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ROLE OF ASCORBATE IN PROTECTION BY NITECAPONE AGAINST CARDIAC ISCHEMIA–REPERFUSION INJURY

NOBUYA HARAMAKI, DAPHNE B. STEWART, SARITA AGGARWAL, TERUYUKI KAWABATA and LESTER PACKER*

Membrane Bioenergetics Group, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, U.S.A.

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Abstract—The antioxidant properties of nitecapone, a catechol derivative and an inhibitor of catechol-*O*-methyltransferase, were reported recently. In the present study, the influence of nitecapone on isolated rat heart ischemia–reperfusion injury was investigated to elucidate its cardioprotective role. Nitecapone, administered in the perfusion buffer from the beginning of the pre-ischemic phase, significantly improved recovery of cardiac mechanical function, suppressed enzyme leakage in the coronary effluent, and minimized loss of ascorbate, compared with the control group. In rats fed a diet containing 4% ascorbate, myocardial ascorbate content in ascorbate-fed rats after ischemia–reperfusion was higher than that in control rats fed a normal diet without ischemia. However, supplemented rats did not show any beneficial effects on cardiac mechanical recovery or enzyme leakage, suggesting that maintenance of tissue ascorbate level is not the cause, but the result of the protective effects of nitecapone against cardiac ischemia–reperfusion injury. The iron-chelating effect of nitecapone was also tested. It was confirmed, using electron spin resonance, that 50 μM nitecapone chelates the same concentration of iron released from the heart into the coronary effluent. Hence, the iron-chelating ability of nitecapone may be responsible, at least in part, for its cardioprotective effects in ischemia–reperfusion injury.

Key words: rat heart; myocardial antioxidant; ascorbate; iron chelation

There is considerable evidence to support the hypothesis that reactive oxygen species such as the superoxide anion, hydrogen peroxide, and hydroxyl radical are key elements in reperfusion injury of the post-ischemic heart [1]. Studies in model systems have shown that exogenous antioxidants protect hearts against the ischemic–reperfused injury [2]. However, each antioxidant has a different property such as directly scavenging radicals (e.g. radical scavengers and reducing agents) or diminishing the generation of radicals (e.g. chelating transition metals and inhibiting enzymes). Therefore, the protective effects and mechanisms of various antioxidants against ischemia–reperfusion injury are expected to be different. This has significance in potential therapy, in which a combination of antioxidants with complementary mechanisms may prove to be useful.

3-(3,4-Dihydroxy-5-nitrobenzylidene)-2,4-pentanedione (nitecapone) is a new, highly potent and selective COMT† (EC 2.1.1.6) inhibitor with gastroprotective properties (Fig. 1) [3–5]. It is well tolerated and does not affect heart rate or blood pressure in humans [5–7]. We recently showed that nitecapone scavenges superoxide anion and peroxyl radical *in vitro*, and may recycle vitamin E through a reaction with ascorbate [8, 9]. In the present study, the protective effect of nitecapone against ischemia–reperfusion injury of the heart was investigated using the isolated, perfused heart model. In addition, some of the possible mechanisms of the cardioprotective effect of nitecapone, including interaction with ascorbate and chelation of transition metals, were explored.

MATERIALS AND METHODS

Animal

Experiment A: Male Sprague–Dawley rats (Bantin & Kingman, Fremont, CA) weighing 250–300 g were allowed at least 3 days of in-house acclimatization with *ad lib.* access to standard laboratory food and water.

Experiment B: Male Sprague–Dawley rats (Bantin & Kingman) (3 weeks old) were fed one of two diets differing only in the presence of ascorbate (control diet: without ascorbate; ascorbate-supplemented diet: 2% ascorbate and 2% sodium ascorbate). Four percent ascorbate supplementation was based on the study by Tsao *et al.* [10] using supplemented mice, which, like rats, are able to synthesize ascorbate. Animals were fed either the control or ascorbate-supplemented diet for 6–7 weeks.

Reagents

Nitecapone (Fig. 1) was a gift of Orion-Farmos Pharmaceuticals (Espoo, Finland). All other chemicals were obtained from standard sources. Double-distilled water was used throughout.

