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The beneficial effects of peroxynitrite on ischaemia–reperfusion arrhythmias in rat isolated hearts

Sedat Altuğ *, A.T. Demiryürek, İclal Çakıcı, İlker Kanzık

Department of Pharmacology, Faculty of Pharmacy, Gazi University, Hipodrom, 06330, Ankara, Turkey

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Abstract

The simultaneous production of nitric oxide (NO) and superoxide leads to the formation of a potent toxic metabolite peroxynitrite ($ONOO^-$). However, $ONOO^-$ at low concentrations has been found to exert cardioprotective effects. The purpose of the present study was to investigate the effects of exogenous $ONOO^-$ on ischaemia–reperfusion arrhythmias. We studied the concentration–response effects of $ONOO^-$ (0.4, 4, 40 μM ml^{-1} min^{-1} for 20 min) in rat isolated hearts perfused with Krebs–Henseleit solution. The 0.4 μM concentration of $ONOO^-$ was selected for further experiments since it did not affect the sinus rhythm. In the hearts subjected to 10 min of ischaemia followed by 10 min of reperfusion during 0.4 μM ml^{-1} min^{-1} $ONOO^-$ infusion, the incidence of ventricular fibrillation was decreased significantly from 93% to 38% ($n = 8$) and none of the hearts had an irreversible ventricular fibrillation. Urate, a $ONOO^-$ scavenger (at 1 mM, $n = 7$), added to the perfusate 5 min prior to the coronary artery occlusion and maintained throughout the experimental period, did not significantly modify the beneficial effects of $ONOO^-$. Although L-N^G-nitroarginine methylester (L-NAME) (100 μM, $n = 8$) had no effect, superoxide dismutase (10 U ml^{-1}) + catalase (100 U ml^{-1}) increased the number of ventricular ectopic beats from 91 ± 32 to 286 ± 83 ($n = 5$) and augmented the incidence of irreversible ventricular fibrillation from 0% to 60%. There were no marked changes in the time of onset of the first arrhythmias in any group. These results suggest that $ONOO^-$ at a low concentration may exert beneficial effects on ischaemia–reperfusion-induced arrhythmias in rat isolated hearts.

Keywords: Peroxynitrite; Ischaemia–reperfusion arrhythmia; Heart; (Rat); Urate

1. Introduction

Although reperfusion of the ischaemic myocardium seems the only way to salvage the myocardium from necrosis, reperfusion after a short period of ischaemia leads to reperfusion arrhythmias (Kane et al., 1984). The cause of myocardial reperfusion injury seems to be multifactorial, but nitric oxide (NO) and reactive oxygen species including superoxide play an important role (Woodward and Zakaria, 1985; Weyrich et al., 1992). NO formation is markedly increased during ischaemia in rat isolated heart (Zweier et al., 1995). Immediately upon reflow NO levels increased to 30-fold higher than pre-ischaemic levels and remained at levels 10-fold above pre-ischaemic values throughout the first 5 min of reflow; however, a gradual decrease was seen over next 15 min but the level was still higher compared to the pre-ischaemic value (Wang and Zweier, 1996). Superoxide radical was also formed immediately upon reperfusion and its maximal levels were observed at 40 s of reperfusion and gradually decreased to pre-ischaemic values after 5 min of reperfusion (Wang and Zweier, 1996). Furthermore, NO can react with superoxide to form peroxynitrite ($ONOO^-$), a potentially toxic molecule that may play a pivotal role in the pathophysiology of myocardial reperfusion injury (Beckman et al., 1994; Liu et al., 1997). There is considerable experimental evidence to suggest that during the early reperfusion period $ONOO^-$ formation occurs parallel to superoxide formation. A marked increase in $ONOO^-$ formation was also observed during the early period of reperfusion (Wang and Zweier, 1996; Yasmin et al., 1997). Although high concentrations of $ONOO^-$ appear to be responsible for most of the NO-mediated toxicity, in recent years, it has become apparent that low levels of $ONOO^-$ are also able to mediate beneficial effects in some physiological processes (Beckman and Koppenol, 1996). $ONOO^-$ relaxes various

* Corresponding author. Tel.: +90-312-2222380; fax: +90-312-2211867.

