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High-performance liquid chromatographic detection of hydroxylated benzoic acids as an indirect measure of hydroxyl radical in heart: its possible link with the myocardial reperfusion injury

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ABSTRACT

The present report describes a method suitable for the indirect assay of hydroxyl radical ($OH^{\cdot}$), which is likely to be produced during reperfusion of ischemic myocardium. Isolated rat heart perfused by the Langendorff technique was subjected to 30 min of ischemia, followed by 30 min of reperfusion. Salicylic acid (2 m*M*) was added to the perfusion circuit to trap any $OH^{\cdot}$ radical generated during the experiment. 2,5- and 2,3-dihydroxybenzoic acids (hydroxylated products of salicylic acid) were identified by authentic standards as well as by pure $OH^{\cdot}$-generating system using high-performance liquid chromatography with electrochemical detection. In addition to serving as a chemical trap for the detection of $OH^{\cdot}$, salicylate attenuated myocardial reperfusion injury as evidenced by reduced formation of creatine kinase, decreased lipid peroxidation, and improved myocardial contractile functions during reperfusion. These results thus provide direct evidence for the presence of $OH^{\cdot}$ in heart and link it to the myocardial reperfusion injury.

INTRODUCTION

Oxygen-derived free radicals have been linked to ischemic and reperfusion injury in a variety of tissues including heart [1–5]. Once these free radicals are formed, they presumably attack the polyunsaturated fatty acids of membrane phospholipids and cause lipid peroxidation [6,7], which has been accepted as an indirect, yet presumptive, marker for free radical production [8–10]. Recently, electron paramagnetic resonance spectrometry (EPR) has been used to directly demonstrate the presence of these free radicals. To date, the EPR method remains the only method of choice for this purpose [11–14].

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However, there are certain disadvantages regarding the use of EPR. First, it is very expensive and is not readily available to many investigators. Second, chemical interconversions between spin-trap radical adducts occasionally lead to confusion in properly identifying radical species. Third, interpretation of the data is extremely difficult and frequently computer assistance is essential. Finally, and most importantly, oxygen free radicals may be generated in biological tissues in such small quantities that they are overpowered by other free radical signals, making the proper identification difficult.

During recent years, a number of investigators, including Floyd *et al.* [15], Grootveld and Halliwell [16], and Pritsos *et al.* [17], described high-performance liquid chromatography (HPLC) methods to detect oxygen-free radicals. These methods are easily adaptable, and some of them are extremely sensitive in detecting very low levels of hydroxyl radical ($OH^{\bullet}$). We have adapted one of these methods using salicylate to trap $OH^{\bullet}$ and subsequent analysis of the hydroxylated products by HPLC using an electrochemical detector as our method of choice to confirm the role of $OH^{\bullet}$ in myocardial ischemic and reperfusion injury.

MATERIALS AND METHODS

Materials

All chemicals used in this study were of analytical grade. Salicylic acid and its hydroxylated derivatives, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), were obtained from the Aldrich (Milwaukee, WI, U.S.A.). Dimethyl thiourea (DMTU), dimethyl sulfoxide (DMSO), hypoxanthine, and xanthine oxidase (XO) were all purchased from Sigma (St. Louis, MO, U.S.A.). Water was purified with a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.). Chelex resin was obtained from Bio-Rad (Richmond, CA, U.S.A.). All of the solutions were made up in Chelex-treated Milli-Q water, unless mentioned otherwise.

Methods

Preparation of isolated heart. Male Sprague-Dawley rats weighing 250–300 g were anesthetized with an intravenous injection of sodium pentobarbital (6.5 mg/100 g). Hearts were removed and perfused with Krebs-Henseleit bicarbonate buffer equilibrated with a gas mixture of 95% oxygen and 5% carbon dioxide (pH 7.4), using Langendorff's non-recirculating mode [18]. Heart was perfused for 15 min at 37°C in the presence of 2 m*M* salicylate at 100 cm water perfusion pressure for stabilization. Flow was reduced to about 0.1 ml/min, and the heart was rendered ischemic for 30 min. Following 30 min of ischemia, reperfusion was performed for 30 min with non-recirculating Krebs-Henseleit bicarbonate buffer containing 2 m*M* salicylate.

To monitor the myocardial functions during the experiment, a polyethylene catheter was inserted into the left ventricle through the apex. This, in turn, was connected to a Model P23 pressure transducer (Gould Inc., Oxnard, CA, U.S.A.) to measure left ventricular developed pressure (LVDP) and its first derivative ($LVdp/dt$), as described previously [5].

