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GENERATION OF SINGLET OXYGEN AND HYDROXYL RADICAL FROM SODIUM CHLORITE AND LACTIC ACID

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Reperfusion of ischemic tissue is associated with the formation of hydroxyl radical (OH·). In this report, a novel mechanism for (OH·) generation from ($^1O_2$) is proposed based on the experimental evidence from the present study. A number of experiments were performed which conclusively demonstrated the formation of $^1O_2$ from the reaction of lactic acid and hypohalite radical. Singlet oxygen attacks the unsaturated olefinic derivatives, which are also formed during reperfusion of ischemic tissue. The reaction between $^1O_2$ and olefinic compounds produces hydroperoxides, which ultimately form OH· radical. The validity of the above mechanism of OH· radical formation is warranted from our experimentsl results.

KEY WORDS: Singlet oxygen, hydroxyl radical, ischemia, reperfusion, heart, polymorphonuclear leukocytes

INTRODUCTION

Current knowledge suggests that OH· may be derived from superoxide anion (O_2^-) as a primary source of oxygen free radicals. It has been speculated that OH· is formed in a biological system such as heart from O_2^- by the action of H_2O_2 catalyzed by a transient metal such as iron (Fenton reaction), as described below:[1,2]

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(i)}$$

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

However, iron is not present in free form in biological systems. Rather, it is always found to be bound with haem and stored in transferrin. It remains unclear how the iron is released, and it is not known whether the Fenton reaction actually occurs in biological tissue during pathophysiological conditions such as ischemia and reperfusion. Recently, an alternate mechanism of OH· formation from O_2^- by the reactions of O_2^- and nitric oxide has been proposed which is independent of the transition metal.[3]

We hypothesized that OH· may be derived from a source other than O_2^-, thus not requiring iron for its formation. A number of recent reports suggest that ischemia and reperfusion cause activation of polymorphonuclear leukocytes (PMN), which

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can generate hypohalite radical and hypochlorous acid (HOCl) according to the following reactions:[4]

$$\text{NADPH} + 2O_2 \xrightarrow{\text{NADPH oxidase}} \text{NADP}^+ + 2O_2 + H^+ \quad \text{(iii)}$$

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH\cdot + O_2 \quad \text{(iv)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \quad \text{(v)}$$

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{myeloperoxidase}} H_2O + HOCl \quad \text{(vi)}$$

The occurrence of these reactions has been demonstrated, and an ischemic reperfused tissue is likely to contain hypohalite radical derived from activated PMN.[4,5] It is also known that ischemia causes the accumulation of lactic acid and protons and leads to lactic acidosis.[6–8] Thus, an ischemic and reperfused tissue may contain both hypochlorite and lactic acid. In this study, we attempted to demonstrate that the reaction between hypochlorite and lactic acid can produce singlet oxygen ($^1O_2$), which can then produce OH· radicals directly.

MATERIALS AND METHODS

Sodium chlorite, lactic acid, 1,3-diphenyl isobenzofuran, anthracene, 2,3-dimethyl-2-butene, salicylic acid (sodium salt), and cholesterol were purchased from Aldrich Chemicals Co., Inc., Milwaukee, WI. Deuterium oxide (99.8 atom% D) was purchased from Sigma Chemical Co., St. Louis, MO. We performed a number of different tests specific for singlet oxygen. The generation of singlet oxygen was initially measured by detecting the chemiluminescence produced with luminol according to the method described by Misra and Squatrito.[9] In this study, we have used a two-pack system, where aqueous solution of 3.03 wt% of sodium chlorite was used as Part 1 and aqueous solution of 16.72 wt% of lactic acid was used as Part 2. For each experiment, we have used Part 1, Part 2, and water in the ratio of 1:1:10 (by volume). Lactic acid and sodium chlorite concentration in the resulting mixture were 154.7 mM and 100 μM, respectively; and the resultant pH was 6.5.

