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COMMENTARY
SALICYLATE TRAPPING OF ·OH AS A TOOL FOR STUDYING POST-ISCHEMIC OXIDATIVE INJURY IN THE ISOLATED RAT HEART

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The use of salicylate as a chemical trap for ·OH represents a simple and convenient alternative to the use of spin trapping techniques to study oxidative injury in isolated perfused organs. In these systems, salicylate is included in the perfusion buffer at concentrations ranging from 0.1 to 2 mM depending on the detection apparatus employed. In our studies, we have used a coulometric detector, which has a theoretical efficiency of 100% as compared to 1-5% for the standard glassy carbon electrode. We have been able to generate reproducible results by inclusion of only 100 μM salicylate, a concentration demonstrated not to affect pre- or post-ischemic cardiac function. In initial studies, we observed an increase in perfusate 2,5-dihydroxybenzoic acid consistent with an early post-ischemic burst of ·OH, not unlike that reported using spin trapping techniques. Since then we and others have used this technique to examine possible relationships between ·OH formation and treatments that alter post-ischemic cardiac functional recovery. For example, preischemic loading of hearts with copper results in increases in post-ischemic dysfunction and LDH release that were associated with an increase in 2,5-dihydroxybenzoate and by inference, ·OH formation. Alternatively, we have reported that the nitroxide spin label, TEMPO, reputed to be a superoxide dismutase mimetic, decreased post-ischemic arrhythmias and 2,5-dihydroxybenzoate formation. Most recently, we have observed that preischemic loading of hearts with zinc-bis-histidinate results in improved post-ischemic cardiac function and decreased LDH release; changes that were associated with decreased 2,5-dihydroxybenzoate formation. These studies indicate that under certain conditions, salicylate is a valuable alternative to spin trapping techniques to probe the role of ·OH in cardiac oxidative injury, particularly when applied to the isolated perfused heart preparation.

KEY WORDS: Salicylate, hydroxyl radical, free radicals, heart, Langendorff, electrochemical detection.

INTRODUCTION

Free radicals and reactive oxygen intermediates (ROI) are thought to be involved in numerous pathologic and toxicologic disease states, such as aging,[1] radiation damage,[2,3] rheumatoid diseases,[4] and xenobiotic-induced tissue damage.[5] The last 15 years has seen publication of innumerable studies linking post-ischemic reperfusion tissue injury with overproduction of ROIs. These intermediates have been implicated in production of ischemic damage to the heart,[6-8] kidney,[9,10] brain,[11,12]

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liver,[13–15] pancreas,[16,17] skin[18,19] and intestine.[20,21] With the aid of electron paramagnetic resonance (ESR) spectroscopy in combination with spin trapping, it has become possible to detect the presence of free radicals in *in vitro*, *in vivo*, and *ex vivo* biological systems. In regards to reflow states, there have been numerous spin trapping studies in many different models, including isolated perfused hearts,[22,23] open and closed chest animal models,[24,26] and humans, *ex vivo*.[27,28] The *in vitro* spin trapping studies utilizing 5,5′dimethyl-pyrroline-N-oxide (DMPO) as the spin trap[22,23] have suggested the formation of ·OH during the early phases of reperfusion.[22] Formation of the DMPO-OH adduct occurred as a burst that peaked after about three minutes of reperfusion and was dependent on the presence of oxygen and arose from decomposition of the O_2^--adduct of DMPO (DMPO-OOH).[22] The *in vivo* and *ex vivo* studies have utilized primarily α-phenylbutylnitrone (PBN) to detect secondary lipid and alkoxyl radicals thought to be the result of primary radical formation.[24–28] While the spin trapping studies have provided evidence for the presence of oxygen intermediates in reperfused tissue, there are certain limitations to the use of this technique. For one, the use of ESR spin trapping is limited by relative low sensitivity of 10^{-6}–10^{-8} molar spins[29] when applied to aqueous solutions. Thus high and potentially toxic concentrations of the spin traps have been necessary to detect the radicals.[23,30,31] Lastly, a major limitation is the metabolism and instability of spin adducts *in vivo* leading to reduction of the stable spin trap-radical adduct and subsequent loss of the spectra.[32–36] Because of these limitations, as well as the high cost of an ESR spectrometer, many investigators, including ourselves, have been using an alternative technique to detect ·OH formation in biological systems.