E-mail address: saltug@tr-net.net.tr (S. Altuğ)

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arteries including coronary arteries through stimulation of cGMP (Liu et al., 1994; Wu et al., 1994; Ku et al., 1995). $ONOO^-$ produces *S*-nitrosothiols which stimulate guanylyl cyclase and release NO (Moro et al., 1994; Wu et al., 1994). $ONOO^-$ has also been found to be cardioprotective in micromolar concentrations both in in vivo and in vitro experiments (Lefer et al., 1997; Nossuli et al., 1997, 1998). Lefer et al. (1997) were the first to describe the cytoprotective effects of low concentrations of $ONOO^-$ against myocardial ischaemia–reperfusion injury in isolated rat heart perfused with rat neutrophils. To our knowledge, there have been no reports on effects of exogenously administered $ONOO^-$ on reperfusion arrhythmias in rat isolated hearts. Therefore, the aim of the present study was to examine the effects of exogenous $ONOO^-$ administration on ischaemia–reperfusion arrhythmias. Another aim was to investigate the concentration–response relationship of exogenous $ONOO^-$ administration on the sinus rhythm of rat isolated hearts.

2. Materials and methods

2.1. Preparation of isolated hearts and coronary artery occlusion / reperfusion

Male Wistar rats obtained from the animal unit of our department weighing 200–400 g were used in this study. The rats were acclimatised to a 12-h light: 12-h dark cycle at 25°C and supplied with standard laboratory diet and tap water ad libitum. Animals were anaesthetised intraperitoneally with thiopental (60 mg kg^{-1}). After the induction of anaesthesia the abdomen was opened and heparin (500 U) was given through the renal vein. After 3 min, the abdominal aorta was cut to reduce the blood volume in the heart. Then the thorax was opened and the heart was quickly excised and put into a Petri dish containing ice-cold Krebs solution of the following composition (mM): NaCl 118; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 1.23; glucose 11 (Woodward and Zakaria, 1985). Then the heart was perfused retrogradely via the aorta by means of a modified Langendorff apparatus at a constant flow of 8–10 ml min^{-1} with Krebs solution at 37°C gassed with 95% O_2 and 5% CO_2 (Piacentini et al., 1993). The perfusion rate was determined according to animal weight using the formula: flow (ml min^{-1}) = $x^{0.56}7.43$ (x is the heart weight) heart weight = $0.0027y + 0.6$ (y is the body weight). A loose ligature was immediately placed round the main left coronary artery, and both ends of the ligature were then passed through a short piece of polythene tubing (1-mm i.d. and 1.5-mm long) to form a snare. Following a stabilisation period of 15 min, the potassium concentration of the perfusate was reduced to 3.2 mM, as low concentrations of potassium enhance the development of reperfusion-induced arrhythmias in the rat isolated heart (Woodward and Zakaria, 1985, Demiryürek et al., 1998). This was achieved by reducing the concentration of KCl from 4.7 to 2.0 mM while maintaining the concentration of KH_2PO_4 at 1.2 mM (Woodward and Zakaria, 1985). After 5 min, the snare around the left coronary artery was tightened and held in place with a small clip. An increase in perfusion pressure indicated successful ligation. Ten minutes later the clip holding the ligature was removed; a decrease in perfusion pressure indicated successful reperfusion which was maintained for 10 min. An electrocardiogram (ECG) was recorded by two electrodes placed on the right atrium and apex throughout the experiment by using computerised data acquisition system (TDA 95 Maycom, Turkey) (Altuğ et al., 1999). Coronary perfusion pressure was measured with a pressure transducer attached to the system and recorded continuously with the same system. Any heart which had ventricular fibrillation or ventricular tachycardia during the ischaemia period were excluded from the study.