Experimental conditions

The rats were anesthetized with diethyl ether, and 400 U of heparin was injected intravenously. The heart was excised and the aorta was immediately attached to the perfusion apparatus using the Langendorff method [11]. A balloon catheter was inserted into the left ventricle via the left atrium and connected to a pressure transducer.

* Corresponding author: Dr. Lester Packer, Department of Molecular and Cell Biology, University of California, 251 Life Sciences Addition, Berkeley, CA 94720-3200. Tel (510)642-1872; FAX (510)642-8313.

† Abbreviations: COMT, catechol-*O*-methyltransferase; LDH, lactate dehydrogenase; DHA, dehydroascorbate; BHT, butylated hydroxytoluene; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; HR, heart rate; and LVDP, left ventricular developed pressure.

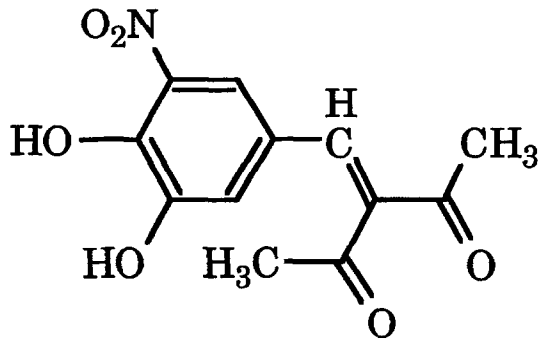

Fig. 1. Structure of nitecapone.

The perfusate was a modified Krebs-bicarbonate buffer with or without nitecapone at several different concentrations [12]. Fresh buffers were prepared on the day of each experiment and were filtered through a 0.22-μm filter [13]. The perfusate was gassed with 95% O_2/5% CO_2 and maintained at pH 7.4 and 37°. During the experiments, left ventricular pressure and heart rate were monitored using a Gould/Stathman P23 pressure transducer connected to a Gilson Duograph, and the coronary effluent was collected. The volume of coronary effluent was measured and immediately separated in two tubes; one of them was stored at −5° for iron measurement, and the other was mixed with an equal volume of 5 g/dL albumin to stabilize the enzyme activities and was stored at −5° until the measurement of enzyme activity [14].

Experimental protocols

An initial 20-min equilibration period was followed by 40 min of "no-flow" normothermic global ischemia and 20 min of reperfusion. Control hearts were subjected to 60-min perfusion without ischemia. Experiment A: Hearts of both the control and the ischemia–reperfusion group were perfused with Krebs-bicarbonate buffer including several concentrations of nitecapone (0, 10, 50, 100 or 200 μM). Experiment B: Hearts from animals fed the two different types of diet were separated further into four additional groups (control 60-min perfusion or ischemia–reperfusion, with or without 50 μM nitecapone). At the end of the experiments, hearts were freeze-clamped and stored in liquid nitrogen.

LDH activity

Myocardial LDH leakage was measured in the coronary effluent. Substrate concentrations were 180 μM nicotinamide adenine dinucleotide (reduced form) (NADH) and 0.6 μM pyruvate. After the addition of 100 μL of sample (total volume 2500 μL), oxidation of NADH was monitored spectrophotometrically at 340 nm. Each value was obtained as IU/min/g wet weight.

Tissue ascorbate assays

Ascorbate and DHA content in cardiac tissue were measured by HPLC using electrochemical detection [15]. Samples were homogenized in an ice-cold 90% methanol, 1 mM EDTA, 50 μM deferoxamine, and 1.5 mM BHT solution bubbled with argon gas. A teflon homogenizer was used at maximum speed for 1 min. After centrifugation (3000 *g* for 3 min), the supernatant was analyzed immediately by HPLC for ascorbate. For total ascorbate, the supernatant of the sample was incubated for 10–15 min with 5 mM 2,3-dimercapto-1-propanol. After incubation, the solution was extracted three times with ethyl ether. Then samples were purged with nitrogen gas for 2 min and injected immediately into the HPLC. DHA was calculated by subtraction of ascorbate from total ascorbate.

