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SHORT COMMUNICATION

Cardiac Microdialysis of Salicylic Acid •OH Generation on Nonenzymatic Oxidation by Norepinephrine in Rat Heart

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ABSTRACT. The effect of pargyline, a monoamine oxidase inhibitor, on the generation of hydroxyl free radicals (•OH) was investigated using cardiac microdialysis. Salicylic acid in Ringer's solution (0.5 nmol · μL^{-1} · min^{-1}) was infused directly through a microdialysis probe to detect the generation of •OH as reflected by the formation of dihydroxybenzoic acid (DHBA) in the myocardium of anesthetized rats. When pargyline (100 nmol · μL^{-1} · min^{-1}) was infused in rat heart, the level of norepinephrine (NE) gradually increased in a time-dependent manner and an increase of DHBA was also observed. When NE was administered to the pargyline pretreated animals, a marked elevation in the levels of 2,3- and 2,5-DHBA formation was obtained, as compared to the group treated with NE only, showing a positive linear correlation between NE and •OH formation trapped as 2,3-DHBA (R^2 = 0.981) or 2,5-DHBA (R^2 = 0.984) in the dialysate. NE clearly produced an increase in •OH formation. These results indicate that accumulation of NE in the extracellular fluid elicited by pargyline can be auto-oxidized, which in turn, leads (possibly by an indirect mechanism) to the formation of cytotoxic •OH free radicals. BIOCHEM PHARMACOL 53;9:1375–1378, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. norepinephrine; pargyline; hydroxyl free radical; salicylic acid; anesthetized rat; microdialysis

Monoamine oxidase (MAO†; EC 1.4.3.4) is one of the main enzymes in the metabolism of various catecholamines. It has been proposed that the deamination of catecholamine by MAO [1] contributes to the formation of cytotoxic free radicals in the presence of transition metals such as iron, copper, and manganese [2–6]. Norepinephrine (NE) released from cardiac sympathetic nerve is thought to play a significant role in the etiology of various cardiac pathophysiological disorders [7–9]. Although free radical reactions are a part of normal metabolism, the overproduction of reactive oxygen species such as superoxide anion (O_2^-), H_2O_2, and hydroxyl free radical (•OH) may contribute to cellular injury [10]. 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 [11, 12].

The •OH reacts with salicylate and generates 2,3- and 2,5-dihydroxybenzoic acid (DHBA) [13–15], which can be measured electrochemically in picomole quantity by a high-performance liquid chromatographic-electrochemical (HPLC-EC) procedure. The formation of DHBA after systemic administration of salicylate is used as an index of •OH generation in myocardium. The present study examined the effect of pargyline, a MAO inhibitor, on •OH generation in heart utilizing a cardiac microdialysis heart perfusion method.

MATERIALS AND METHODS

Experimental Protocol

Adult male Wistar rats weighing 300–400 g were housed in an environmentally controlled room (20–25°C, 50–60% humidity) with available food and water *ad libitum* for 4 days prior to our experiments. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO, USA). The level of anesthesia was maintained with continuous i.v. infusion of chloral hydrate (20 mg · kg^{-1} · hr^{-1}). Artificial ventilation was maintained with constant-volume respiration using room air mixed 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, Medical University of Oita.

We designed the microdialysis probe-holding system that

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† *Abbreviations:* •OH, hydroxyl free radical; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid; NE, norepinephrine; XO; xanthine oxidase; O_2^-, superoxide anion; DOPGAL, 3,4-dihydroxyphenylglycolaldehyde; SA, salicylic acid.

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enabled a loose fixation of the probe and its synchronized movement with each motion [16]. 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 hour to prevent blood coagulation. When a perfusion flow of 1 μL/min was used, the relative recovery rate of 1 μM standard solution of NE was 17.0 ± 0.7%. The drugs were dissolved in Ringer's solution containing 147 mM NaCl, 2.3 mM $CaCl_2$, and 4 mM KCl (pH 7.4).