Myocardial cellular injury was monitored by assaying the release of creatine kinase (CK) from heart. CK was assayed enzymatically using an assay kit obtained from Sigma [5].

The extent of lipid peroxidation was measured in the perfusate buffer by assaying malondialdehyde (MDA) formation as described previously [10]. In brief, 1 ml of perfusate was treated with 1 ml of ice-cold 30% $HClO_4$ and 1 ml of 0.75% thiobarbituric acid (TBA) dissolved in 0.5% sodium acetate. The samples were boiled for 20 min and centrifuged to remove the pellet. The color of the supernatant was read at 535 nm. The concentration of the supernatant was measured using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

To examine whether the generated free radical was indeed $OH^{\cdot}$, parallel experiments were performed by simultaneously adding a specific scavenger of $OH^{\cdot}$ (deferoxamine) along with salicylate. The experiments were terminated at different points: prior to ischemia, after 30 min of ischemia, and after 1, 2, 5, 15 and 30 min of reperfusion. At least ten experiments were performed for each time frame. Perfusates and hearts were processed as described below.

Subcellular fractonation of heart. Hearts were homogenized in Krebs-Henseleit bicarbonate buffer using a Polytron homogenizer (Brinkman, NY, U.S.A.). The homogenate was centrifuged at 100 *g* to remove nuclear debris. The supernatant was then centrifuged at 10000 *g* to collect mitochondria. Mitochondria-free supernatant was centrifuged at 100000 *g* to precipitate microsomal fraction. Mitochondria (500–6500 *g*) and microsomes (10000–100000 *g*) were further purified by repeated precipitation and washing as described elsewhere [19]. Marker enzymes were assayed to judge the purity of the preparation [20]. Nuclear fraction was purified by sucrose density gradient as described previously [20].

Analyis of hydroxylated benzoic acids by HPLC. Each subcellular fraction as well as whole homogenate were suspended in 1 ml Tris–sucrose buffer and 800 μl homogenate was treated with 40 μl 3 *M* hydrochloric acid. The precipitate was removed by centrifugation and the supernatant was filtered though Rainin nylon-66 membrane filter (0.22 μm pore size) (Rainin, Woburn, MA, U.S.A.). To examine the presence of hydroxylated benzoic acids in the perfusate buffer, 1 ml of the perfusate was similarly treated. A 20-μl volume of the sample was injected onto an Altex Ultrasphere 3 μm ODS (75 mm × 4.6 mm) (Rainin) equipped in a Waters Assoc. (Millford, MA, U.S.A.) HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. 2,5-DHBA and 2,3-DHBA (hydroxylated products of salicylic acid after interaction with $OH^{\cdot}$ were eluted by buffer containing 0.03 *M* sodium acetate and 0.03 *M* citric acid (pH 3.6) at a flow-rate of 1 ml/min [21]. The detection potential was maintained at 0.6 V, employing the Ag/AgCl reference electrode. Peaks were identified by authentic standards as well as by injecting the hydroxylated products of salicylic acid from a pure $OH^{\cdot}$-generating system [7].

Statistical analysis. For comparison between two groups, the Student's *t*-test was used for the statistical analysis. For the comparison between several independent groups, analysis of variance followed by Scheffe's test was performed. Results were considered significant when $p < 0.05$.

RESULTS

Generation of $OH^{\cdot}$ during reperfusion of ischemic myocardium

Whole heart as well as each subcellular fraction were subjected to HPLC for the detection of $OH^{\cdot}$ radical by measuring hydroxylated benzoic acids. Both heart (Fig. 1)