Monitoring $^1O_2$ Production using D_2O

Luminol was added to the sodium chlorite/lactic acid system at pH 10. The light generated during the reaction was measured using a luminometer, Model 1250 (LKB Instruments, Inc., Piscataway, NJ, USA). We have studied the sodium chlorite/lactic acid system by irradiating the sample with a Xenon lamp through a methyl orange cut-off filter. During each chemiluminescence experiment, 800 μl of phosphate buffer, 200 μl luminol solution, and 10 μl of sodium chlorite/lactic acid system (final concentration 0.5 μM sodium chlorite, 0.75 mM lactic acid) were used in the presence or absence of other additives. It is reported that the lifetime of $^1O_2$ is longer in D_2O than in H_2O by a factor of ten or fifteen.[10] In addition, the kinetics of the reaction was followed as a function of pH up to a period of 3 hr. Identical experiments were carried out individually in D_2O and in H_2O.

Use of 1,3-diphenyl isobenzofuran as $^1O_2$ quencher

1,3-diphenyl isobenzofuran is well known as a specific $^1O_2$ quencher. It reacts with $^1O_2$ to produce corresponding endoperoxide.[11] Both 0.25 mM and 0.5 mM solutions of 1,3-diphenyl isobenzofuran successfully quenched singlet oxygen, which was measured indirectly using a luminometer. In addition, 1,3-diphenyl isobenzofuran shows an absorption maximum at 415 nm. A 25 μM solution of 1,3-diphenyl isobenzofuran in ethanol/acetone (1:1) was taken, and 50 μl of a sodium chlorite/lactic acid sample solution was added to it. The absorption spectra as a function of time was recorded on a Beckman DU-40 spectrophotometer.

Specific reaction for $^1O_2$ with 2,3-dimethyl-2-butene

2,3-dimethyl-2-butene reacts with singlet oxygen to produce the corresponding 2,3-dimethyl-3-hydroperoxybutene-1.[11,12] To a solution containing 200 mg (0.0024 mol) of 2,3-dimethyl-2-butene in 5 ml of methylene chloride and 3 ml of acetone was added 2 ml of the sodium chlorite/lactic acid system. The resultant mixture was vigorously stirred in an ice bath under aerobic conditions. The reaction was carried for 8 h, and the reaction mixture was filtered and the solvent removed under vacuum. The product was vacuum-distilled and dried. The IR spectra of the starting material and the reaction product were taken on a Perkin-Elmer 177 IR spectrophotometer.

Reaction of $^1O_2$ with cholesterol

Cholesterol reacts with $^1O_2$ to produce its corresponding 5-α-hydroperoxide derivative, which is also a specific reaction for $^1O_2$.[13,14] Radical auto-oxidation of cholesterol leads to 7-α and β-hydroperoxides, alcohols, ketones and epoxides, but not the 5-α-hydroperoxide derivative. During the experiment, we chose rose bengal as a potential source of $^1O_2$ and compared the reaction product derived individually from cholesterol and rose bengal with that of cholesterol and the sodium chlorite/lactic acid system under identical reaction conditions. For this purpose, two different experiments were carried out. In the first experiment, we took 100 mg (0.26 mmol) of cholesterol in a 6 ml solvent mixture of methylene chloride and methanol (1:1), to which 200 mg of polymerized rose bengal sensitizer beads were added. The resultant mixture was stirred vigorously in an ice bath under oxygen atmosphere. The reaction was carried out for 25 h, then filtered and the solvent removed under vacuum. Similarly, in the second experiment 100 mg of cholesterol in a 6 ml solvent mixture of methylene chloride and methanol was treated with the sodium chlorite/lactic acid system (1 ml) in the same way as described in the first experiment. The product was spotted on a TLC plate and developed using the solvent system (benzene: ethyl acetate = 68:32)