Pargyline · HCl, sodium salicylate, and its hydroxylated metabolites were purchased from Sigma Chemical Co. Ringer's solution containing salicylic acid (0.5 nmol · μL^{-1} · min^{-1}) was perfused by a microinjection pump (Carnegie Medicine CMA/100, Sweden) to determine the basal levels of the formation of 2,3- or 2,5-DHBA during a definite period of time. Samples (1 μL/min) were collected every 15 min into small collecting tubes containing 15 μL of 0.1 N $HClO_4$ to prevent amine oxidation and assayed immediately for 2,3- and 2,5-DHBA by an HPLC-EC procedure. The formation of 2,3- or 2,5-DHBA by NE (Wako Pure Chemical Industries, Osaka, Japan) was examined *in vivo*. In cumulative dose–response experiments, three different concentrations of NE, 5×10^{-5}, 2.5×10^{-6}, 5×10^{-5} M were infused directly through the dialysis probe in the rat myocardium for 15 min each.

Analytical Procedures

The dialysate samples were immediately injected for analysis into an HPLC-EC equipped with a glassy carbon working electrode (Eicom Corp., Kyoto, Japan) and an analytic reverse-phase column on an Eicompak MA-50ODS 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 heptane sulfonic acid sodium salt (Sigma), 0.1 g Na_2EDTA, 3 mL triethylamine (Wako), 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). The results were reported as mean ± SE of output. Data were analyzed for significance by Mann-Whitney *U*-test. A *P*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

The present study suggests that some extracellular autooxidation of NE, occurring in the presence of oxygen and some transition metal, could lead to the formation of •OH free radicals. In an *in vivo* perfusion system, time-dependent changes in the level of NE and the formation of 2,3- and 2,5-DHBA were monitored in the dialysates from rat heart after pargyline treatment. Dialysate NE level gradually decreased, reaching an almost steady-state level of 0.146 ± 0.019 nmol · mL^{-1} at 135–150 min after probe implantation. This value was significantly different from the value at 75–90 min after probe implantation ($P < 0.05$). When pargyline (100 nmol · μL^{-1} · min^{-1}) was directly infused in the rat heart through a microdialysis probe for 105 min, the level of NE increased in a time-dependent manner (Fig. 1, top). However, an increase in NE level in the absence of pargyline treatment was not observed (data not shown). The basal levels of 2,3- and 2,5-DHBA in the dialysate samples of control following the cardiac infusion of salicylic