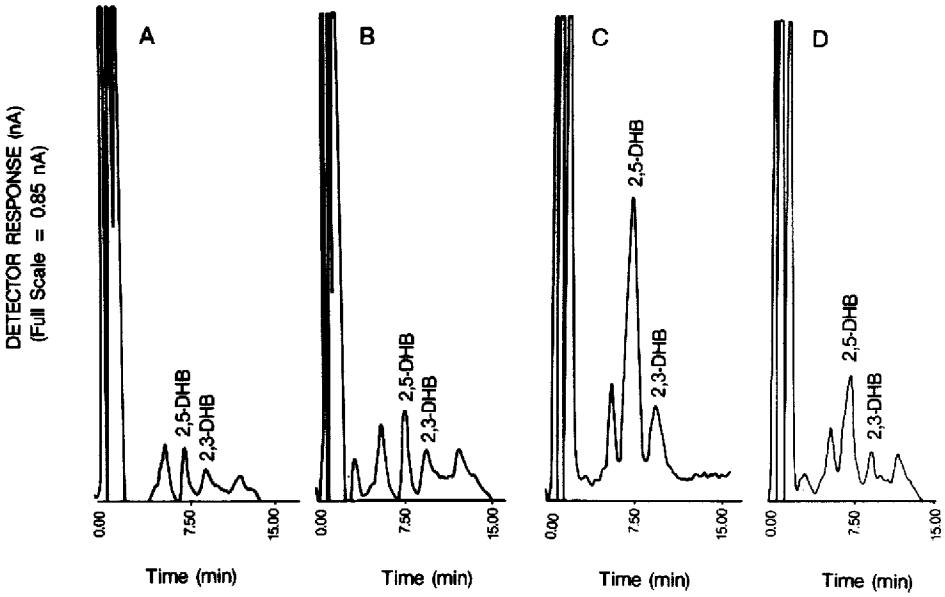

Fig. 1. Indirect detection of OH$^{\cdot}$ as 2,3- and 2,5-dihydroxybenzoic acid (DHB) in the ischemic and reperfused rat heart. (A) Isolated rat heart was perfused in the presence of 2 m*M* salicylate for 30 min; (B) Isolated rat heart was perfused in the presence of 2 m*M* salicylate for 15 min, and then the heart was made ischemic for 30 min; (C) Isolated rat heart was preperfused for 15 min, followed by 30 min of ischemia and 2 min of reperfusion. Salicylic acid (2 m*M*) was added to the perfusion circuit after ischemia and at the onset of reperfusion; (D) Isolated rat heart was preperfused for 15 min, followed by 30 min of ischemia. Ischemic heart was then reperfused for a further period of 2 min with a buffer containing 2 m*M* salicylate and 0.6 m*M* deferoxamine.

and mitochondrial fraction (not shown) demonstrated peaks during reperfusion corresponding to OH$^{\cdot}$ (Table I). The intensity of the 2,3- and 2,5-DHBA signals was higher in the mitochondria compared to that for the whole heart. The cell-free cytosolic fraction also exhibited a very small peak corresponding to OH$^{\cdot}$ (not shown). The peaks corresponding to the 2,3- and 2,5-DHBA signals were reduced significantly when hearts were perfused in the presence of deferoxamine, which inhibits OH$^{\cdot}$ formation by chelating free iron. This suggests that the DHBA signals generated in the reperfused hearts were indeed due to the formation of OH$^{\cdot}$.

We also examined the perfusate for the presence of 2,3- and 2,5-DHBA. In concert with the heart, perfusates from reperfused heart also demonstrated the presence of hydroxylated benzoic acid peaks when analyzed by HPLC (Table I). The intensities of these peaks, however, were smaller compared to those found in heart. These peaks were also completely abolished when hearts were preperfused in the presence of deferoxamine.

Analysis of OH$^{\cdot}$-salicylate reaction products by HPLC

To further confirm the identity of the hydroxylated benzoic acid signals found in heart and in perfusate samples, we produced pure OH$^{\cdot}$ radical from the reaction of hypoxanthine (100 μM), XO (8 mU),$FeCl_3$ (100 μM), and EDTA (100 μM). When these reactions were carried out in the presence of salicylate (2 m*M*) and injected into

TABLE I

GENERATION OF OH$^{\cdot}$ IN ISCHEMIC MYOCARDIUM AS A FUNCTION OF THE DURATION OF REPERFUSION

Results are expressed as means ± S.E. of at least 5 experiments in each group. Generation of OH$^{\cdot}$ is measured indirectly by measuring 2,3- and 2,5-DHBA using HPLC.

	Concentration of 2,3- and 2,5-DHBA (nmol/ml)				
	Duration of reperfusion (min)				
	1	2	5	15	30
Whole heart	0.69 ± 0.07	0.63 ± 0.05	0.47 ± 0.09	0.31 ± 0.06	0.18 ± 0.03
+ deferoxamine	0.16 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	0.8 ± 0.01	0.08 ± 0.01
Mitochondrial fraction	2.11 ± 0.44	2.0 ± 0.39	1.65 ± 0.25	0.82 ± 0.17	0.30 ± 0.09
+ deferoxamine	0.11 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.07 ± 0.01	0.08 ± 0.02
Perfusate	0.28 ± 0.05	0.27 ± 0.06	0.17 ± 0.04	0.09 ± 0.02	0.08 ± 0.02
+ deferoxamine	0.07 ± 0.02	0.07 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.02

the HPLC system, peaks for hydroxylated benzoic acids emerged at the identical positions having the same retention times (Fig. 2). Since OH$^{\cdot}$ reacts with salicylic acid and produces 2,3-DHBA and 2,5-DHBA, we also analyzed the authentic standards of