2.2. Experimental protocol

After the completion of surgical procedures, all hearts were allowed to stabilise for 15 min prior to the experimental protocol. In the first group of experiments, the concentration of $ONOO^-$ which did not cause rhythm disturbances was determined. Three different concentrations of $ONOO^-$, 0.4, 4 and 40 μM ml^{-1} min^{-1} for 20 min, at pH = 8.4 (Nossuli et al., 1997) were infused through the rubber tubing placed just proximal to the heart. Stock solutions were kept on ice, and they and the infusion lines were wrapped in aluminum foil to reduce exposure to light. For the rest of the experiments, the concentration of 0.4 μM ml^{-1} min^{-1} was chosen since $ONOO^-$ induced no arrhythmias at this concentration. In order to study the

Table 1
Effects of $ONOO^-$ infused for 20 min in three different concentrations (0.4, 4, 40 μM ml^{-1} min^{-1}) on sinus rhythm
VEBs, Ventricular ectopic beats defined as discrete and identifiable premature QRS complexes; VT, ventricular tachycardia; VF, ventricular fibrillation.

	n	Total VEBs	Time of onset of first arrhythmias (s)	VT time (s)	Irreversible VF time (s)	Total VF time (s)
$ONOO^-$ (40)	5	119 ± 54	96 ± 77[a]	77 ± 24	–	226 ± 140
$ONOO^-$ (4)	5	209 ± 76[a]	393 ± 92[a]	150 ± 52	296 ± 42	455 ± 92
$ONOO^-$ (0.4)	8	18 ± 5	732 ± 84	–	–	–

[a] $P < 0.05$ compared to $ONOO^-$ 0.4.

Table 2
Effects of $ONOO^-$ infused for 20 min at 0.4 μM ml^{-1} min^{-1} on reperfusion-induced arrhythmias
VEBs, Ventricular ectopic beats defined as discrete and identifiable premature QRS complexes; VT, ventricular tachycardia; VF, ventricular fibrillation.

	n	Total VEBs	Time of onset of first arrhythmias (s)	% VT	% Irreversible VF	% Total VF
Control	15	95 ± 32	3 ± 0.2	100	47	93
$ONOO^-$ (0.4)	8	91 ± 32	2 ± 0.2	88	0	38[a]

[a] $P < 0.05$ compared to $ONOO^-$ 0.4.

effects of $ONOO^-$ on ischaemia–reperfusion arrhythmias, $ONOO^-$ (0.4 μM ml^{-1} min^{-1}) was infused into the perfusion solution at the beginning of occlusion during both ischaemia and reperfusion periods (for 20 min) through the rubber tubing placed just proximal to the heart. In the third group of experiments, the effects of $ONOO^-$ on reperfusion-induced arrhythmias were investigated by administration of several drugs. L-N^G-nitroarginine methylester (L-NAME) (100 μM) (Pabla and Curtis, 1995), urate (1 mM) (Lacursiere and Kingma, 1991) and superoxide dismutase (10 U ml^{-1}) in combination with catalase (100 U ml^{-1}) (Demiryürek et al., 1998) were added to perfusion solution 5 min before the coronary artery occlusion and perfused until the end of reperfusion period in the presence of $ONOO^-$ infusion (0.4 μM ml^{-1} min^{-1}).

2.3. Measured parameters

Heart rate was measured from the recordings of ECG with 15-s cycles. The incidence of arrhythmias was registered in accordance with the Lambeth conventions (Walker et al., 1988) as ventricular fibrillation and ventricular ectopic beats. Ventricular tachycardia was diagnosed as four or more consecutive ventricular ectopic beats. Ventricular fibrillation was diagnosed when ECG recording showed chaotic activity with an amplitude less than that of the normal ECG. Irreversible ventricular fibrillation was defined as ventricular fibrillation which did not reverse within the 10-min period of reperfusion. The onset and duration of arrhythmias were also measured.

2.4. $ONOO^-$ synthesis

$ONOO^-$ was synthesised in our laboratory from acidified nitrite and hydrogen peroxide according to the method of Beckman et al. (1994). Fresh dilutions were made with Krebs solution just before use and the pH of this solution was adjusted to 8.4 by addition of an appropriate volume of 0.1 N NaOH (Nossuli et al., 1997). The stock solutions were aliquoted and stored at −18°C for a week.