Fluorescence quenching by the sodium chlorite/lactic acid system

Singlet oxygen is reported to quench the fluorescence of several organic molecules, including anthracene[15] in both the aqueous and the vapor phases. Quenching of these reactions is generally diffusion-controlled. Fluorescence spectra of anthracene (10^{-5} M) in ethanol was recorded at 365 nm in the absence and presence of various

concentrations of the sodium chlorite/lactic system. Fluorescence quenching experiments of anthracene with the sodium chlorite/lactic acid system was carried out on a Perkin-Elmer spectrofluorimeter, Model MPF 44B, using a 1 cm quartz cell. The photosensitive detector used was a photomultiplier tube, Model R446F, from the Hamamatsu Corporation, Japan, which is sensitive to both blue and red regions of the spectrum. It was observed that the extent of fluorescence of anthracene decreases with the addition of a sodium chlorite/lactic acid mixture. With the increasing concentration of the mixture, more and more fluorescence quenching of anthracene was observed.

Detection of OH· radical formation from sodium chlorite and lactic acid reaction by HPLC

The presence of OH· was confirmed by HPLC, as described previously.[2,16–17] The sodium chlorite/lactic acid system was mixed to generate $^1O_2$ and reacted with 2 mM of salicylic acid (sodium salt). The resultant mixture was degassed and filtered through a Rainin Nylon 66 membrane filter (0.45 μm syringe). A 20 μl sample was injected into an Altex Ultrasphere 3 μ ODS (75 × 4.6 mm) equipped in a Waters Associates HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. The hydroxylated products of salicylic acid (i.e., 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid) emerged as sharp peaks with the buffer, 0.03 M sodium acetate and 0.03 M citric acid (pH 3.6) (filtered and degassed), at a flow rate of 1.0 ml/min. Detector potential was maintained at 0.6 V, employing an Ag/AgCl reference electrode. The time required for each analysis was about 10 min. Dimethyl sulfoxide (DMSO) was successfully used as a scavenging agent.

RESULTS

Monitoring of $^1O_2$ Production using D_2O

Table I shows the chemiluminescence peak in D_2O to be about five times higher compared to that in water, suggesting the production $^1O_2$ from the sodium chlorite/lactic acid system. Chemiluminescence experiments were also carried out using different polyhydroxyl/sugar solutions, which led to an increase in the peak intensities. Results are given in Table I.

TABLE I
Chemiluminescence response from the reactions of lactic acid and sodium chlorite in the presence and absence of sugars and D_2O

Additives (dose)	Chemiluminescence response
Lactic acid/sodium chlorite system	100
D_2O in lieu of H_2O	492
Glycerol (0.1 mM)	108
Sucrose (0.1 mM)	121
Sorbitol (0.1 mM)	132
Mannitol (0.2 mM)	149

Use of 1,3-diphenyl isobenzofuran as $^1O_2$ quencher

The variation of absorbance at 415 nm vs. time is shown in Figure 1. A decrease in absorbance was observed with time, which is an indirect measurement for the formation of endoperoxide by $^1O_2$.

Specific Reaction for $^1O_2$ with 2,3-dimethyl-2-butene

Table II shows the IR bands of the starting material 2,3-dimethyl-2-butene as well as of its reaction product with $^1O_2$. 2,3-Dimethyl-2-butene shows a band at 1672 cm^{-1} corresponding to a tetrasubstituted alkene C–H stretching vibration, whereas the reaction product shows a band at 1651 cm^{-1} representing the presence of gem disubstituted alkene ($C{=}CH_2$) moiety in the reaction product. The C–O stretching vibration at 1725 cm^{-1} further strengthens the formation of the hydroperoxide. In addition, the band at 3580 cm^{-1} (broad) indicates the O–H and OOH band which further strengthen the formation of the hydroperoxide derivative. The refractive index of the reaction product was found to be 1.4402, which is very close to the literature data.

