Protective Effect of Histidine on Hydroxyl Radical Generation Induced by Potassium-Depolarization in Rat Myocardium

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ABSTRACT—We investigated the efficacy of histidine on potassium-depolarization induced hydroxyl radical (·OH) generation in the extracellular fluid of rat myocardium by a flexibly mounted microdialysis technique (O system). After the rat was anesthetized, a microdialysis probe was implanted in the left ventricular myocardium, and then sodium salicylate in Ringer's solution (0.5 nmol/μl per minute) was infused to detect the generation of ·OH as reflected by the nonenzymatic formation of 2,3-dihydroxybenzoic acid (DHBA). Infusion of KCl (70 mM) clearly produced an increase in ·OH formation. However, when KCl in the presence of a high concentration of histidine (25 mM) was infused through the microdialysis probe, KCl failed to increase the 2,3-DHBA formation. To examine the effect of histidine on ischemia-reperfusion of the myocardium, the heart was subjected to myocardial ischemia for 15 min by occlusion of the left anterior descending coronary artery (LAD). When the heart was reperfused, a marked elevation of the levels of 2,3-DHBA was observed in the heart dialysate. However, when corresponding experiments were performed with histidine (25 mM)-pretreated animals, histidine prevented the ischemia-reperfusion induced ·OH formation trapped as 2,3-DHBA. These results indicate that histidine may protect against K^+-depolarization-evoked ·OH generation in rat myocardium.

Keywords: Depolarization, Potassium chloride, Histidine, Hydroxyl radical, Microdialysis

It is well known that in the case of acute myocardial infarction or ischemia, there is a marked increase in extracellular potassium concentration ($[K^+]_o$) and the resulting membrane potential of the ventricular muscle in the infarcted area is remarkably depolarized (1). However, the release of norepinephrine was induced by depolarization (2). Norepinephrine may also have a deleterious effect on the myocardium by serving as a source of free radicals (3, 4). We hypothesized that increased $[K^+]_o$ as a consequence of depolarization of the ventricular muscles induced free radical generation. Although free radical reactions are a part of normal metabolism, the interaction between depolarization and oxygen free radicals in the myocardium is not clear. The generated reactive oxygen species have been implicated as important mediators of tissue injury (5–7). These reactive species include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and extremely reactive hydroxyl radical (·OH) or singlet oxygen ($^1O_2$). Of these, $^1O_2$ is thought to be one of the most damaging species (8). The xanthine oxidase (XO) pathway may produce large amounts of O_2^- with introduction of O_2 at reperfusion (7), and XO activity actually increases during ischemia but intensifies after reperfusion (9). $^1O_2$ can damage (10) constituents of biological membranes and can lead to inactivation of many enzymes (11). DNA damage (11) and oxidation of mitochondrial components (12) due to $^1O_2$ have also been demonstrated. Histidine, a free amino acid, has been demonstrated to have an antioxidative action (6, 13). Although histidine is known to be an $^1O_2$ scavenger (14), the effect of histidine on injury uncertain. Histidine is also a chelator of metal ions. Therefore, histidine can remove transition metals from the reactive site, resulting in a markedly decreased rate of ·OH formation (15).

The ·OH reacts with salicylate and generates 2,3- and 2,5-dihydroxybenzoic acid (DHBA), which can be measured by high performance liquid chromatography with electrochemical detection (HPLC-EC). However, 2,3-DHBA can be nonenzymatically formed by the aromatic hydroxylation of ·OH and can provide an assay for ·OH

formation (16, 17). The present study focused on the possible use of salicylate hydroxylation as an in vivo trapping procedure (18, 19). In the present study, to investigate the antioxidant effects of histidine, we examined the protective effect of histidine on ·OH formation induced by potassium-depolarization in the extracellular fluid of rat myocardium using a microdialysis technique (O system) (20).

MATERIALS AND METHODS

Animal preparation

Adult male Wistar rats weighing 300–400 g were kept in an environmentally controlled room (20–23℃, 50–60% humidity, illuminated from 7:00 to 19:00) with food and water ad libitum. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), and the level of anesthesia was maintained by intraperitoneal injection of chloral hydrate (20 mg/kg). Artificial ventilation was maintained by constant-volume respiration with room air mixed with oxygen. The heart rate, arterial blood pressure and electrocardiogram (ECG) were monitored and recorded continuously. This study was approved by the Ethical Committee for Animal Experiments, Medical University of Oita.