2.5. Materials

L-NAME, urate, superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver), and sodium nitrite were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide was purchased from Merck (Darmstat, Germany).

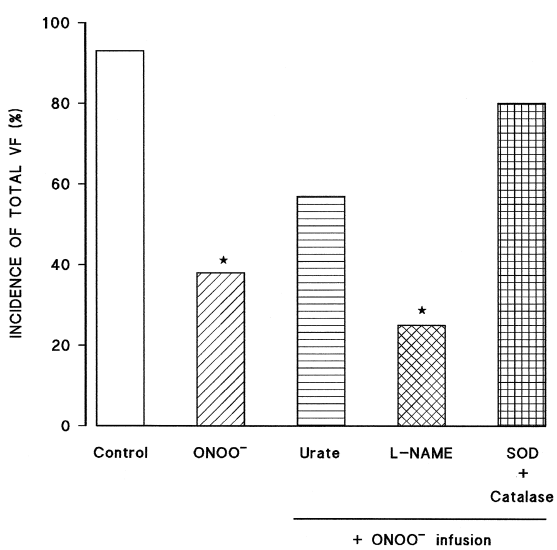

Fig. 1. Effects of urate ($n = 7$), L-NAME ($n = 8$), superoxide dismutase (SOD) and catalase ($n = 5$) on the incidence of ventricular fibrillation (VF) on rat isolated hearts infused with $ONOO^-$ ($n = 8$) (0.4 μM ml^{-1} min^{-1} for 20 min). *$P < 0.05$ compared to control group.

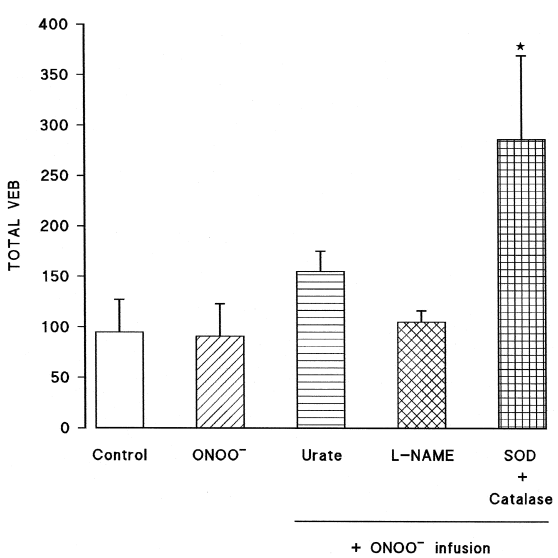

Fig. 2. Effects of urate ($n = 7$), L-NAME ($n = 8$), SOD and catalase ($n = 5$) on the number of total ventricular ectopic beats (VEB) in rat isolated hearts infused with $ONOO^-$ ($n = 8$) (0.4 μM ml^{-1} min^{-1} for 20 min). *$P < 0.05$ compared to control group.

Table 3
Effects of urate (1 mM), L-NAME (100 μM) and superoxide dismutase (10 U ml^{-1}) + catalase (100 U ml^{-1}) infused for 20 min with $ONOO^-$ (0.4 μM ml^{-1} min^{-1}) on reperfusion-induced arrhythmias
VT, ventricular tachycardia; VF, ventricular fibrillation.

	n	Time of onset of first arrhythmias (s)	% VT	% Irreversible VF
$ONOO^-$	8	2 ± 0.2	88	0
Urate + $ONOO^-$	7	3 ± 0.2	100	14
L-NAME + $ONOO^-$	8	3 ± 0.2	100	0
SOD + Catalase + $ONOO^-$	5	4 ± 0.6	100	60*

*$P < 0.05$ compared to $ONOO^-$.