THE SALICYLATE TECHNIQUE (AROMATIC HYDROXYLATION)

The use of salicylate as a reporter molecule is based on the ability of ·OH to attack and add to aromatic compounds. Hydroxyl radical adds to salicylate with a reported rate constant of $1.2 \times 10^{10} M^{-1} \cdot s^{-1}$.[37] There is a large body of literature on aromatic hydroxylation that has been extensively reviewed by Halliwell *et al.*[38] Although many investigators tend to credit Floyd and coworkers[39] with development of the salicylate technique, in fact use of aromatic hydroxylation in biochemical systems dates to 1976 when Halliwell and Ahluwalia[40] used *p*-coumaric acid to detect ·OH formation in the horseradish peroxidase system. To this authors knowledge, credit for first use of aromatic hydroxylation *in vivo* should probably be shared by Floyd and coworkers,[41] who used a loading dose of salicylate to detect ·OH formation in mice that had been treated with Adriamycin®; with Grootveld and Halliwell,[42] who detected dihydroxybenzoates in plasma from rheumatoid arthritis patients who had consumed aspirin (acetylsalicylic acid). Since then, aromatic hydroxylation, primarily of salicylate, has been used by many investigators to probe the role of ·OH in many different pathologic and toxicologic conditions, including, but not limited to: ischemia/reflow states in the heart,[43–45] brain,[46–48] retina[49] and intestine;[50] diabetes;[51] head trauma;[52] diaphragm fatigue;[53] and MPTP intoxication.[54,55] As another example of aromatic hydroxylation, phenylalanine has recently been used to detect post-ischemic ·OH formation in an *in vivo* dog preparation.[56]

In regards to salicylate, the dihydroxylated products formed will depend on several factors, including pH, oxygen tension and the presence of metals.[38,57,58]

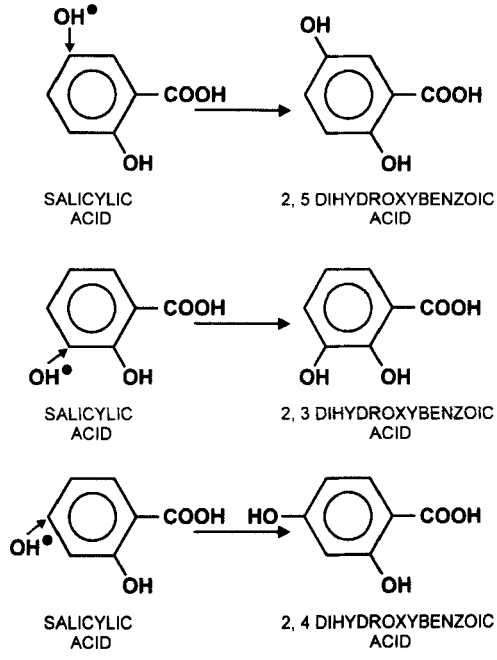

FIGURE 1 Chemical trapping of ·OH by salicylate.

The mechanism appears to involve electrophilic attack of the ring by the ·OH to form a hydroxycyclohexadienyl radical which can decay by oxidation to form a hydroquinone or by dehydration to form a phenoxyl radical.[59,60] Under physiologic conditions, the major products of ·OH attack on salicylate are 2,3-DHBA, 2,5-DHBA and catechol (Figure 1). Other minor products include the 2,4- and 2,6-DHBA, an example of which is shown in Figure 1. The ratios of each will vary according to the experimental conditions, but have been reported to be approximately 5:4:1 (2,3-DHBA:2,5-DHBA:catechol).[39,42] Studies by Maskos *et al.*[57] have indicated that the presence of the OH group in the two position acts as an ortho-para director, thus accounting for the product distribution.

The initial step in detection is separation of the different DHBAs by high performance liquid chromatography (HPLC). Although column lengths and sizes may vary, the stationary phase usually consists of a C_{18}, reverse phase packing.[39,42,43] Likewise, relative proportions of the mobile phase may vary, but in general is a combination of acetate, citrate and methanol, in the pH range of 3.5.[39] The individual DHBAs can then be detected by one of three methods. Because of the quinone/hydroquinone nucleus, DHBAs can undergo oxidation-reduction reactions, in which an electron is either lost or gained. This type of chemistry makes them ideal candidates for detection by electrochemical or amperometric means. The vast majority of investigators using this technique utilize this means of detection and use the standard glassy carbon electrode. For this electrode, hydrodynamic voltammograms generally indicate that an oxidation potential of +0.6 to +0.80 V versus Ag/AgCl reference electrode yields optimal detector response with minimal background noise.[39,42] Because the analyte passes over only one surface of the electrode,