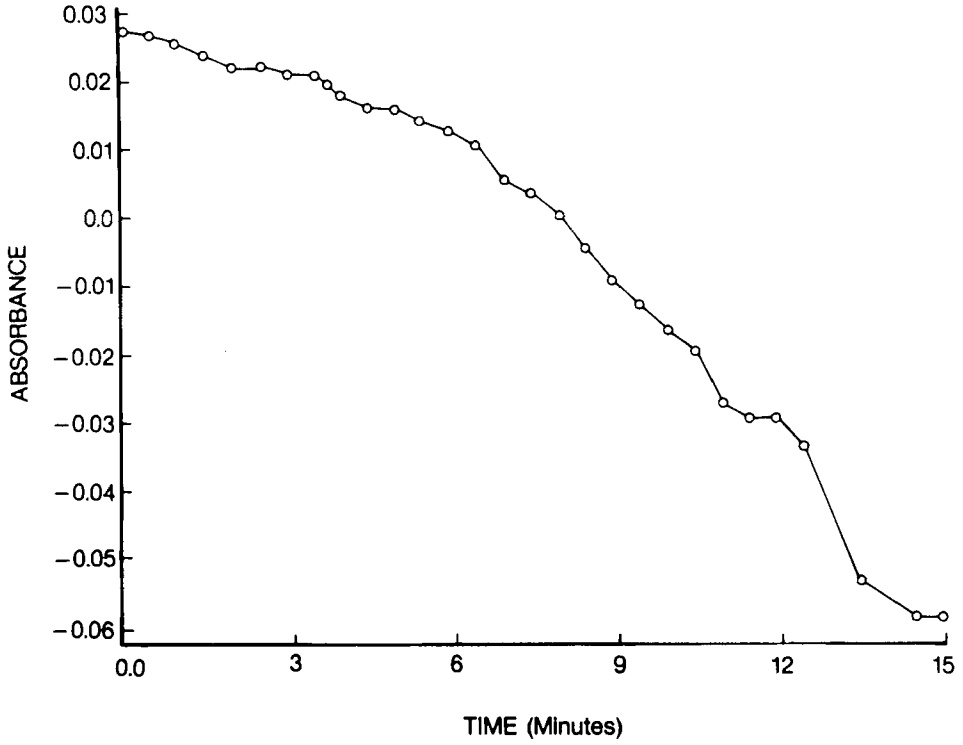


FIGURE 1 Effects of 1,3-diphenylisobenzofuran on $^1O_2$. $^1O_2$ was generated by the action of sodium chlorite/lactic acid reaction, and 1,3-diphenylisobenzofuran was added to the reaction mixture at zero time. The changes in absorption spectra were recorded as a function of time using a Beckman DU-40 spectrophotometer.

TABLE II
Infra-red spectral band of 2,3-dimethyl-2-butene and its reaction product with singlet oxygen

Products	Infra-red bands (cm^{-1})
2,3-dimethyl-2-butene	2950, 1672, 1380, 1165
Reaction product of 2,3-dimethyl-2-butene with sodium chlorite/lactic acid system	3580, 1725, 1651, 1435, 1220, 1100, 998

