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SHORT COMMUNICATION

Effect of Iron (II) on the Generation of Hydroxyl Free Radicals in Rat Myocardium

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ABSTRACT. We examined the effect of iron (II) on the generation of hydroxyl radicals (•OH) in the extracellular fluid of rat myocardium. Salicylic acid in Ringer's solution (0.5 nmole · μL^{-1} · min^{-1}) was directly infused through a microdialysis probe to detect the generation of •OH as reflected by the formation of dihydroxybenzoic acid (DHBA) in the myocardium. Iron clearly produced a dose-dependent increase in •OH formation. A positive linear correlation between iron (II) and the formation of 2,3-DHBA (R^2 = 0.970) or 2.5-DHBA (R^2 = 0.983) was observed. However, when desferrioxamine (DES) was infused through a dialysis probe, a marked increase in DHBA formation was obtained. The present results suggest that iron (III) may reduce •OH formation by the Fenton reaction. BIOCHEM PHARMACOL 51;10:1411–1413, 1996.

KEY WORDS. hydroxyl radical; Fenton reaction; Harber-Weiss reaction; iron (II); desferrioxamine

Although the function of iron content in heart is not known, its homeostasis is important for normal heart function. Hemorrhage or disorders of iron metabolism may cause high levels of free iron in various tissues. Iron has been implicated in cell degeneration more often than any other metal [1]. There is considerable evidence that intracellular iron mediates the toxicity of an excess of O_2 and H_2O_2 to the cells. Although free radical reactions are a part of normal metabolism, the overproduction of reactive oxygen species, such as O_2^-, H_2O_2, and $•OH^+$, may contribute to their cellular injury. It is important to explain the role played by radicals and metals in some disease states.

The •OH is extremely reactive, reacting as soon as it comes into contact with another molecule in solution. Because it is so reactive, •OH generated *in vivo* does not persist for even a microsecond, but rapidly combines with molecules in its immediate vicinity, such as lipids and proteins [2, 3].

The •OH reacts with salicylate and generates 2,3-DHBA and 2,5-DHBA [4, 5], which can be measured electrochemically in picomole quantities by HPLC [6, 7]. The formation of DHBA after systemic administration of salicylate is used as an index of •OH generation in myocardium.

We investigated the effect of Fe^{2+} on •OH generation in the presence of DES, a strong iron chelator.

MATERIALS AND METHODS

Experimental Protocol

Wistar rats weighing 300–400 g were anesthetized with an i.v. injection of chloral hydrate (400 mg/kg i.p.). The level of anesthesia was maintained with continuous i.v. infusion of chloral hydrate (20 mg · kg^{-1} · h^{-1}). Artificial ventilation was maintained with constant-volume respiration using room air mixed with oxygen. The heart rate, blood pressure, and electrocardiogram (ECG) were monitored and recorded continuously. This study was approved by the Ethical Committee for Animal Experiments, Oita Medical University.

We designed the microdialysis probe holding system that enables a loose fixation of the probe and its synchronized movement with each motion [8]. The dialysis probe was implanted in the area of the left anterior descending coronary artery (LAD). Heparin sodium (200 U/kg) was administered i.v. before probe implantation; 100 U/kg was then given every 1 hr to prevent blood coagulation. When a perfusion flow of 1 μL/min was used, the relative recovery rate of 1 μM standard solution of 2,3- and 2,5-DHBA was approximately 10 and 11%, respectively. Dialysate norepinephrine levels reached a steady-state level at 150–165 min after probe implantation. Therefore, we started the measurements of 2,3- and 2,5-DHBA at 150 min after probe implantation.

DES, sodium salicylate, and its hydroxylated metabolites were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) Ringer solution containing salicylic acid (0.5 nmole · μL^{-1} · min^{-1}) and DES (50 p mole · μL^{-1} · min^{-1}) were perfused by a microinjection pump (Carnegie Medicine CMA/100) to determine the basal levels of the formation of 2,3- or 2,5-DHBA during a definite period of time.

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†*Abbreviations:* •OH, hydroxyl free radical; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; DES, desferrioxamine.

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Samples (1 μL/min) were collected every 15 min into small collecting tubes containing 15 μL of 0.1 N $HClO_4$ and assayed immediately for 2,3- and 2,5-DHBA by a high-performance liquid chromatograph with an electrochemical (HPLC-EC) procedure. The formation of 2,3- or 2,5-DHBA by iron (II) was examined *in vivo*. In a cumulative dose-response experiment, 3 different concentrations of ferrous ammonium salt, 5×10^{-6}, 2.5×10^{-5}, 5×10^{-5} M (Sigma) were infused directly through the dialysis probe in the rat myocardium for 15 min each.

Analytic Procedure

The dialysate samples were immediately injected for analysis into an HPLC-EC system equipped with a glassy carbon working electrode (EICOM CORP., Japan) and an analytic reverse-phase column on an Eicompak MA-50DS column (5 μm 4.6 × 150 mm; EICOM). The working electrode was set at a detector potential of 0.75 V. Each liter of mobile phase contained 1.5 g l-heptansulfonic acid sodium salt (Sigma), 0.1 g Na. EDTA, 3 mL triethylamine (Wako Pure Chemical Industries, Japan) and 125 mL acetonitrile (Wako) dissolved in H_2O. The pH of the solution was adjusted to 2.8 with 3 mL phosphoric acid (Wako). Values are presented as means ± SE.