ESR measurement

DMPO (100 mM) was used as a spin trap to detect hydroxyl radicals [16]. Ten micromolar $FeSO_4$ and Krebs-bicarbonate buffer (pH 7.4) with or without 50 μM nitecapone were mixed, and then the reaction was initiated by the addition of 100 mM hydrogen peroxide. ESR spectra were recorded by a Bruker IBM ER200 D-SRC spectrometer in 50-μL capillary tubes at room temperature 5 min after the addition of hydrogen peroxide under the following conditions: microwave power, 20 mW; gain, 5×10^5; modulation, 0.125 mT; scanning field, 348 ± 5 mT; sweep time, 50 sec; time constant, 100 msec.

Other assays

The concentration of total iron in the collected coronary effluent was measured by the ferrozine method [17]. Protein determination was performed by the method of Lowry *et al.* [18] with lyophilized bovine serum albumin as the standard.

Statistical analysis

All data are expressed as means ± SEM. An analysis of variance was used for the comparison of more than two means; when a significant *F*-value was obtained, comparisons were carried out using the Student–Newmann–Keuls test. A difference was considered statistically significant at $P < 0.05$.

RESULTS

Results of functional recovery and enzyme leakage during reperfusion suggest that 50 μM nitecapone is the most efficient concentration to protect against the injury to the heart caused by ischemia–reperfusion. Nitecapone at a concentration of 50 μM improved cardiac mechanical recovery after 20 min of reperfusion following 40 min of global ischemia (Fig. 2); neither the higher concentrations (100 and 200 μM) nor the lower concentration (10 μM) provided significant protection. It was confirmed that nitecapone does not interfere with this enzyme assay. LDH leakage after ischemia–reperfusion was suppressed by nitecapone perfusion at all concentrations ($P < 0.05$, Fig. 3); at and above 50 mM nitecapone, LDH activity in the effluent was almost the same as that in the pre-ischemic conditions. Rate-pressure products and LDH leakage of the pre-ischemic phase and after 60 min of perfusion without ischemia were unchanged by any nitecapone concentration tested (data not shown).

Tissue total and reduced ascorbate contents were decreased after 20 min of reperfusion following 40 min of global ischemia without nitecapone (Fig. 4). The decrease in ascorbate was suppressed ($P < 0.05$) by perfusion with 50 μM nitecapone. Nitecapone had no effect on ascorbate levels in 60-min perfusion without ischemia.

In rats fed a diet containing 4% ascorbate to elevate

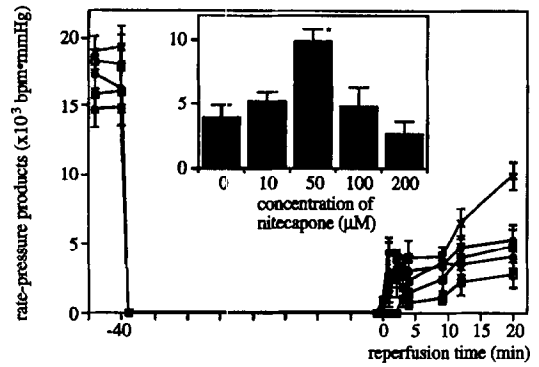

Fig. 2. Effect of various concentrations of nitecapone on cardiac mechanical recovery during reperfusion following ischemia. Rate-pressure products, indicating cardiac function, were calculated as [heart rate; HR (bpm)] × [left ventricular developed pressure; LVDP (mm Hg)]. Symbols: control (●), 10 μM (○), 50 μM (**X**), 100 μM (■), and 200 μM (□) nitecapone. Rate-pressure products at 20 min of reperfusion are shown in the bar graph. Each value is the mean ± SEM; N ≧ 4 for all points. Key: (*) $P < 0.05$ compared with the absence of nitecapone.