Drugs used

Histidine, sodium salicylate and its hydroxylate metabolites were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The drugs were dissolved in Ringer's solution consisting of 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl (pH 7.4).

Microdialysis technique

We measured ·OH in rat hearts by the flexibly mounted microdialysis technique (O system), which involves the synchronized movement of the tip of the probe with the beating heart to reduce tissue injury (20). Details of the flexibly mounted microdialysis technique and its application to measure biological substances in the interstitial space have been described previously (20, 21). We created a suitable microdialysis probe. The tube of the dialysis probe (approx. 15-cm-long) was supported loosely at the mid-point on a rotatable stainless-steel wire, so that its movement was totally synchronized with the rapid up-and-down movement of the tip caused by the heart beats. The probe was implanted from the epicardial surface into the left ventricular myocardium to a depth of 3 mm for perfusate administration through the inlet tube. The synchronized movement of the tip of the microdialysis probe with the beating ventricle minimized tissue injury that would otherwise be caused by friction between the probe and the muscle tissue. The tip of a microdialysis probe (3-mm length and 220-μm o.d. with the distal end closed) was made of dialysis membrane (cellulose hollow fiber (10-μm-thick) with 50,000 molecular weight cut-off). Two fine silica tubes (150-μm o.d.) were inserted from the open-end into the tip of the microdialysis tube consisting of a cylinder-shaped dialysis membrane and which served as an inlet for the perfusate and an outlet for the dialysate. The inlet tube was connected to a micro-injection pump (CMA/100; Carnegie Medicine, Stockholm, Sweden), and the outlet tube was connected a HPLC pump (Fig. 1).

Experimental protocol

In an in vivo perfusion system, dialysate norepinephrine levels almost reach a steady-state level at 120-min after probe implantation. Therefore, we started the measurement of 2,3-DHBA at 120 min after probe implantation. When a perfusion flow of 1 μl/min was used, the relative recovery of 10^{-6} M standard solution of norepinephrine was 17.0±0.7%. Histidine was infused directly through a microdialysis probe in the rat heart. We measured the time-dependent changes of the level of 2,3-DHBA after implantation of the probe, in the absence and presence of histidine. In the preparation of ischemic

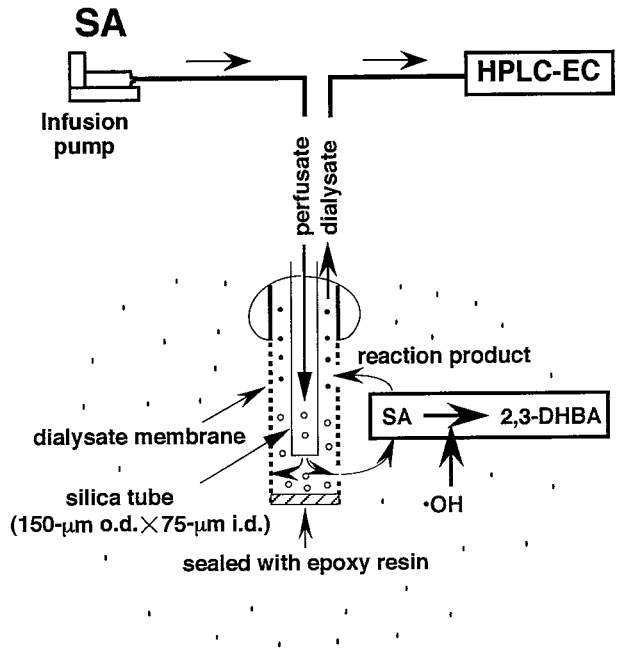

Fig. 1. Microdialysis probe in heart. The microdialysis probe was implanted from the epicardial surface into the left ventricular myocardium to the depth of 3 mm and perfused with Ringer's solution by a microinjection pump. The probe (3-mm exposure) was implanted from the epicardial surface into the left ventricular myocardium to the depth of 3 mm. The hydroxyl radical (·OH) reacts with salicylate (SA) and generates 2,3-DHBA, which can be measured electrochemically in picomole quantity by HPLC-EC.

rat, after the microdialysis probe implantation in the ischemic zone, the left anterior descending 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 occlusion of the LAD of the coronary artery followed by reperfusion for 60 min.