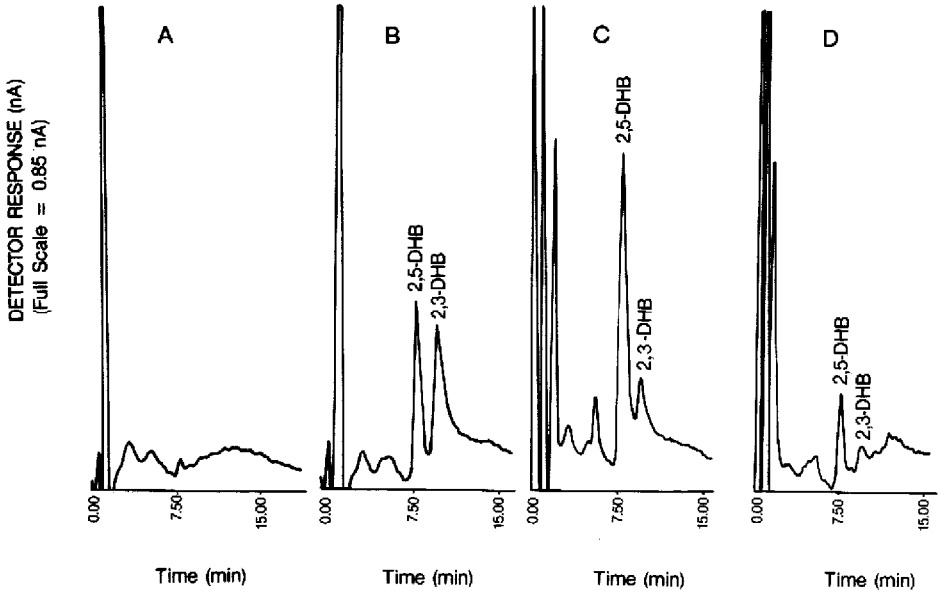

Fig. 2. Indirect *in vitro* detection of OH$^{\cdot}$ radical as 2,3- and 2,5-dihydroxybenzoic acid (DHB) by the use of HPLC. (A) 25 μl of reaction blank: hypoxanthine + $FeCl_3$ + EDTA + salicylate; (B) 25 μl of 125 μM 2,5-DHB and 250 μM 2,3-DHB authentic standards; (C) 25 μl of chemically generated OH$^{\cdot}$ (hypoxanthine + xanthine oxidase + $FeCl_3$ + EDTA) + salicylate; (D) Same reaction mixture as C + 0.6 mM deferoxamine.

these two hydroxylated benzoic acids. As show in Fig. 2, these two standards had identical retention times. Adding deferoxamine to the OH•-generating system removed these peaks completely. We also spiked the hydroxylated benzoic acid peaks obtained from the heart extract with authentic standards. The results shown in Fig. 3 demonstrate that these peaks indeed are identical with 2,5-DHBA and 2,3-DHBA.

Generation of OH• as a function of duration of reperfusion

Since oxygen free radicals are known to appear at the onset of reperfusion, we examined the presence of OH• by measuring 2,3- and 2,5-DHBA as a function of reperfusion time. As expected, OH• was reduced as reperfusion progressed (Table I). A slight reduction of hydroxylated benzoic acids was noted after 2 min of reperfusion in heart as well as in its mitochondrial fraction. Reduction of hydroxylated benzoic acid signals was significant after 15 and 30 min of reperfusion in both whole heart and mitochondria. The intensity of hydroxylated benzoic acids in the perfusate followed a different pattern. The amount of hydroxylated benzoic acids remained unchanged after 15 min of reperfusion. No hydroxylated benzoic acid signal was recovered from hearts after 30 min of reperfusion.

Effects of salicylate on myocardial preservation

Since the release of CK directly relates to cellular injury, the perfusate from perfused heart was assayed for the presence of CK activities. Release of this enzyme was markedly increased during reperfusion in both groups, as expected (Table II).