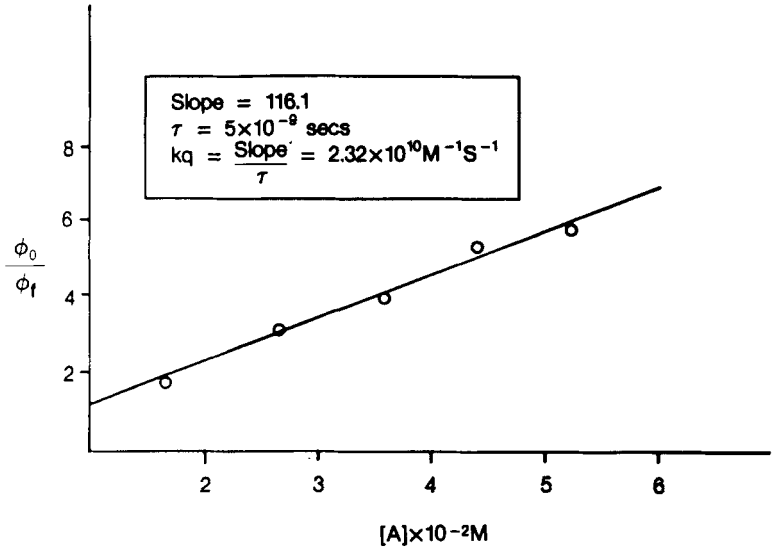


FIGURE 2 Stern-Volmer plot of $^1O_2$ generating system. Fluorescence spectra of anthracene (10^{-5} M) in ethanol was recorded at 365 nm in the absence (ϕ_0) and presence (ϕ_f) of various concentrations [A] of $^1O_2$ generating system, sodium chlorite/lactic acid. Fluorescence quenching experiments were carried out using a Perkin-Elmer MPF 44B spectrofluorimeter. ϕ_0/ϕ_f was plotted against A. The slope of the line is represented by K_q/τ, where τ is the fluorescence of anthracene in ethanol.

Reaction of $^1O_2$ with cholesterol

As described under Methods, cholesterol reacted with $^1O_2$ generated from the sodium chlorite/lactic acid system as well as that from rose bengal (results not shown). In this study, rose bengal served as the control source of $^1O_2$. On reaction with the sodium chlorite/lactic acid system, cholesterol yielded a product which, when run by TLC, gave an Rf value equivalent to 5-α-hydroperoxide derivative obtained from cholesterol-$^1O_2$ reaction products produced from rose bengal. Thus, this result suggests that the sodium chlorite/lactic acid system also produced $^1O_2$.

Fluorescence Quenching by the Sodium Chlorite/Lactic Acid System

Figure 2 shows the Stern-Volmer plot of ϕ_0/ϕ_f (where ϕ_0 is the fluorescence without addition of the sodium chlorite/lactic acid system, and ϕ_f is the fluorescence after addition of the sodium chlorite/lactic acid system) *versus* increasing concentration

of the sodium chlorite/lactic acid system (A). The quenching constant κ_q was determined from the slope of the Stern-Volmer plot (where slope = $\kappa_q \times \tau$ and τ is the fluorescence life-time of anthracene in ethanol, which is 5×10^{-9} sec.[18] In the present study with the sodium hypochlorite/lactic acid system, κ_q is 2.32×10^{10} $M^{-1} S^{-1}$, as calculated from the slope in Figure 2, which proves the quenching to be diffusion controlled. The κ_q value obtained in this experiment is identical to the value reported.[19] This result clearly indicates that the sodium chlorite/lactic acid system generates $^1O_2$, which in turn quenches the fluorescence of anthracene, since sodium chlorite or lactic acid alone do not quench the fluorescence of anthracene.

Effects of Substrates and pH on $^1O_2$ Generation

To further confirm the optimum conditions of the reaction, the substrates were monitored for chemiluminescence response in various combinations. As shown in Figure 3, no chemiluminescence response was observed for lactic acid or $NaClO_2$ alone. The generation of $^1O_2$ was maximal when 0.75 mM of lactic acid was added to 0.5 μM $NaClO_2$ at pH 6.5. At physiologic pH (7.4) also a significant amount of the response was observed. No chemiluminescence response was observed when lactic acid was replaced by acetic acid.