In vivo trapping of ·OH by salicylate

The ·OH radicals generated when captured as the hydroxylated derivative of salicylic acid was measured by HPLC. The authentic standards of 2,3-DHBA (reaction products of salicylic acid and ·OH) had an identical retention time. For trapping ·OH radicals (17, 20) in the myocardium, sodium salicylate in Ringer's solution (0.5 nmol/μl per minute) was perfused by a micro-injection pump (CMA/100, Carnegie Medicine) and the basal level of 2,3-DHBA during a definite period time was determined. Samples (1 μl/min) were collected after 15 min into small collecting tubes containing 15 μl of 0.1 N $HClO_4$.

Analytical procedure

The dialysate samples were immediately injected for analysis into an HPLC-EC system equipped with a glassy carbon working electrode (Eicom, Kyoto) and an analytic reverse-phase column on an Eicompak MA-5ODS 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 1-heptansulfonic acid sodium salt (Sigma), 0.1 g Na_2 EDTA, 3 ml triethylamine (Wako Pure Chemical Industries, Osaka) 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).

Statistical analyses

All values are presented as means±S.E.M. Differences between the time courses of the levels of 2,3-DHBA were analyzed by means of the Mann-Whiteney *U*-test. The significance of difference was determined by using ANOVA with Fisher's *post hoc* test. A P value of less than 0.05 was regarded as being statistically significant.

RESULTS

The level of 2,3-DHBA in the heart dialysate samples of control animals following infusion of salicylate (0.5 nmol/μl per minute) was 0.038±0.012 μM. The effect of KCl on the sequential change of ·OH formation trapped as 2,3-DHBA in the dialysate obtained from six rats are shown in Fig. 2. KCl significantly increased ($P<0.05$) the ·OH formation, trapped as 2,3-DHBA, by the action of K^+ depolarization. When KCl (70 mM) was infused into rat heart through a microdialysis probe at 150 min after probe implantation, the level of 2,3-DHBA at 180–195 min after probe implantation significantly increased in relation to the level at 135–150 min after probe implantation. However, in the presence of a high concentration of histidine (25 mM), KCl failed to increase the levels of 2,3-DHBA. Equivalent increases in osmotic concentration of the Ringer's solution, made by adding sucrose (150 mM), did not affect the level of 2,3-DHBA. However, in the presence of a high concentration of histidine (25 mM), KCl failed to increase the levels of 2,3-DHBA (Fig. 2). Similar experiments were repeated using various concentrations of histidine (0, 5, 25 and 50 mM) in the presence of KCl (70 mM) and the results are summarized in Fig. 3. Histidine reduced the K^+ depolarization-induced 2,3-DHBA formation in a concentration-dependent manner over the concentration range of 5–50 mM; the maximum effect (i.e., 39.8±9.0% of control ($n=6$, $P<0.05$)) was attained at 25 mM histidine.

The presence of ·OH was observed in ischemic-reper-

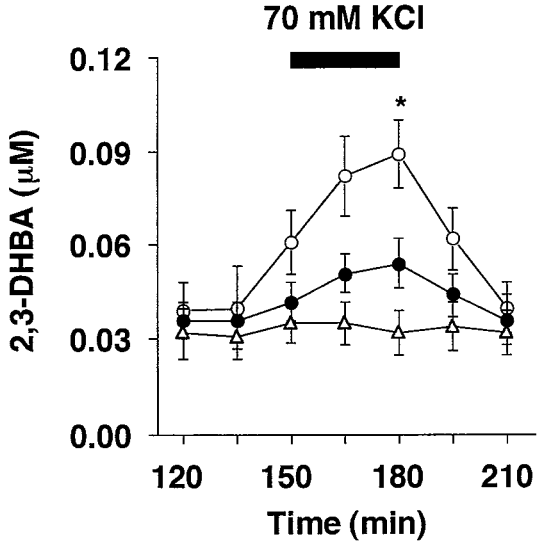

Fig. 2. The ·OH generation in rat myocardium after high KCl treatment. After a 120-min washout with Ringer's solution (pH 7.4), sodium salicylate (0.5 nmol/μl per minute) was infused for 105 min to trap ·OH. Thereafter, KCl (closed column, 70 mM) added in salicylic acid solution at 150 min after probe implantation (30 min after administration of salicylic acid) was infused directly through the microdialysis probe into the rat heart for 45 min. Histidine-pretreated rat (●) was compared with KCl-only treated rats (○). Equivalent increases in osmotic concentration of the Ringer's solution, made by adding sucrose (150 mM, △), did not affect the level of 2,3-DHBA. Dialysate samples were collected every 15 min into 0.1 N $HClO_4$ and immediately assayed for 2,3-DHBA by HPLC-EC. Values are each the mean±S.E.M. for 6 animals. Differences between the time courses of the levels of 2,3-DHBA were analyzed by means of the Mann-Whiteney *U*-test. *$P<0.05$ vs levels at 135–150 min after probe implantation. Abscissa, after a 120-min washout with Ringer's solution, infusion of salicylic acid a was started.