efficiency of charge transfer is limited to only 1–5%. Nonetheless, the glassy carbon electrode is sensitive to the ρM range. To detect ·OH in the isolated perfused rat heart using this method of detection generally requires inclusion of salicylate at concentrations in the range of 1 mM.[45,61] An example of a electrochemical chromatogram obtained from pulmonary artery effluent from an isolated rat heart treated with salicylate is shown in Figure 2. A second means of detection is use of a standard UV detector set at 315 ηm.[44,62] This means of detection appears to be the least sensitive (ηM range for both 2,3- and 2,5-DHBA),[44] but nonetheless seems to give reproducible results with inclusion of 1 mM salicylate in the buffer.[44,62,63] An example of a UV chromatogram obtained from pulmonary artery effluent is shown in Figure 3.

The last means of detection, and the one used in our laboratory, is the coulemetric technique, which is a form of amperometric detection. The difference is that the analyte flows through a porous graphite electrode, thus charge transfer is virtually 100%.[64] This method takes advantage of the ability of hydroquinone/quinone compounds to undergo reversible oxidation-reduction reactions. By applying an oxidizing potential at the initial electrode and then a reducing potential at the downstream electrode, selectivity is increased because interfering compounds lacking the property of reversibility are screened out. In our laboratory, we use a Coulechem coulemetric detector (ESA model 5100A) equipped with a porous

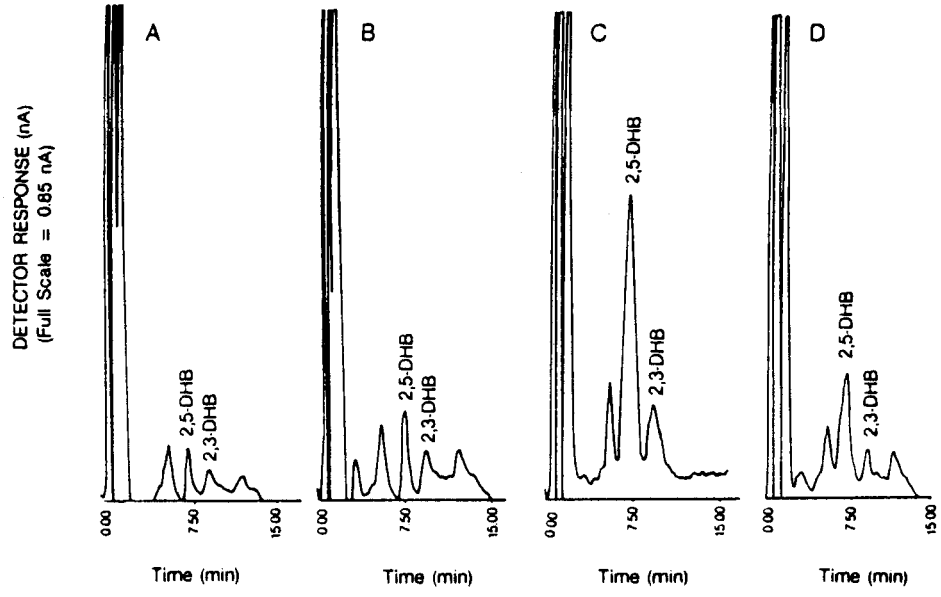

FIGURE 2 Indirect detection of ·OH as 2,3- and 2,5-dihydroxybenzoic acid (DHB) in the ischemic and reperfused rat heart. (A) Isolated rat heart was perfused in the presence of 2 mM salicylate for 30 min; (B) Isolated rat heart was perfused in the presence of 2 mM salicylate for 15 min, and then the heart 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 mM) 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 mM salicylate and 0.6 mM deferoxamine. (Reprinted from reference #45 with permission.)