2.6. Statistical analysis

Values are presented as means ± standard error of the mean or as percentage incidence. A Fischer's extract χ^2-test was used to detect significant differences in the incidence of ventricular tachycardia, ventricular fibrillation, and irreversible ventricular fibrillation between control and drug-treated groups. Statistical comparison of more than two groups was performed by a One-way analysis of variance followed by Student–Newman–Keuls test. In all tests, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of $ONOO^-$ on sinus rhythm

In order to examine the effects of $ONOO^-$ infusion on sinus rhythm, three different concentrations of $ONOO^-$ were studied (0.4, 4, 40 μM ml^{-1} min^{-1}). At 40 and 4 μM ml^{-1} min^{-1}, $ONOO^-$ produced severe arrhythmias (Table 1). The total ventricular fibrillation time was 226 ± 140 s ($n = 5$) at 40 μM ml^{-1} min^{-1} and 455 ± 92 ($n = 5$) at 4 μM ml^{-1} min^{-1}. However, at 0.4 μM ml^{-1} min^{-1}, $ONOO^-$ only produced a small number of ventricular ectopic beats, 18 ± 5 ($n = 8$) (Fig 2). Therefore, this concentration was chosen for the further studies with ischaemia–reperfusion experiments since it did not affect the sinus rhythm markedly.

3.2. Effects of $ONOO^-$ infusion on reperfusion-induced ventricular arrhythmias

The number and the incidence of ventricular arrhythmias over a 10-min reperfusion period in control and $ONOO^-$-treated rat isolated hearts are shown in Table 2. Although $ONOO^-$ at 0.4 μM ml^{-1} min^{-1} had no effect on the time of onset of the first arrhythmia, it caused a significant reduction in the incidence of ventricular fibrillation. None of the hearts had an irreversible ventricular fibrillation attack in this group (Table 2). $ONOO^-$ administration did not cause any significant change in coronary perfusion pressure and pressure rate index measured at certain time points throughout the experiment (data not shown).

3.3. Effects of urate, L-NAME, superoxide dismutase + catalase treatments on reperfusion-induced ventricular arrhythmias in $ONOO^-$-infused groups

Administration of urate (1mM) did not change the $ONOO^-$ induced beneficial effects on reperfusion-induced arrhythmias, although it appeared to increase the number of ventricular ectopic beats from 91 ± 32 ($n = 8$) to 155 ± 20 ($n = 7$) and the incidence of ventricular fibrillation from 38% to 57%. However, these differences were not statistically significant (Figs. 1 and 2). L-NAME (100 μM) treatment reduced the incidence of total ventricular fibrillation from 38% to 25% but did not affect the incidence of irreversible ventricular fibrillation whereas superoxide dismutase (10 U ml^{-1}) + catalase (100 U ml^{-1}) administration significantly increased the incidence of irreversible ventricular fibrillation from 0% to 60%. Also, the number of ventricular ectopic beats was significantly increased with superoxide dismutase + catalase administration from 91 ± 32 ($n = 8$) to 286 ± 83 ($n = 5$). None of the drug treatments changed the time of onset of the first arrhythmias. Urate, L-NAME and superoxide dismutase + catalase administration did not cause any significant change in coronary perfusion pressure and pressure rate index measured at certain time points throughout the experiment (data not shown) (Table 3).

4. Discussion

In the present study, we found that exogenously administered $ONOO^-$ at a low concentration showed no marked effect on sinus rhythm and produced cardioprotective effects against ischaemia–reperfusion-induced arrhythmias in rat isolated hearts. To our knowledge, this study is the first investigation of the effects of $ONOO^-$ on reperfusion-induced arrhythmias in physiological salt-perfused heart. Our results are, in part, in agreement with those of

Schulz et al. (1997), who found that $ONOO^-$ infusion at a high concentration of 40 μM, but not 4 μM, over a 60-min period caused a marked depression in cardiac work and a marked loss of cardiac efficiency. Our results also showed that $ONOO^-$ infusion at high concentrations (4 and 40 μM) over 20-min-induced arrhythmias while at a low concentration of $ONOO^-$ (0.4 μM) markedly reduced ventricular fibrillation in rat hearts. It has been proposed that the maximally achievable concentration of $ONOO^-$ formed under in vivo conditions is in the low micromolar range (i.e., 2–5 μM), and that concentrations above these levels are probably not be formed in vivo (Nossuli et al., 1998). Therefore, the concentrations of $ONOO^-$ (0.4 and 4 μM) used in the present experiment are highly likely to be reached in vivo.