fused rat heart. After the dialysate probe was implanted in the left ventricular myocardium, the levels of 2,3-DHBA remained unchanged until reperfusion. When the heart was reperfused, a marked elevation of the level of 2,3-DHBA was observed in the heart dialysate. However, this elevation of 2,3-DHBA was not observed outside of the ischemic area. However, when histidine (25 mM) was administered, the elevation of 2,3-DHBA was not observed in ischemic-reperfused rat heart (Fig. 4).

DISCUSSION

The results of our present study showed that histidine, a scavenger of highly active $^1O_2$, may protect against K^+ depolarization evoked ·OH generation in the rat myocardium. The microdialysis technique was recently introduced for in vivo heart experiments in order to measure interstitial biological substances such as catecholamines, hydroxyl radical and purine metabolites (20, 22–25). We measured the ·OH radical when captured as the hydroxylated derivative of salicylic acid (16, 17, 20, 22) in rat hearts by use of the flexibly mounted microdialysis technique, which involves the synchronized movement of the tip of the probe with the beating heart to reduce tissue injury (20, 22). With this technique, it is feasible to make stable and long-term measurements of ·OH generation. The control of 2,3-DHBA formation in cardiac dialysate was about 10% higher than the in vitro perfusion reagent background (data not shown). Accordingly, the concentration profile of the administered compounds in the surrounding interstitial space is unknown; in general, the extracellular concentration of a compound given through the probe would never reach the concentration in the dialysis probe (21). This is an unavoidable limitation of the microdialysis technique that should be kept in mind when interpreting the experimental data.

It is known that K^+ depolarization induces Ca^{2+} overload (26, 27). We previously reported (3) that Ca^{2+} overload generates ·OH radicals in rat heart. This finding is agreement with our data that K^+ depolarization evokes ·OH radicals. Free radical reactions are a part of normal metabolism. When produced in excess, radicals can cause tissue injury (7). The enzyme xanthine oxidase resulting from xanthine dehydrogenase during ischemia (7) is thought to be a potential source of O_2^- in rat myocardium. Theoretically, ·OH may be formed in vivo during enzymatic oxidation. O_2^- has an extremely short half-life (28) 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 (29–31). In addition, ·OH can arise from an interaction between H_2O_2 and O_2^- (Haber-Weiss reaction). The

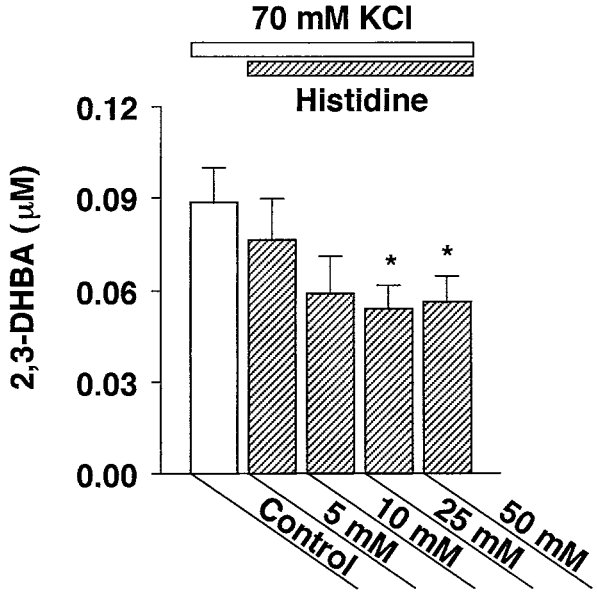

Fig. 3. Inhibitory effect of histidine on KCl-induced ·OH formation. When histidine (diagonal shaded column) was administered to KCl (70 mM)-pretreated animals, dialysate 2,3-DHBA levels at 180–195 min after probe implantation (at 30–45 min after administration of KCl) were compared with that in the control group (open column). Values are each the mean±S.E.M. for 6 animals. The significance of difference was determined by ANOVA with Fisher's *post hoc* test. *$P<0.05$ vs control group.