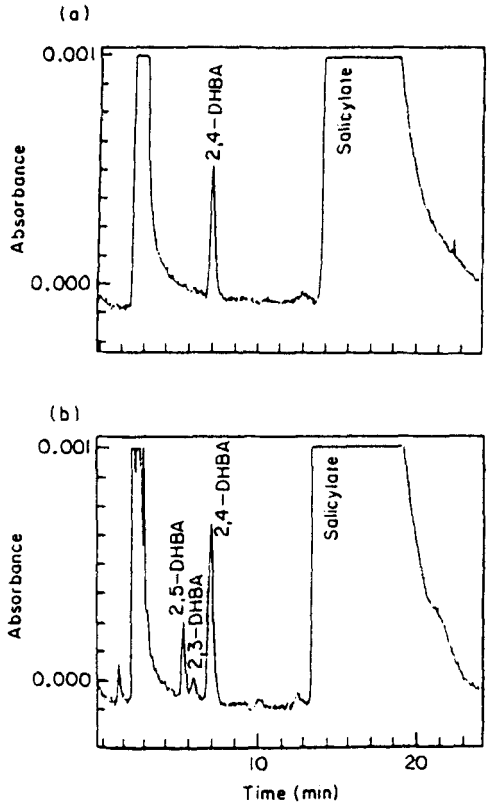

FIGURE 3 Chromatograms of the effluent from the ischemic heart after aerobic reperfusion. (a): Chromatogram of the effluent before ischemia. The peaks of 2,5- or 2,3-DHBA were not observed. 2,4-DHBA was used as an internal standard. (b): Chromatogram of the effluent 60 s after reperfusion. The peaks of 2,5- and 2,3-DHBA were detected. (Reprinted from reference #44 with permission.)

graphite electrode (ESA Model 5011 Analytical Cell). Cyclic voltammograms have indicated that application of an oxidizing potential of +0.4 V at electrode 1 and a reducing potential of −0.25 V at electrode 2 yields optimal detector response with minimal background noise. We routinely use electrode 2 (reducing electrode) as our detector. Also, we apply a guard potential of −0.30 V by placing a guard cell (ESA Model 5021) after the column, but before the electrodes. This configuration and reducing potential allows for scrubbing of the analyte prior to coulemetric analysis and basically results in removal of all electroactive contaminants that are not capable of undergoing reversible reduction, thus increasing selectivity and sensitivity as background noise is reduced.[64] Because of the virtual 100% efficiency of charge transfer, coulemetric analysis is very sensitive. We have been able to detect standard solutions of 2,5-DHBA in the high *f*M range, although as applied to the isolated heart preparation, it is only necessary to be able to detect the products of salicylate hydroxylation in the high ρM to low ηM range. Unlike investigators using the standard glassy carbon electrode or UV detector, we have been able to accomplish this through inclusion of only 100 μM salicylate in our buffer (Figure 4). Obviously,