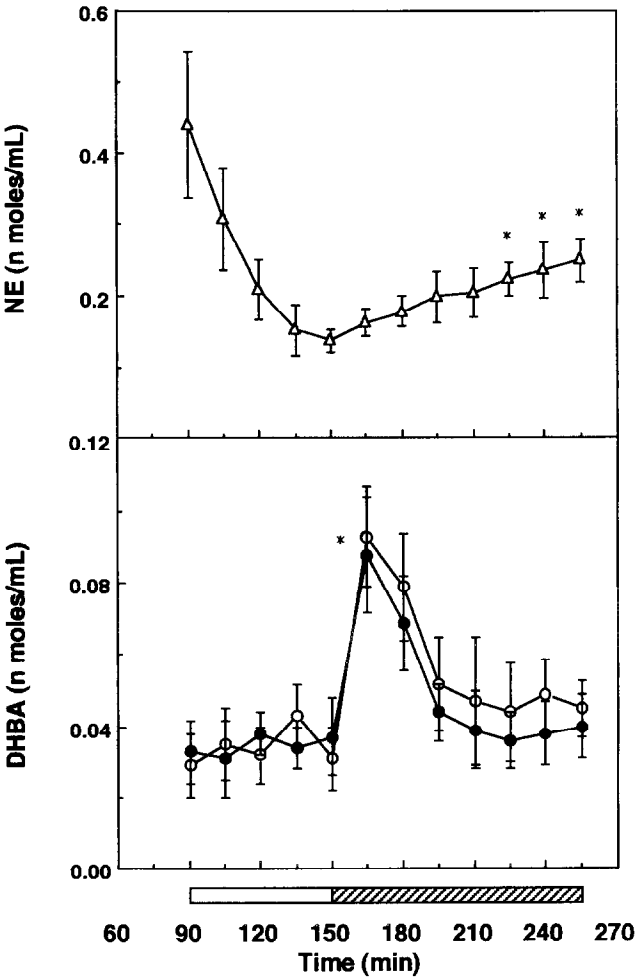

FIG. 1. Relationship between NE and •OH generation after pargyline treatment. After a 90-min washout with Ringer's solution, the time course of dialysate NE (top; △) and *in vivo* trapping of highly reactive •OH (bottom; ○ 2,5-DHBA, ● 2,3-DHBA) in extracellular fluid of myocardium was investigated by infusing salicylic acid in Ringer's solution (□; 0.5 nmol · μL^{-1} · min^{-1}) through myocardial microdialysis probe placed in a rat heart for 60 min. Thereafter, pargyline (slanted rule column; 100 nmol · μL^{-1} · min^{-1}) added in salicylic acid solution at 150 min after probe implantation (60 min after administration of salicylic acid) was infused directly through microdialysis probe in the rat heart for 105 min to trap •OH formed during sustained NE. The level of NE after pargyline treatment increased in a time-dependent manner. Dialysis samples were collected at 15-min intervals and immediately assayed for NE and 2,3- and 2,5-DHBA using an HPLC-EC procedure. Differences in time course between 2,3- and 2,5-DHBA levels were statistically studied by Mann-Whitney *U*-test. Values are expressed as mean ± SE from six rats. *$P < 0.05$ vs. levels at 135–150 min. Abscissa, after 90-min washout with Ringer's solution, infusion of salicylic acid was started.

acid (0.5 nmol · μL^{-1} · min^{-1}) were 0.036 ± 0.008 and 0.031 ± 0.010 nmol · mL^{-1}, respectively. When the level of NE after pargyline treatment increased, a marked transient elevation in the levels of 2,3- and 2,5-DHBA was observed in the cardiac dialysates (Fig. 1, bottom). The level at 150–165 min (or 15 min after administration of pargyline) was significantly increased in relation to the level at 135–150 min. NE clearly produced an increased •OH formation. Moreover, when NE was administered to the pargyline-pretreated animals, a marked elevation in the levels of 2,3- and 2,5-DHBA was obtained, as compared with the NE-only treated group, showing a positive linear correlation between NE and •OH formation trapped as 2,3-DHBA (R^2 = 0.981) or 2,5-DHBA (R^2 = 0.984) in the dialysate (Fig. 2).

The formation of such products of DHBA and especially 2,3-DHBA following systemic administration of salicylate is being used as an index of •OH generation [17–20] both *in vitro* and *in vivo*. MAO is one of the heart enzymes playing a role in the metabolism of various catecholamines. Some investigators reported [15, 16, 21, 22] a linear correlation between the formation of •OH products of salicylate and efflux/oxidation of catecholamine. When the level of NE after pargyline treatment increased, a marked elevation in the levels of 2,3- and 2,5-DHBA was observed in the heart dialysate (Fig. 1). NE enhanced not only the formation of 2,3-DHBA, the nonenzymatic •OH adduct of salicylate, but also that of 2,5-DHBA. The effects of NE were dose dependent, and the results demonstrate a positive linear correlation between NE and •OH formation trapped as 2,3- or 2,5-DHBA in the heart dialysate (Fig. 2). However,