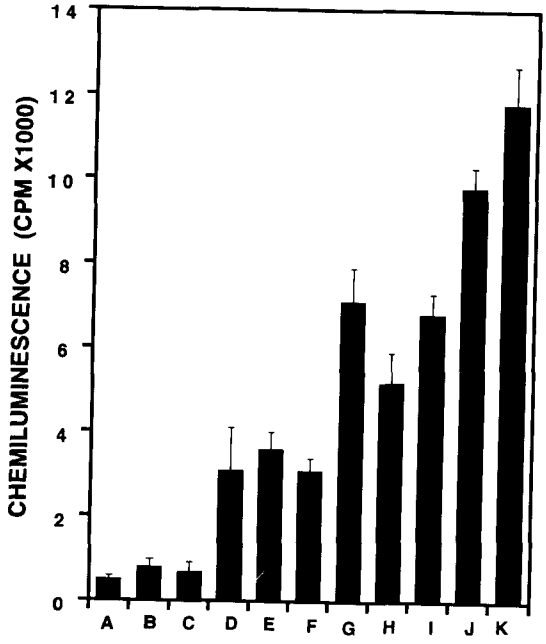


FIGURE 3 Effects of substrates and pH on the $^1O_2$ generation as measured by chemuluminescence response. A, $NaClO_2$ (0.5 μM) only; B, Lactic acid (0.75 μM) only; C, $NaClO_2$ (0.5 μM) + acetic acid (0.75 mM); D, $NaClO_2$ (0.05 μM) + lactic acid (0.075 mM), pH 11.2; E, $NaClO_2$ (0.5 μM) + lactic acid (0.75 mM), pH 11.2; F, $NaClO_2$ (0.5 μM) + lactic acid (0.075 mM), pH 11.2; G, $NaClO_2$ (0.5 μM) + lactic acid (0.75 mM), pH 8.0; H, $NaClO_2$ (0.5 μM) + lactic acid (0.075 mM), pH 8.0; I, $NaClO_2$ (0.05 μM) + lactic acid (0.75 mM), pH 8.0; J, $NaClO_2$ (0.5 μM) + lactic acid (0.75 mM), pH 7.4; K, $NaClO_2$ (0.5 μM) + lactic acid (0.75 mM), pH 6.5.

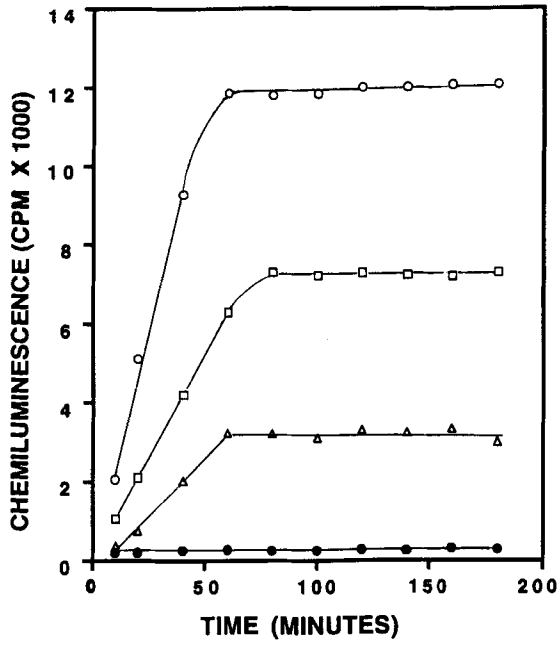


FIGURE 4 Effects of pH on the kinetics of $^1O_2$ generation – as measured by chemiluminescence response. ○–○, Lactic acid (0.075 mM) + $NaClO_2$ (0.5 μM), pH 6.5; □–□, Lactic acid (0.75 mM) + $NaClO_2$ (0.5 μM), pH 7.4; Δ–Δ, Lactic acid (0.75 mM) + $NaClO_2$ (0.5 μM), pH 11.2; ●–●, Lactic acid (0.75 mM) only.

pH dependence of the reaction as a function of time is shown in Figure 4. The kinetics of the reaction followed a mixed order mode. Initially, the reaction was of the first order since it followed a straight line. After 1 min the reaction became a second order. Again, no chemiluminescence was observed with lactic acid alone.

Detection of Hydroxyl Radical Formation from Sodium Chlorite and Lactic Acid Reaction by HPLC

Figure 5 shows the HPLC of the standard 2,3- and 2,5-dihydroxybenzoic acid mixture, and the reaction products of hydroxyl radical generating from sodium chlorite/lactic acid system with salicylic acid in the presence and absence of DMSO, a known hydroxyl radical scavenger, at two different concentrations. In this figure, the standard hydroxybenzoic acids have the same retention time as the reaction products of sodium chlorite/lactic acid, indicating the product to be OH· radical.