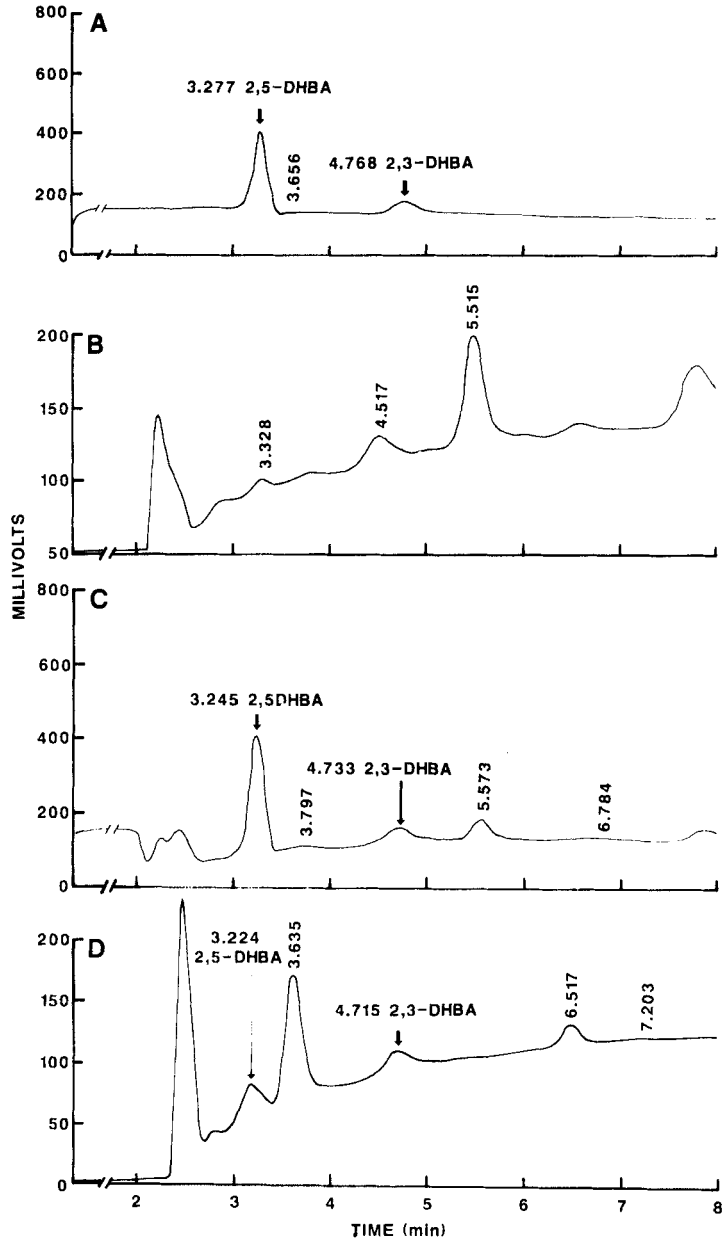

FIGURE 4 Representative chromatograms of 2,5- and 2,3-dihydroxybenzoic acid (coulemetric detection). (A) Standards in buffer. 125 *f*mol in 20 μL injected into HPLC. (B) Cardiac tissue from heart that was not treated with salicylate. 20 μL tissue extract injected into HPLC. The scale on the ordinate has been expanded. (C) Standard in cardiac tissue extract. 125 *f*mol in 20 μL tissue extract injected into HPLC. (D) Cardiac tissue subjected to ischemia and reperfusion. 20 μL tissue extract injected into HPLC. The scale on the ordinate has been expanded. (Reprinted from reference #43 with permission.)

salicylate on the isolated heart or related preparations have been reported. One of the earliest studies examined the effect of salicylate on the activity of *phosphorylase a* in the isolated rat heart with the purpose of examining the glycogenolytic action of this compound.[81] In this study, within 10 minutes of perfusion, 5 mM salicylate was observed to result in a three-fold increase in *phosphorylase a* activity and a four-fold increase in lactate production, as well as a dramatic decrease in glycogen content. Several studies have examined the effect of salicylate on cardiac electrophysiology. In studies on sheep cardiac purkinje fibers, Cohen *et al.*[82,83] have demonstrated that inclusion of salicylate in the superfusion media results in reversible changes in resting potential and prolongation of the action potential, as well as a reversible increase in the action potential threshold. However, these changes were only significant at concentrations of salicylate greater than 10 mM, which in the isolated perfused heart would be considered to be a toxic level. Other studies on isolated atrial muscle from rabbit have demonstrated that salicylate, in the concentration range of 300–500 μM causes a dose dependent decrease in discharge frequency of sino-atrial nodal cells.[84] More recently, studies have examined the effect of salicylate on pre- and post-ischemic function of the isolated heart preparation, keeping in mind that the optimal reporter molecule should have no effect. In the case of post-ischemic function this is an important question, since any reporter molecule essentially scavenges the toxic species. Depending on whether sufficient scavenging occurs prior to reaching the reaching the toxic level, theoretically a protective effect should be observed, similar to what has been reported for the spin trapping agents DMPO[73] and PBN.[85,86] In our initial study,[43] although we did not examine the post-ischemic effects, we did report that perfusion of the isolated rat heart with up to 100 μM salicylate for 30 minutes had no effect on pre-ischemic cardiac function (Table 1), a result which has subsequently been confirmed in another study using a concentration of 1 mM.[66] Post-ischemic effects of salicylate were examined by Liu *et al.*,[66] who reported that in the range of 0.5 to 1 mM, salicylate decreases post-ischemic release of creatine kinase as well as incidence and duration of reperfusion arrhythmias following 30 min of normothermic global ischemia. At 0.1 mM, no post-ischemic effects were apparent, while concentrations of 5 mM or greater were toxic.[66] Since the concentration of salicylate will depend on the detector used (coulemetric vs. amperometric vs. UV detector), it will be incumbent upon investigators who wish to use this technique to consider the possibility that salicylate may have an effect on their preparation.

TABLE 1
he effect of 100 μM salicylate on the function of isolated perfused rat hearts

Functional Parameter	Control	Salicylate
Heart Rate (bpm)	330 ± 19	334 ± 22
Coronary Flow (mL/min)	21.8 ± 1.4	21.3 ± 1.5
LV Peak Systolic Pressure (mm Hg)	95 ± 5	90 ± 6
$+dP/dt_{max}$ (mm Hg/s)	1952 ± 259	2142 ± 200

Isolated rat hearts were perfused with either KH buffer or KH buffer containing 100 Mμ salicylate for 30 minutes and several functional parameters were monitored. The values are the mean (±SEM) of 5 to 6 determinations at 30 min. (Reprinted from reference #43 with permission).