RESULTS AND DISCUSSION

The present study focused on the possible use of salicylate hydroxylation as an *in vivo* trapping procedure [9, 10] for monitoring DHBA generation in rat myocardium utilizing an *in vivo* microdialysis technique [8]. Iron (II) clearly produced a dose-dependent increase in •OH formation. The authentic standards of 2,3- and 2,5-DHBA (reaction products of salicylic acid and •OH) had an identical retention time. The basal levels of 2,3- and 2,5-DHBA in the heart dialysate samples of control animals following infusion of salicylate were 0.036 ± 0.007 and 0.039 ± 0.012 nmoles/mL, respectively. Infusion of DES (50 p mole · μ^{-1} · min^{-1}) through the dialysis probe increased the generation of ·OH as reflected by 2,3- and 2,5-DHBA, but this change was not significant. However, when iron (II) was administered to the DES-pretreated animals, a marked increase in 2,3- and 2.5-DHBA was obtained, as compared with the iron (II) only- treated group, showing a positive linear correlation between iron (II) and •OH formation trapped as 2,3-DHBA (R^2 = 0.970) or 2,5-DHBA (R^2 = 0.983) in the dialysate (Fig. 1). Iron enhanced not only the formation of 2,3-DHBA, the nonenzymatic •OH adduct of salicylate, but also that of 2,5-DHBA. The present results demonstrated an increase in both 2,3- and 2,5-hydroxylations of salicylate by DES-treated iron (II) in the myocardial microdialysis perfusion experiment during treatment with DES and iron (II).

The superoxide anion radical itself is somewhat poorly

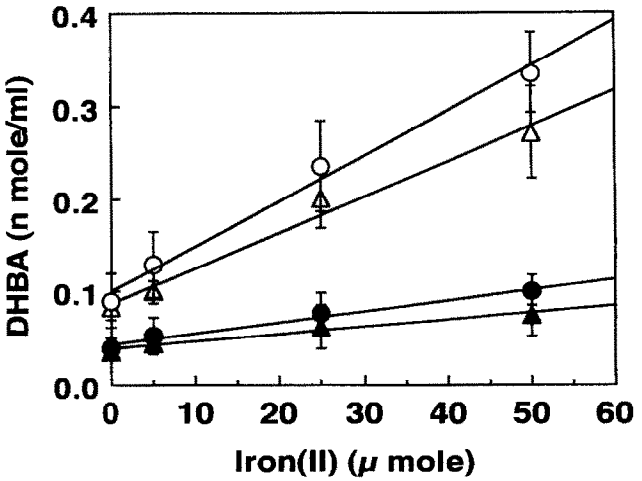


FIG. 1. Cumulative dose-response relationships between the formation of iron (II) and the efflux of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) in the rat heart. Iron (II) and sodium salicylate (0.5 nmole · μL^{-1} · min^{-1}) were infused through the dialysis probe. The dialysate samples for the determination of 2,3- (▲) and 2,5-DHBA (●) were collected every 15 min and assayed immediately by HPLC-EC. When iron (II) was infused in the DES pretreated animals, 2,3- (△) and 2,5-DHBA (○) levels markedly elevated vs the iron (II) only-treated group. Each value represents the mean SEM of 6 animals.

reactive in aqueous solution, but does participate in the reactions in which iron ions are involved, leading to the generation of more damaging •OH species. The pertinent reactions are presented below [9] where reaction (3) is the sum of (1) and (2):

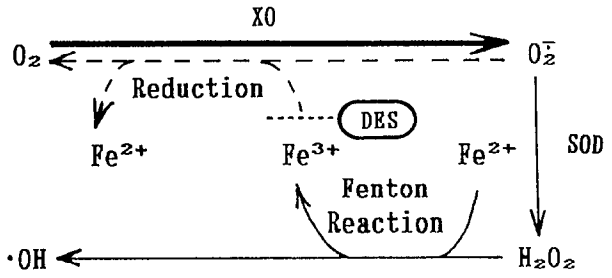
$$O_2^{\cdot-} + Fe^{3+} \xrightarrow{\text{Reduction}} O_2 + Fe^{2+} \quad (1)$$

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

$$O_2^{\cdot-} + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 \quad \text{(Harber-Weiss reaction)} \quad (3)$$

Theoretically, •OH may be formed *in vivo* during enzymatic oxidation (Fig. 2). $O_2^{\cdot-}$ has an extremely short half-life [1] and rapidly undergoes dismutation, yielding H_2O_2, H_2O_2 then undergoes Fenton-type reactions in the presence of iron and yields highly cytotoxic •OH [11, 12]. In addition, •OH can arise from an interaction between H_2O_2 and $O_2^{\cdot-}$ (Harber-Weiss reaction). However, iron (III) can be reduced further to iron (II) by the $O_2^{\cdot-}$, whereas superoxide anion may not be reduced by the DES treatment, a strong iron (III) chelator. When the level of DHBA markedly increases due to DES treatment, O_2, in turn, leads to the formation of $O_2^{\cdot-}$. Therefore, iron (II) in the presence of H_2O_2 results in further formation of •OH. This is, perhaps, why DES markedly increases •OH formation.

Free radical reactions are a part of normal human metabolism. When produced in excess, radicals can cause tis-



a) xanthine + O_2 —XO→ O_2^- + H_2O_2 + urea

b) 2 O_2^- + 2 H^+ —SOD→ H_2O_2 + O_2

c) For the Fenton reaction : H_2O_2 ⟶ $^{\cdot}OH^-$ + OH^-

FIG. 2. The reaction pathway in rat heart illustrates the formation of hydroxyl radical in the presence of iron (II) and oxygen. O_2^-, superoxide anion; DHBA, dihydroxybenzoic acid; ·OH, hydroxyl radical; DES, desferrioxamine; XO, xanthine oxidase.

sue injury. The results of the present study may be useful in elucidating the actual mechanism of free radical formation in heart disorders such as myocardial infarction.

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