Endogenous NO generation does not appear to be involved in the effects of $ONOO^-$ in our experiments. Our results may support the finding that inhibition of NO production improves myocardial function and reduces arrhythmias after ischaemia–reperfusion in rat-isolated perfused hearts (Naseem et al., 1995).

Reperfusion of an ischaemic myocardium provides an excellent environment for the genesis of oxygen-derived free radicals (Manning et al., 1988). It is known that oxygen-derived free radicals play an important role in the genesis of reperfusion-induced arrhythmias (Woodward and Zakaria, 1985; Bernier et al., 1986; Vergely et al., 1998) and removal of superoxide and hydrogen peroxide can protect hearts against ischaemia–reperfusion arrhythmias (Woodward and Zakaria, 1985). However, superoxide dismutase and catalase administration in the presence of $ONOO^-$ infusion in our experiments caused an augmentation of the severity of reperfusion-induced arrhythmias. The underlying mechanism of this augmentation is presently unknown.

The cardioprotective effect of $ONOO^-$ infusion at 0.4 μM ml^{-1} min^{-1} was not abolished with urate treatment although uric acid is a well-known scavenger of $ONOO^-$ (Hooper et al., 1998). Our findings are in agreement with Lacursiere and Kingma (1991), who showed that a high concentration of urate was ineffective in ischaemia–reperfusion arrhythmias in rat hearts. In contrast, 1 mM urate effectively quenched $ONOO^-$-induced luminol chemiluminescence in rat hearts (Wang and Zweier, 1996). Recently, it has been shown that uric acid can react with $ONOO^-$ to form a NO donor (Skinner et al., 1998). This may explain the cardioprotective effects seen in the presence of uric acid and $ONOO^-$ in our experiments.

Since $ONOO^-$ administration did not cause any significant change in coronary perfusion pressure and pressure rate index measured at certain time points throughout the experiment, the cardioprotective effect of $ONOO^-$ was not due to effects on these variables.

Although the exact mechanism of cardioprotective action of $ONOO^-$ is not known, there are some possibilities (Nossuli et al., 1998). Firstly, $ONOO^-$ can *S*-nitrosylate glutathione or other thiol-containing substances in tissues, causing the formation of *S*-nitrosothiols (Moro et al., 1994; Wu et al., 1994). *S*-nitrosothiols can directly activate guanylyl cyclase and also release NO over sustained periods of time (Wu et al., 1994). Secondly, $ONOO^-$ may form intermediates that act as NO donors in the presence of plasma, proteins, glucose or glutathione (Moro et al., 1994, 1995). Elevation of NO levels results in elevation of intracellular cGMP levels, which may be responsible for the anti-arrhythmic activity. Also, myocardial cAMP plays an important role in the genesis of ventricular arrhythmias in the ischaemic heart. Modulation of cAMP concentrations can take place at the level of adenylyl cyclases or cAMP phosphodiesterases. The level of intracellular cGMP controls the activity of phosphodiesterases (Have-Madsen et al., 1996). Thus, elevation of the myocardial cGMP concentration by NO may produce an anti-arrhythmic effect by decreasing the cAMP content and this may be responsible for the cardioprotective effects of $ONOO^-$ seen in our experiment. Lastly, $ONOO^-$ can directly activate guanylyl cyclase, leading to cGMP accumulation in vascular smooth muscle (Tarpey et al., 1995), resulting in coronary relaxation.

In summary, the results of this study show that exogenously administered $ONOO^-$ at low concentrations may exert cardioprotective effects on ischaemia–reperfusion-induced arrhythmias in rat isolated hearts. Although there are some possibilities which may account for this protection, the underlying mechanism remains unclear and requires further investigation.

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