THE CONTROVERSY (2,3- versus 2,5-DHBA)

In 1991, a cautionary note was published on the use of salicylate as an assay for detection of ·OH, suggesting that only 2,3-DHBA, not 2,5-DHBA, is a reliable indicator of ·OH formation.[87] The authors of this cautionary note based this suggestion on the well described *cytochrome* P_{450}-mediated metabolism of salicylate to gentisic acid (2,5-DHBA)[88,89] and the subsequent demonstration that large amounts of 2,3-DHBA are not formed by rat liver microsomes or reconstituted vesicles containing various *cytochrome* P_{450s}, unless conditions are present that allow for formation of ·OH.[90] On the surface, this study appears to cast doubt on the interpretations of other studies that utilized salicylate to study ·OH formation, under a variety of conditions and in a variety of systems, that reported changes in 2,5-DHBA without reporting changes in 2,3-DHBA, since these changes could have been the result of salicylate metabolism. However, on further examination of the *cytochrome* P_{450} study,[90] it is apparent that the amount of 2,3-DHBA formed was anywhere from 10–25% of the amount of 2,5-DHBA formed in the microsomal systems and equal amounts of each were formed in the reconstituted *cytochrome* P_{450} systems. This then suggests that 2,3-DHBA may be a product of salicylate metabolism, albeit probably less than the 2,5-derivative. This interpretation is consistent with a past publication by this same group rigorously demonstrating that 2,3-DHBA is a minor product of human acetylsalicylic acid (aspirin) metabolism.[89] Thus it seems that the argument against reporting 2,5-DHBA values may also hold true for 2,3-DHBA. To further fuel the controversy, a very recent study, utilizing salicylate to probe post-traumatic ·OH formation in an *in vivo* mouse head injury model, failed to demonstrate any effect of the *cytochrome* P_{450} inhibitor, SKF-525A, on the magnitude of increase in post-traumatic brain levels of 2,5-DHBA.[52] Furthermore, in direct contrast to the *in vitro* study using microsomes,[90] no effect of SKF-525A was observed on plasma levels of 2,5-DHBA following administration of salicylate.[52] These contrasting results suggest that either the *in vitro* and *in vivo* preparations respond very differently to SKF-525A treatment, or that *in vitro*, *cytochrome* P_{450}-mediated formation of 2,5-DHBA does not contribute significantly to brain or plasma levels of this derivative. This latter interpretation would be consistent with the reported metabolism of salicylate, in that less than 1% of this compound undergoes oxidative biotransformation[44,88] to the di- or trihydroxybenzoic acid species. Of equal concern is the report that increases of 2,5-DHBA, exclusively, with no changes in 2,3-DHBA might be indicative of $^1O_2$ attack on salicylate.[91] Feix and Kalyanaram[91] have suggested that this might occur by cycloaddition, forming an unstable epoxide which then decays to the peroxide or hydroxy adduct or possibly by a charge-transfer reaction, both of which might lead to formation of exclusively 2,5-DHBA.

In the isolated perfused heart, all of the available evidence indicates that post-ischemic increases in 2,5-DHBA are secondary to ·OH attack. For example, several studies have been published in the isolated perfused rat heart which indicate that formation of 2,5-DHBA (and 2,3-DHBA) during early reperfusion is inhibited by the inclusion in the buffer of antioxidants, such as mannitol,[44] deferoxamine[45] (see figure 3D), or zinc,[78] thus suggesting that production is a ·OH-mediated event. Moreover, the general time course of ·OH production in all of these salicylate studies[43–45,78] is in general agreement with that reported in the numerous Langendorff studies using the spin trap, DMPO.[22,73,74] Furthermore, because the activity of the *cytochrome* P_{450} system is extremely low in myocardium, it is highly

inclusion of this low concentration makes this technique semi-quantitative at best, as it is unlikely that the salicylate could compete effectively with other components of the perfusate (glucose, HCO_3^-, etc.) for ·OH. Nonetheless, we have found that even this low concentration of salicylate is useful to detect relative changes in flux of this species.[65] When considering possible effects of salicylate on the preparation (discussed in detail in a separate section), this becomes important as this low concentration has been shown not to affect pre- or post-ischemic function of the isolated heart.[43,66] Moreover, studies by Gelvan *et al.*[67] have demonstrated that salicylate (and to a greater extent, phenylalanine) can chelate copper(II). This results in the introduction of a positive bias as system metal distribution is perturbed with redistribution of the metal from the tissue binding site to the reporter molecule and subsequent localization of ·OH formation to this site. These investigators[67] further demonstrated that only salicylate in the 0.1–0.2 mM range caused negligible perturbation of metal ion distribution. Nonetheless, it is apparent that either of the three detectors (coulemetric, amperometric or UV) can be used to detect ·OH formation in the isolated heart, with the difference being the concentration of salicylate that must be included in the buffer.

