,100]. However, this theory is under consideration [101]. NO may mediate $\bullet\text{OH}$ -induced ischaemia/reperfusion via depolarization of the ventricular muscle. Cytotoxic free radicals such as ONOO^- and $\bullet\text{OH}$ may also be implicated in NO-mediated cell injury [99]. It is known that NOS inhibition may inhibit depolarization-induced NOS activation by Ca^{2+} influx through blockade of Na^+ and Ca^{2+} channels [102]. The reactive oxygen species causes excessive Na^+ entry through the fast Na^+ channel, leading to intracellular Ca^{2+} overload through the Na^+ - Ca^{2+} exchange system [94]. However, NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, attenuate $\bullet\text{OH}$ generation by ischaemia/reperfusion [27]. It is possible that NO mediates ischaemia/reperfusion induced $\bullet\text{OH}$ generation via depolarization in ventricular muscle. Accordingly to the reaction pathway in (Figure 3). $\bullet\text{OH}$ was generated by the presence of NOS and O_2^- . NO may possibility induce $\bullet\text{OH}$ generation after reaction with O_2^- and NO. NO mediates $\bullet\text{OH}$ generation via NOS inhibition.

Controversy exist concerning possible neurotoxicity and/or neuroprotective roles of NO, which may prevent $\bullet\text{OH}$ -induced oxidative injury [103,104]. Moreover, iron complex-induced $\bullet\text{OH}$ generation and associated oxidative cellular injury was blocked by the co-administration of NO and dihydrolipoic acid (a potent antioxidant compound). Some NO donor (e.g., sodium nitroprusside) stimulated, while others (nitroglycerin, diethylamine/NO, NO in Ringer's solution) suppressed $\bullet\text{OH}$ generation *in vivo* [105]. Exogenous NO can act as $\bullet\text{OH}$ scavenger and protect neurons from oxidative injury [106].

LDL oxidation and $\bullet\text{OH}$ generation

Several experimental studies have shown that oxygen radical contributes to myocardial damage induced by ischaemia/reperfusion [6,107]. It is well known that

ischaemia induces depolarization [108,109]. NO may mediate ischaemia/reperfusion-induced $\bullet\text{OH}$ generation via depolarization in ventricular muscle. NO is responsible for tissue damage during ischaemia. L-NAME (NG-nitro-L-arginine methyl ester, a NOS inhibitor) attenuated $\bullet\text{OH}$ generation by ischaemia/reperfusion of rat heart [27]. It is known that NOS inhibition prevents depolarization-induced NOS activation by affecting Ca^{2+} influx through blockade by $\text{Na}^+/\text{Ca}^{2+}$ channel [102]. Oxidative modification of low-density lipoprotein (LDL) is thought to contribute to the production of oxygen-derived free radicals [110]. Oxidative LDL (Ox-LDL) may be important in neurotoxicity in the brain [111]. It is well known that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors reduce the oxidizability of LDL [112]. The inhibitory effect on the oxidizability of LDL oxidation can reduce $\bullet\text{OH}$ formation. The blockage of LDL oxidation by fluvastatin (an inhibitor of LDL oxidation) can reduce $\bullet\text{OH}$ generation. However, L-NAME did not affect NA-induced $\bullet\text{OH}$ formation. Fluvastatin is associated with a cardioprotective effect due to the suppression of NA-induced $\bullet\text{OH}$ formation by inhibiting LDL oxidation [27]. LDL oxidation may be related to NA-induced $\bullet\text{OH}$ generation, but LDL oxidation may be unrelated $\bullet\text{OH}$ generation K^+ depolarization-induced $\bullet\text{OH}$ generation [27].

Effect of histidine on the formation of $\bullet\text{OH}$ in ischaemic injury

Histidine, a free amino acid, has been demonstrated to have an antioxidative action [113,114]. Although histidine is known to scavenge singlet oxygen ($^1\text{O}_2$) [115,116], the effect of histidine on ischaemic reperfusion injury still remains uncertain. Histidine is also a chelator of metal ions. Therefore, histidine could remove transition metals from the reactive site, resulting in a markedly decreased rate of $\bullet\text{OH}$ formation [117].

Ischaemia-reperfusion increased the level of NA. However, in the presence of histidine, ischaemia-reperfusion failed to increase the level of NA. Moreover, when the ischaemic arrhythmia appeared, the presence of free radicals was observed in the ischaemic-reperfused rat heart [6]. However, neither typical changes in Electrocardiogram (ECG) nor $\bullet\text{OH}$ generation in the ischaemic-reperfused rat heart was observed after administration of histidine [29]. Histidine is a poor scavenger of $\bullet\text{OH}$. Unfortunately, it is difficult to separate $\bullet\text{OH}$ radical from that by $^1\text{O}_2$ because of the nonspecificity of histidine in scavenging both of the oxidant species. In addition, most of the $^1\text{O}_2$ scavengers react with $\bullet\text{OH}$ radical, often with a greater rate constant than the reaction with $^1\text{O}_2$. Histidine appears to scavenge most O_2 species, as indicated by the present and previous studies [118]. More, Feix and Kalyanaraman [119] reported that $^1\text{O}_2$ could also attack salicylic acid to form 2,3-DHBA and 2,5-DHBA, which implies that salicylic acid is not a specific trapping agent for $\bullet\text{OH}$, but can also trap $^1\text{O}_2$. Histidine is thus an excellent scavenger of reactive O_2 species in vivo conditions. Histidine protects myocardium against ischaemia-reperfusion-induced $\bullet\text{OH}$ generation.

Conclusion

In vivo microdialysis techniques enable monitoring of generation of free radicals in myocardial tissues and could answer fundamental questions about the clinical implications of ROS. Data obtained in human are needed with the conduction of clinical studies investigating the effects of medications in physiological *in vivo* conditions. Although the cardioprotective effect of NO is discussed, NO also has a role in the pathogenesis of cellular injury. These experiments with cardiac microdialysis technique have versatile applications and offer new possibilities for the *in vivo* study of cardiac physiology.

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