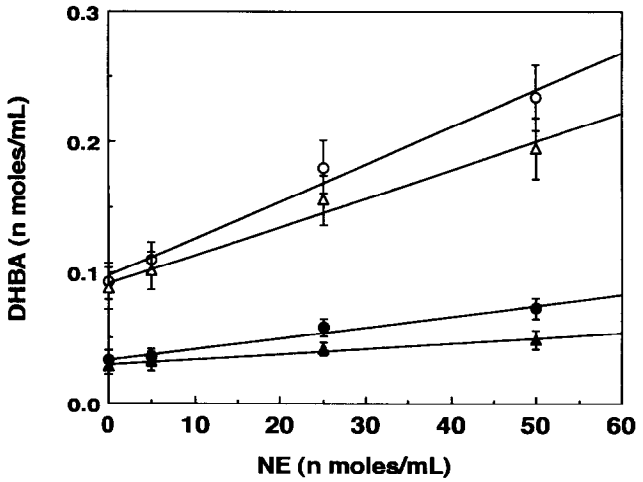

FIG. 2. Cumulative dose–response relationship between NE and the formation of •OH products of salicylate. NE and sodium salicylate (0.5 n mol · μL^{-1} · min^{-1}) were infused through the dialysis probe. The dialysis samples for the determination of 2,3-DHBA (▲) and 2,5-DHBA (●) were collected every 15 min and assayed immediately by the HPLC-EC procedure. When NE (0, 5, 25, and 50 μM) was infused in the pargyline-pretreated animals, 2,3-DHBA (△) and 2,5-DHBA (○) levels markedly elevated vs. the NE only-treated group. Each value represents the mean SEM of six rats.

when the same experiment was previously performed in rat liver, well known for its very low level of catecholamines, no elevation in the levels of DHBA products was observed (data not shown). This finding shows that extracellular NE is needed for the observed effect of pargyline on salicylic hydroxylation. Dopamine or NE is known to be auto-oxidized in the presence of oxygen and transition metals [2–4]. The enzyme xanthine oxidase (XO) is also thought to be a source of O_2^-. The sustained elevation of NE in the extracellular fluid elicited by pargyline can be auto-oxidized, which in turn, leads (possibly by an indirect mechanism) to the formation of cytotoxic •OH free radicals. The present results demonstrated an increase in both 2,3- and 2,5-dihydroxylation of salicylate following administration of NE in the cardiac microdialysis heart perfusion experiment using salicylic acid as the •OH trapping agent.

The O_2^- itself is somewhat poorly reactive in aqueous solution, but does participate in the reaction in which the iron ions are involved, leading to the generation of more damaging •OH species. O_2^- has an extremely short half-life [5] and rapidly undergoes dismutation yielding H_2O_2. H_2O_2 then undergoes a Fenton-type reaction in the presence of iron to yield cytotoxic •OH. In addition, •OH can also arise from an interaction between H_2O_2 and O_2 (Haber-Weiss reaction). Theoretically, •OH may be formed *in vivo* during nonenzymatic oxidation [3, 23, 24] and/or enzymatic oxidation of NE. Our data indicate that the elevation in NE may cause •OH generation, as reflected by 2,3- and 2,5-DHBA levels in the myocardial dialysate. Free radical reactions are a part of normal metabolism. According to the reaction pathway in Fig. 3, •OH was generated by the presence of NE and oxygen. When produced in excess, radical can cause tissue injury. These results suggest that nonenzymatic oxidation of NE in the extracellular fluid may play a key role in the generation of •OH free radicals in the heart.

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. These experi-

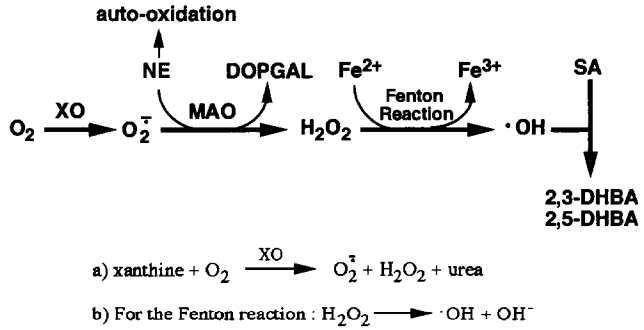

FIG. 3. The reaction pathway in rat heart illustrates the formation of hydroxyl radical in the presence of NE and oxygen. Abbreviations: XO, xanthine oxidase; O_2^-, superoxide anion; DOPGAL, 3,4-dihydroxyphenylglycolaldehyde; SA, salicylic acid; DHBA, dihydroxybenzoic acid; •OH, hydroxyl radical; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 2,5-DHBA, 2,5-dihydroxybenzoic acid.

ments in 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 hydroxylation of salicylate to detect •OH generation 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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