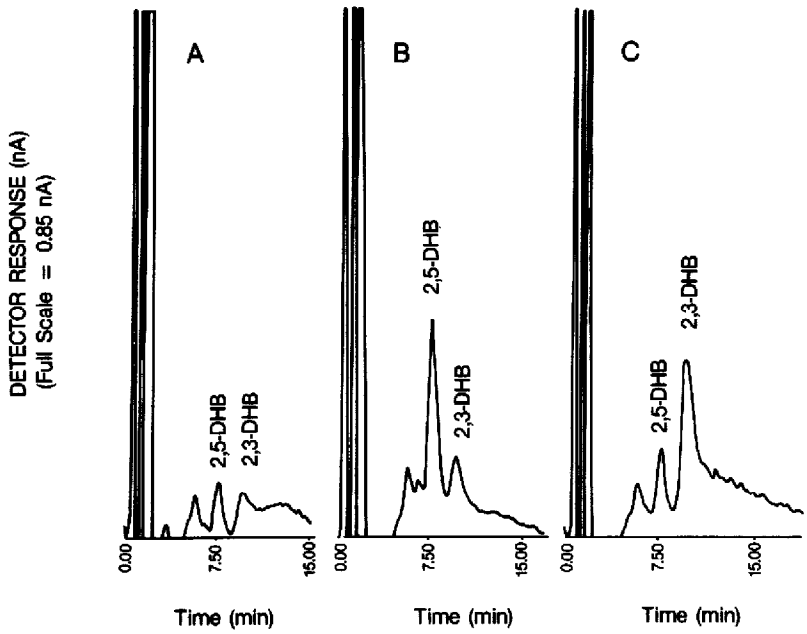

Fig. 3. 2,3- and 2,5-dihydroxybenzoic acid (DHB) in reperfused heart in the presence of spiked standards. (A) Isolated rat heart was perfused for 15 min in the presence of 2 m*M* salicylate; (B) Same as A, but spiked with 2,5-DHB authentic standard; (C) Same as A, but spiked with 2,3-DHB authentic standard.

TABLE II

EFFECTS OF SALICYLATE ON MYOCARDIAL CELLULAR INJURY, LEFT VENTRICULAR FUNCTIONS AND LIPID PEROXIDATION DURING ISCHEMIA AND REPERFUSION

Results are expressed as means ± S.E. of seven experiments in each group.

	CK Release[a] (IU/l)		LVDP[b] (mmHg)		LV Max dp/dt[b] (mmHg/s)		MDA[a] (nmol/g)	
	−sal[b]	+sal	−sal	+sal	−sal	+sal	−sal	+sal
Baseline	0.05 ± 0.04	0.07 ± 0.05	95 ± 2	92 ± 6	1298 ± 75	1177 ± 101	21.5 ± 2.2	20.3 ± 1.9
30 min ischemia	11 ± 2.5	10 ± 2.7	59 ± 7	57 ± 9	1005 ± 118	987 ± 135	24.1 ± 3.5	23.6 ± 2.8
30 min reperfusion	32 ± 5.2	17[c] ± 3.4	55 ± 4	68[c] ± 3	742 ± 89	1169[c] ± 78	62.7 ± 9.4	38.0[c] ± 7.6

[a] CK = Creatine kinase; MDA = malondialdehyde.

[b] LVDP = Left ventricular developed pressure; LV max dp/dt = maximum first derivative of left ventricular developed pressure; sal = salicylic acid.

[c] $p < 0.05$ compared to corresponding untreated group.

However, the amount of CK release was significantly lower in the salicylate group compared to the untreated control group.

Similarly, both LVDP and LV$dp/dt$ were decreased after ischemic insult and during reperfusion. Again, reduction in the left ventricular functions was sigificantly inhibited by salicylic acid, indicating the ability of salicylic acid to preserve myocardial contractile functions.

Malondialdehyde formation, a presumptive marker for lipid peroxidation, was also enhanced during reperfusion of ischemic myocardium in both groups. Similar to other parameters malondialdehyde formation was also lower in the salicylic acid group, suggesting that this compound interfered with $OH^{\cdot}$–lipid interaction.

DISCUSSION

Reports linking the oxygen-derived free radicals with myocardial ischemic and reperfusion injury are overwhelming [1–5]. However, direct evidence demonstrating oxygen free radicals as mediators of reperfusion injury has yet to be shown. Most of the evidence is based on the fact that reperfusion of ischemic myocardium is associated with the formation of malondialdehyde, a presumptive marker for lipid peroxidation [6,7], and that scavengers of free radicals such as superoxide dismutase (SOD) and catalase can attenuate reperfusion injury [22–24]. Although spin-trapping of the free radicals and subsequent determination by EPR confirmed their presence in reperfused heart [11–14], direct demonstration of linking these free radicals with reperfusion injury is not possible by this technique. Spin-trapped free radicals are still extremely short-lived, and they must be handled in a special way in order to show their presence with EPR.