DISCUSSION

In this study, we demonstrated the formation of OH· from the reactions of sodium chlorite and lactic acid. As mentioned earlier in reactions (iii) to (vi), activated PMN represent a potential source for the HOCl and OCl· radical. Activated PMN produce O_2^- by the membrane-associated enzyme, NADPH oxidase (reaction iii).

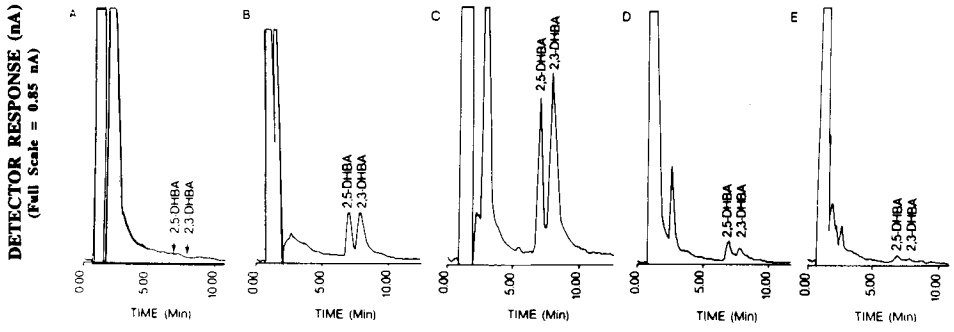


FIGURE 5 HPLC of OH·-salicylic acid adducts. The method has been described in the text. A, Salicylate only; B, Lactate acid/chlorite system + salicylate; C, Chlorite/lactate irradiated as (C) + DMSO (5 mM) + salicylate; D, Chlorite/lactate irradiated as (C) + DMSO (10 mM) + salicylate.

Dismutation of O_2 yields H_2O_2, which oxidizes the Cl· to yield HOCl (reaction vi). This last reaction is catalyzed by myeloperoxidase, an enzyme released from activated PMN. Thus, the "myeloperoxidase-H_2O_2Cl-system" can produce hypohalite radical during phagocytosis, which may also contribute to the inflammatory response which may occur during ischemia and reperfusion. In a previous study, we demonstrated a possibility of PMN being the source of such free radicals in ischemic reperfused heart.[4] Several other reports point to a similar possibility.[20–22]

Lactate is a substance known to accumulate in the ischemic myocardium.[23] Increased lactate and the associated rise in the cytosolic NADH have been shown to inhibit glycolysis and reduce anaerobic ATP production.[24] Transport of lactic acid into the cell can occur as that of a protonated acid which dissociates in the intracellular space, releasing H^+.

From the above discussion, it should be clear that both lactate and chlorite are present during reperfusion of ischemic tissue. In addition, increase in lactic acid concentration in conjunction with increased H^+ concentration leads to intracellular acidosis, which is a salient feature of myocardial ischemia.[25] In acidic pH, chlorite and lactic acid undergo the following chemical reaction:

$$NaClO_2 \rightleftharpoons Na^+ + ClO_2^-$$

$$ClO_2 \xrightarrow{H^+} HClO_2$$

It is known that chlorous acid undergoes spontaneous decomposition to the more stable oxidation states. The ratio of chlorite and chlorous acid is strictly dependent upon pH.

$$HClO_2 \rightleftharpoons ClO_2 + HCl + H_2O$$

The rate of ClO_2 generation increases in the presence of hydroxyl-bearing compounds in the following order: ethyl alcohol < glycerol < dextrose < galactose/mannose < ribose, which was confirmed from the results of this study.