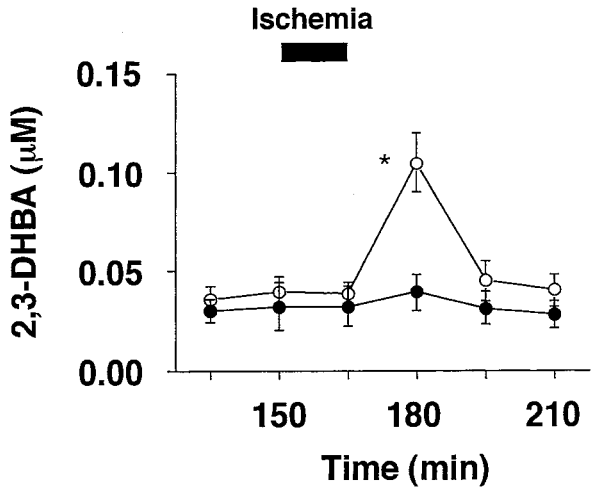

Fig. 4. Effect of histidine on the formation of ·OH in ischemic injury. In vivo trapping of highly reactive ·OH in the extracellular fluid of the myocardium was investigated by infusing sodium salicylate in Ringer's solution (0.5 nmol/μl per minute) through a myocardial microdialysis probe placed in a rat heart for 60 min. Histidine (25 mM)-treated group (●) of ischemia (closed column) was compared with the control group (○). Dialysate samples were collected and immediately assayed for 2,3-DHBA by HPLC-EC. Values are each the mean±S.E.M. for 6 animals. Differences between the time courses of the levels of 2,3-DHBA were studied by means of the Mann-Whiteney *U*-test. *$P<0.05$ vs levels at 135–150 min.

mechanisms of contribution of $^1O_2$ to postischemic reperfusion injury remain uncertain. As a consequence, there have been numerous studies that have sought to elevate the ability of antioxidants such as SOD to limit such injury. High concentration of histidine (25 mM) prevented the ·OH formation by the action of K^+ depolarization (Fig. 2). Oxygen-derived free radicals are thought to be responsible for postischemic reperfusion injury (5–7). Although controversial, a considerable effect on ischemia-reperfusion exists (32) to support the notion that oxygen radicals play a role in ischemia-reperfusion injury. It is well known that ischemia increases $[K^+]_o$ (33). Therefore, it is possible that ischemia increases ·OH generation. However, we could not measure that ischemia-induced increase in ·OH formation. This is an unavoidable limitation of the microdialysis technique. Although histidine has been shown to have antioxidative properties, the effect of histidine on myocardial postischemic reperfusion injury and on reactive O_2 species is not well characterized. Therefore, we investigated the scavenging effects of histidine on ischemia-reperfusion-induced ·OH generation. Our studies showed that histidine prevented the ischemia-reperfusion-induced ·OH generation trapped as 2,3-DHBA. Ischemia-reperfusion increased the level of norepinephrine. However, in the presence of histidine (25 mM), ischemia-reperfusion failed to increase the level of norepinephrine (data not shown). Moreover, when the ischemic arrhythmia appeared, the presence of free radicals was observed in the ischemic-reperfused rat heart. However, neither typical changes in ECG nor ·OH generation in the ischemic-reperfused rat heart were observed after administration of histidine (data not shown). Histidine is a poor scavenger of ·OH. Unfortunately, it is difficult to separate injury induced by ·OH radicals from that by $^1O_2$ because of the nonspecificity of histidine in scavenging both of the oxidant species. In addition, most of the $^1O_2$ scavengers react with ·OH radical, often with a greater rate constant than the reaction with $^1O_2$. Histidine appears to scavenge most O_2 species, as indicated by the present and previous studies (34). Hydroxyl radicals were measured with salicylic acid as a trapping agent (16, 35). More recently, Feix and Kalyanaraman (36) reported that $^1O_2$ could also attack salicylic acid to form 2,5-DHBA and 2,3-DHBA, which implies that salicylic acid is not a specific trapping agent for ·OH, but can also trap $^1O_2$.

The present results indicate that histidine protects myocardium against ischemia-reperfusion damage induced by ·OH generation. These experiments using cardiac microdialysis have versatile applications and offer new possibilities for the in vivo study of cardiac physiology. In the future, cardiac microdialysis heart perfusion experiments using the microdialysis technique (O system) (20) may be useful in answering some of the fundamental questions concerning the relevance of oxidant damage in the pathogenesis of heart disorders.

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