The method described in this study utilized a chemical trap, such that the free radical interaction products are extremely stable and can be subjected to various treatments in order to study their direct role in reperfusion injury. The results of our study confirmed the previous reports concerning the presence of $OH^{\cdot}$ in the ischemic-reperfused heart and further demonstrated their presence in the perfusate, suggesting that $OH^{\cdot}$ is indeed formed during reperfusion of ischemic myocardium. In addition, this study demonstrated for the first time the presence of $OH^{\cdot}$ in the mitochondrial fraction of heart. The reduction of reperfusion injury by salicylic acid with the simultaneous presence of hydroxylated products strongly indicates a direct link of $OH^{\cdot}$ with myocardial reperfusion injury.

A substantial body of evidence now suggests that O_2^- produced initially from molecular oxygen undergoes dismutation, forming hydrogen peroxide as indicated below:

$$O_2 + e^- \rightarrow O_2^- \quad (1)$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (2)$$

The O_2^- was thought to undergo a Haber-Weiss reaction in the presence of hydrogen peroxide, forming $OH^{\cdot}$:

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot} \quad (3)$$

Although this reaction is thermodynamically feasible, and the overall stoichiometry is widely accepted under *in vitro* conditions, the reaction is kinetically very slow or even negligible. The modified reaction has been suggested to occur in two steps by iron-catalyzed Fenton-type reactions, as described below:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\cdot \quad (5)$$

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot \quad (4+5)$$

With present technology, it is virtualy impossible to identify O_2^- in an *in vivo* system, even if it may be produced in mitochondia. O_2^- has a extremely short half-life which rapidly undergoes dismutation, yielding hydrogen peroxide. O_2^- than undergoes a Fenton-type reaction catalyzed by a metal ion such as iron(III). The exact source of iron to catalyze this reaction remains highly speculative [25]. A recent study from our laboratory suggested oxygen-carrying heme proteins as a potential source of iron [26].

Chemically generated $OH^\cdot$ radical (Fenton system), when allowed to react with salicylate, produces three different products: catechol, 2,3-DHBA, and 2,5-DHBA [16]. The hydroxylated benzoic acids can be detected in femtomoles using the above-described HPLC technique [15]. The rate constant of the interaction between $OH^\cdot$ and salicylate is about $5 \cdot 10^9$ to 10^{10} M^{-1} s^{-1} [27]. Because we used an isolated rat heart weighing approximately 1 g, 2 m*M* salicylic acid is expected to react with most of the $OH^\cdot$ formed in this system. Our results not only showed the presence of $OH^\cdot$ in the reperfused myocardium, but it further demonstrated that the mitochondrial fraction was the locus of free radical production. The intensity of generated $OH^\cdot$ drops as reperfusion progresses. The presence of hydroxylated benzoic acid products in the perfusate further suggests that $OH^\cdot$ is indeed formed in the heart during reperfusion, and that it leaches out of heart as a result of the washout.

The attenuation of reperfusion injury as indicated by the reduction of CK release and better preservation of myocardial contractile functions suggests that salicylic acid itself may have some cardioprotective effect. Furthermore, it reduced the extent of lipid peroxidation, indicating its effectiveness in interfering with free radical–lipid interactions. Salicylic acid is a well-known anti-inflammatory agent which functions by inhibiting cyclooxygenase pathway [28]. It is possible that the beneficial effects of salicylate observed during reperfusion of ischemic myocardium are due to its anti-inflammatory actions. However, at a rate constant on the order of $5 \cdot 10^9$ to 10^{10} M^{-1} s^{-1}, salicylate is likely to react with $OH^\cdot$ before it attacks any other compounds and functions by some other mechanisms. $OH^\cdot$ is extremely reactive, and it reacts with virtually almost any compound including lipids, proteins and nucleic acids. Breakdown of phospholipids and its peroxidation has been indicated in ischemic-reperfused myocardium [29]. Lipid peroxidation is presumably one of the major causes for myocardial dysfunction observed during reperfusion.

The major finding of this study is that salicylate can be used as a chemical trap to demonstrate the presence of $OH^\cdot$ in the pathogenesis of myocardial reperfusion injury. The attenuation of reperfusion injury with salicylate in concert strongly suggests a direct link of $OH^\cdot$ as a mediator of reperfusion injury.

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