The generation of ClO_2 also depends upon the number and orientation of hydroxyl function(s) in a compound. Accordingly, chlorous acid undergoes disproportionation reaction to produce chlorine dioxide and other by-products in the presence of cells

or surface active sites.

$$HClO_2 \rightarrow ClO_2 + Cl^+ + ClO_3^-$$

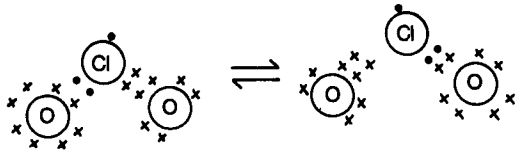
or

$$HClO_2 \rightarrow ClO_2 + HCl + H_2O$$

A single electron on ClO_2 can make it free radical (ClO_2) during its disproportionation step from chlorous acid.

$$HClO_2 \rightarrow ClO_2 + \text{other}$$

The ClO_2 free radical is stable due to the available resonating forms.

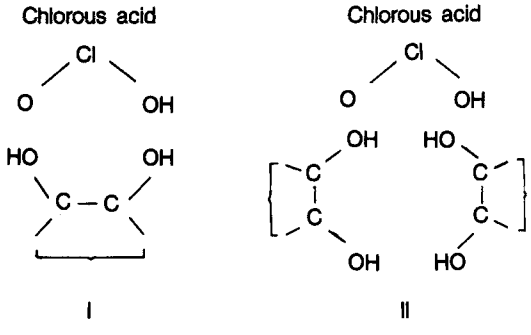


ClO_2 can then undergo further disproportionation at low pH to form $^1O_2$.

$$ClO_2 + H_2O + H^+ \rightarrow Cl^- + {}^1O_2 + HCl + H_2O$$

Another possible mechanism for the $^1O_2$ production may be the disproportionation reaction of ClO_2^- to ClO^- and ClO_3^- following the formation of unstable HOCl. $^1O_2$ can then be derived from HOCl.

A third possible mechanism for the formation of singlet oxygen from sodium chlorite/lactic acid system may be through a cyclic intermediate in the presence of di- or multihydroxyl bearing compounds as shown below:



I II

The $^1O_2$ can be formed from these transition cyclic intermediates I or II and during the reaction of chlorous acid in the presence of hydroxyl-bearing compounds. It has been observed that singlet oxygen generation of ClO_2 production is more in the presence of reducing carbohydrates than ordinary di- or trihydroxy compounds that implies to the greater stability of the six-membered intermediate with chlorous acid than linear dihydroxy compound.

The results of our study confirmed the formation of $^1O_2$ oxygen from chlorite and lactic acid reactions. However, the mechanism of $^1O_2$ oxygen formation is not clear, as it can be formed by any of the mechanisms described above. Once $^1O_2$ is formed,

it can form hydroperoxides with unsaturated olefinic derivatives. Accumulation of unsaturated fatty acids such as arachidonic, linoleic, and oleic acids have been demonstrated in ischemic and reperfused heart.[26,27] The presence of hydroperoxides has also been indicated during reperfusion of ischemic myocardium.[28] Hydroperoxy radical can lead to the formation of OH· according to the following scheme:

$$ClO_2 + H_2O + H^+ \rightarrow Cl^- + {}^1O_2 + HCl + H_2O$$

$$^1O_2 \rightarrow HO_2$$

$$HO_2 + HO_2 \rightarrow HO\cdot + HO^- + O_2$$

$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2$$

The results of our study confirmed the formation of OH· from the reaction of chlorite and lactic acid. When chlorite was allowed to react with lactic acid in the presence of salicylate, the presence of hydroxylated benzoic acids was demonstrated by using HPLC, further supporting our hypothesis.

In summary, our study indicates the formation of both $^1O_2$ and OH· from sodium chlorite and lactic acid reactions. Our results further suggest that OH· may be derived from $^1O_2$. A number of recent studies indicated the presence of $^1O_2$ in ischemic and reperfused heart.[29] Our study, thus, demonstrates an alternate mechanism for the formation of OH· via $^1O_2$.

Acknowledgements

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