THE ISOLATED PERFUSED HEART (LANGENDORFF) PREPARATION AND RESULTS OF STUDIES

The Langendorff isolated heart[68] is an extensively used preparation to study basic cardiac physiology and is useful as a tool for the screening of agents or conditions that may effect myocardial function. To prepare this preparation, a heart is cannulated via the aorta and then is orthogradely perfused through the coronary arteries with Krebs-Henseleit (KH) buffer (crystalloid), or with KH supplemented with either hemoglobin or red blood cells. Investigators are cautioned that Krebs-Henseleit buffer may contain significant adventitious copper and iron that may have an effect on the outcome of their experiment.[69] In this preparation, coronary perfusion is maintained through one of two ways. Constant pressure perfusion is achieved by holding the column of perfusate at a known height, depending on the species from which the heart is obtained. Under these conditions coronary vascular tone is the major determinate of flow rate. Alternatively, the flow rate can be held constant through the use of a calibrated roller pump. In either case, the perfusate is oxygenated and pH maintained at approximately 7.4, via continuous gassing with 95% O_2/5/% CO_2, and temperature is maintained at 37°C by passing the buffer through a heat-exchange column. With the realization that ROIs are involved in the pathogenesis of post-ischemic reperfusion injury of the heart[70,71] and other organs,[21,47] this preparation has become widely used to understand the mechanisms and effects of cardiac oxidative injury. For those investigators interested in a more detailed discussion of this preparation, I recommend obtaining a copy of the English edition of "*The Isolated Perfused Heart According to Langendorff*".[72]

Our initial study,[43] in 1990, utilized salicylate to determine the formation of ·OH in the post-ischemic isolated rat heart. We demonstrated that formation of ·OH appeared to occur as a "burst" in the early phases of reperfusion (Figure 5), a result that was in general agreement with the spin trapping studies of the time.[22,73,74] Subsequently, in the following year two reports were published from different laboratories that basically confirmed and extended our initial studies.[44,45] The interesting aspect was that each of us had used different detectors and

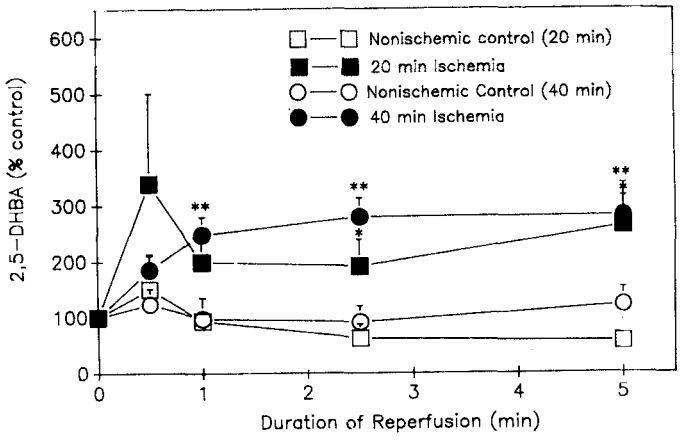

FIGURE 5 Detection of 2,5-Dihydroxybenzoic acid in the effluent from ischemia/reperfusion treated hearts. Isolated rat hearts were perfused with KH buffer containing 100 μM salicylic acid. The hearts were equilibrated for 10 min and then subjected to either 20 or 40 min of "noflow" normothermic global ischemia followed by 5 min of reperfusion. During the reperfusion period, samples of pulmonary artery effluent were collected and assayed for 2,5-DHBA. Control effluent samples were from hearts perfused for the duration of the experiment and not subjected to ischemia. Values are expressed as percentage of the value at 10 min equilibration. Each value represents the mean (±SEM) of five to eight determinations. *P < 0.05 (t-test) when compared with the corresponding control. **P < 0.025 (t-test) when compared with the corresponding control. (Reprinted from reference #43 with permission.)