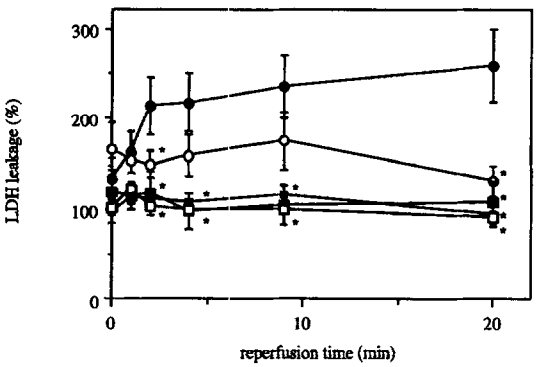

Fig. 3. Effect of various concentrations of nitecapone on LDH leakage in the coronary effluent during reperfusion after 40 min of ischemia. Time course of LDH leakage in coronary effluent during reperfusion by: control (●), 10 μM (○), 50 μM (**X**), 100 μM (■), or 200 μM (□) nitecapone. LDH activities were calculated as IU/min/g wet weight, and compared with activities of the pre-ischemic period. Each value is the mean ± SEM; N ≧ 4 for all points. Key: (*) $P < 0.05$ compared with the absence of nitecapone.

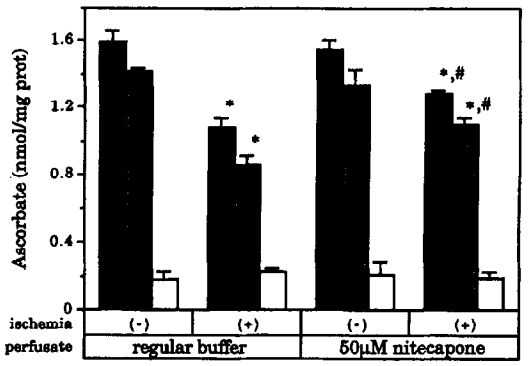

Fig. 4. Influence of nitecapone on myocardial tissue ascorbate content. Total ascorbate, black bar; ascorbate (reduced form), gray bar; and DHA (oxidized form), white bar. Each value is expressed as the mean ±SEM; N ≧ 4 for all points. Key: (*) $P < 0.05$ compared with 60-min control perfusion of each group; and (#) $P < 0.05$ compared with ischemia–reperfusion group perfused by control buffer.

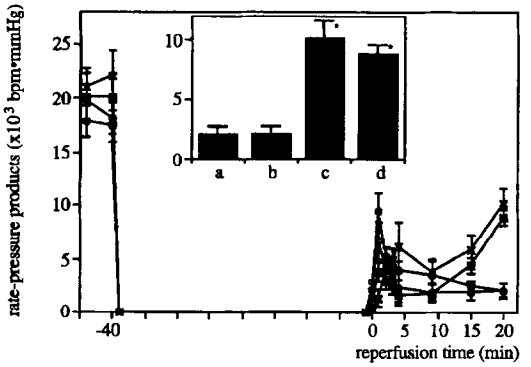

Fig. 5. Effect of ascorbate supplementation and nitecapone perfusion on cardiac mechanical recovery. Rate pressure products which indicate cardiac function were calculated as [HR (bpm)] × [LVDP (mm Hg)]. (a) Normal diet/regular buffer, ●. (b) Ascorbate-supplemented diet/regular buffer, ○. (c) Normal diet/50 μM nitecapone, **X**. (d) Ascorbate-supplemented diet/50 μM nitecapone, ■. Rate-pressure products at 20 min of reperfusion are shown in the bar graph. Each value is expressed as the mean ± SEM; N ≧ 5 for all points. Key: (*) $P < 0.05$ compared with normal diet/regular buffer.

tissue ascorbate concentration, ascorbate supplementation did not influence the growth of the rats (normal diet: 356 ± 4 g body wt; ascorbate diet: 353 ± 4 g body wt), and did not cause significant changes in cardiac recovery (Fig. 5) or LDH leakage (Fig. 6), with or without 50 μM nitecapone perfusion. Myocardial ascorbate content exhibited a number of significant changes (Fig. 7). Hearts of ascorbate-supplemented rats perfused without ischemia showed about a 30% higher value of total ascorbate compared with that of normal fed rats (2.282 vs 1.771 nmol/mg protein). Myocardial total ascorbate was decreased significantly by ischemia–reperfusion, and the degree of decrease in supplemented rats (0.383 nmol/mg protein; 17%) was almost the same as that in rats fed a normal diet (0.409 nmol/mg protein; 23%). Although ascorbate was decreased by ischemia–reperfusion in both groups, the decreased value (1.899 nmol/mg protein) was still higher than the value of 60-min control perfused hearts of rats fed a normal diet (1.771 nmol/mg protein). The same trends were seen for reduced ascorbate. In hearts perfused with 50 μM nitecapone, the myocardial content of total or reduced ascorbate did not decrease after ischemia–reperfusion in either normal or ascorbate-supplemented rats.

In all ischemia–reperfusion groups, a peak iron concentration of 4.8 μM occurred in the first 1 min of reperfusion (data not shown); as a preliminary experiment, it was confirmed that 50 μM nitecapone did not affect the measurement of iron concentration (data not shown). The assay used does not distinguish between chelated and free iron. Using 10 μM iron, which is almost twice the highest concentration of iron found in coronary effluent, the Fenton reaction was initiated with hydrogen peroxide, and generation of hydroxyl radical was detected by DMPO spin-trapping methods using ESR. A

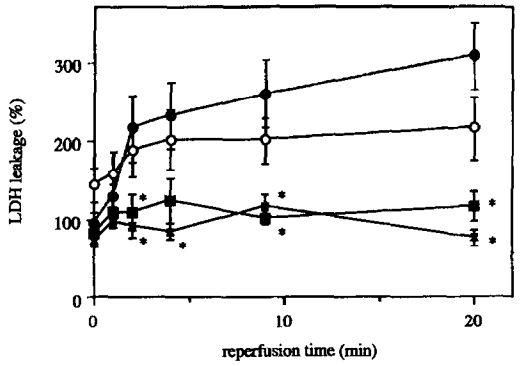

Fig. 6. Effect of ascorbate supplementation and nitecapone perfusion on LDH leakage during reperfusion following 40-min ischemia. Symbols: normal diet/regular buffer (●); ascorbate-supplemented diet/regular buffer (○); normal diet/50 μM nitecapone (X), and ascorbate-supplemented diet/50 μM nitecapone (■). LDH activities were calculated as IU/min/g wet weight, and compared with activities of the pre-ischemic period. Each value is expressed as the mean ± SEM; N ≧ 5 for all points. Key: (*) $P < 0.05$ compared with normal diet/regular buffer.

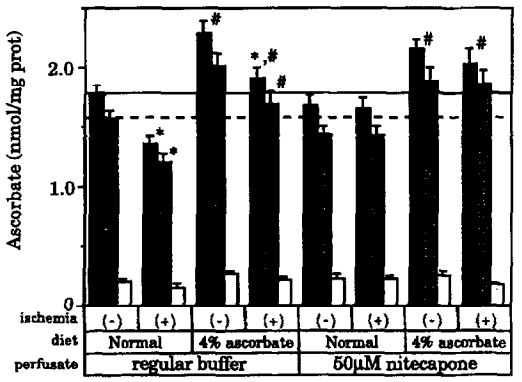

Fig. 7. Influence of ischemia, ascorbate supplementation, and nitecapone perfusion on myocardial ascorbate content. Total ascorbate, black bar; ascorbate (reduced form), gray bar; DHA (oxidized form), white bar. Each value is the mean ± SEM; N ≧ 5 for all points. Key: (*) $P < 0.05$ compared with 60-min control perfusion of each group; and (≠) $P < 0.05$ compared with the same condition in rats fed a normal diet. Total ascorbate and reduced ascorbate contents in hearts of rats fed a normal diet and perfused by regular buffer without ischemia are shown as a solid line and a broken line, respectively.

DMPO-OH adduct, characteristic of the hydroxyl radical with hyperfine splitting constants of $ab^H = aa^H = 1.48$ mT, was formed during reaction in Krebs-bicarbonate buffer (Fig. 8). In contrast, almost no DMPO-OH adduct was detected in the same reagent with 50 μM nitecapone.

DISCUSSION

It is thought that oxygen-derived free radical species play an important role in cardiac ischemia–reperfusion injury [19]. In the present study, the influence of nitecapone, which possesses antioxidant properties, on ischemia–reperfusion injury of the isolated rat heart was investigated using the Langendorff aortic retrograde perfusion system. Nitecapone protected against ischemia–reperfusion injury, as shown by improved cardiac functional recoveries (Fig. 2) and decreased LDH leakage (Fig. 3), with an optimum concentration of 50 μM. Ischemia–reperfusion also caused a decrease in cardiac ascorbate concentration, as seen in previous studies [12, 20], and 50 μM nitecapone significantly reduced this decrease (Fig. 4).