concentrations of salicylate, yet had obtained very similar results.[43-45] Using subcellular fractionation, Das *et al.*[45] further demonstrated that a major site for post-ischemic ·OH formation appeared to be the mitochondria. Since these initial reports we have used the salicylate technique to probe the relationship between agents that affect severity of post-ischemic injury and formation of ·OH. We have demonstrated that preischemic loading of isolated hearts with copper greatly enhances post-ischemic reperfusion injury, and likewise increases ·OH formation.[75] Further, 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), a nitroxide spin label reported to possess superoxide dismutase-mimetic and other antioxidant properties,[76] decreased post-ischemic cardiac injury which was associated with a diminution of ·OH formation.[77] Most recently, we have demonstrated that the cardioprotective effects of zinc-bis-histidinate on the post-ischemic heart are related to decreases in ·OH formation.[78] Studies by Ashraf and coworkers,[62,63] utilizing salicylate in the isolated perfused heart, have questioned the relationship between formation of ·OH and injury associated with calcium paradox, and likewise, between ·OH formation and graded ischemia. Most recently, this group examined the role of ·OH in H_2O_2-induced cardiac injury[79] and has been applying the salicylate technique to studies utilizing cardiomyocytes in culture.[80] These studies illustrate the usefulness of this technique to study the role of ·OH in cardiac oxidative injury.

EFFECTS OF SALICYLATE ON THE PREPARATION

One of the important characteristics of a reporter molecule is that it should not affect the preparation at the concentration being employed. Yet, various effects of

unlikely that it could account for the increases in 2,5-DHBA (or 2,3-DHBA) that have been reported. In regards to the possibility that $^1O_2$ is the species being detected,[91] the lack of exclusivity (increases in both 2,5- and 2,3-DHBA formation have been reported[44,45]), as well as the inhibitory effects of antioxidants and ·OH scavengers, indicate that the principal species being detected is ·OH. Obviously, this does not preclude the possibility that a small portion of the increase in 2,5-DHBA is secondary to $^1O_2$. In our own studies, we report only 2,5-DHBA levels, even though we may detect both. The primary reason for this is that in choosing our oxidizing and reducing potentials we have optimized for the 2,5-isomer. As reported by Floyd and coworkers,[39] as well as ourselves[43] and others,[44,45] sensitivity for detection of DHBAs is highest for this isomer. Because the coulometric detector, as opposed to the standard amperometric detector, increases sensitivity by about 10–100-fold, we can use very low concentrations of salicylate (100 μM versus 1–2 mM) and still maintain *f*mole sensitivity for 2,5-DHBA. Unfortunately, under these conditions, the sensitivity for detection of 2,3-DHBA is only about one tenth of that for the 2,5-isomer.

Nonetheless, the authors of the cautionary note[87] were correct, in that one should always be careful in how experimental results are interpreted. In designing experiments that use either salicylate, or spin traps for that matter, proper controls should always be included. For example, in our original study,[43] we compared the results obtained from the post-ischemic hearts with non-ischemic hearts that had been perfused for an equal time period and observed that background 2,5-DHBA does not change with time in this group (Figure 5). This observation suggests that increases in 2,5-DHBA were secondary to ischemia and subsequent reperfusion and not biotransformation. From all of this evidence, this author can only conclude that as long as proper controls are employed, reporting either 2,5-DHBA and/or 2,3-DHBA as indicators of ·OH formation in the isolated perfused rat heart system is appropriate and the use of salicylate in general remains a very valuable tool.

CONCLUSIONS

In this review, I have tried to illustrate the usefulness of salicylate as a probe to detect ·OH in the isolated perfused heart. This method is meant as an alternative to ESR spin trapping studies and has several advantages over the latter. For one, it is up to 10,000-fold more sensitive, depending on the detector, thus allowing for the use of concentrations of salicylate as low as 100 μM. Secondly, as long as it is understood that biologic transformation of salicylate may result in formation of dihydroxybenzoates, and that proper controls are used, this technique can be used without experiencing difficulties of end-product loss associated with metabolism of spin trapped adducts. Finally, from an economical standpoint, an HPLC and appropriate detector costs far less than an ESR spectrometer. Of course, the major limitation is its presumed specificity for ·OH , thus would be useful only to study conditions in which this radical is suspected of being formed. In conclusion, the use of salicylate as a probe to study the role of ·OH in cardiac oxidative injury is a valuable alternative to the use of ESR spin trapping in preparations not affected by low concentrations of salicylic acid.

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