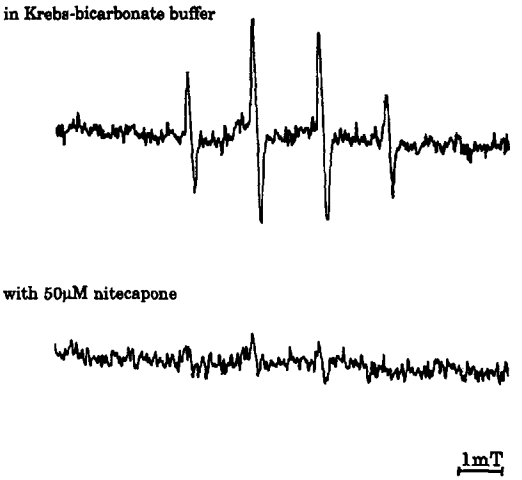

Fig. 8. Chelating effect of nitecapone against iron detected by suppressing the Fenton reaction. DMPO (100 mM) was used as a spin trap to detect hydroxyl radicals. $FeSO_4$ (10 μM) and Krebs–bicarbonate buffer (pH 7.4) with or without 50 μM nitecapone were mixed, and then the reaction was initiated by the addition of 100 mM hydrogen peroxide. ESR spectra were recorded by a Bruker IBM ER200 D-SRC spectrometer in 50-μL capillary tubes at room temperature 5 min after the addition of hydrogen peroxide.

We hypothesized that nitecapone may prevent ischemia–reperfusion injury through its effects on ascorbate; ascorbate is the primary preventative antioxidant in plasma [20–22], and also nitecapone may recycle vitamin E by way of reduction of ascorbate [8]. If this hypothesis is correct, then a higher tissue ascorbate level should show a cardioprotective effect, even without nitecapone. When rats fed an ascorbate-supplemented diet were compared with those fed a normal diet, the values of total and reduced ascorbate in the hearts of the supplemented rats subjected to ischemia were maintained at higher levels than those of 60-min control perfused hearts of the rats fed a normal diet (Fig. 7). However, ascorbate supplementation did not alter cardiac functional recovery (Fig. 5) and LDH leakage (Fig. 6) during reperfusion following ischemia in the presence or absence of nitecapone. These results suggest that increased ascorbate levels are not the mechanism whereby nitecapone exerts its cardioprotective effects, and are also in contrast with previous reports that show cardioprotective effects of ascorbate perfusion against ischemia–reperfusion damage [23, 24]. Hence, the effect of intracellular ascorbate is different from that of extracellular ascorbate.

Another possible mechanism for the cardioprotective effects of nitecapone may be through chelation of transition metals. There are some reports of leakage of transition metals during reperfusion after ischemia as well as protective effects of metal chelators [25–28]. Nitecapone

is a derivative of catechol, which is known to have a strong iron-chelating ability. Nitecapone (50 μM) significantly suppressed the generation of hydroxyl radicals by the Fenton reaction using 10 μM iron, twice the highest concentration found in coronary effluent (Fig. 8). Other studies in our laboratory have demonstrated that nitecapone strongly binds to iron with a ratio (nitecapone:iron) of 3:1 (Kawabata *et al.*, manuscript in preparation). Since 50 μM nitecapone was perfused in this study, it is not surprising that 10 μM iron was chelated easily.

In summary, nitecapone was found to be an effective protective agent in cardiac reperfusion injury, with an optimum concentration in the perfusate of 50 μM. Although nitecapone maintains tissue ascorbate levels, elevated tissue ascorbate is not in itself protective against ischemia–reperfusion. Nitecapone scavenges many free radical species such as superoxide anion and peroxyl radicals [8, 9]. In addition to these scavenging activities, the iron-chelating ability of nitecapone may be responsible for the cardioprotective effects of nitecapone in ischemia–reperfusion injury.

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