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INHIBITION OF NITRIC OXIDE LIMITS INFARCT SIZE IN THE *IN SITU* RABBIT HEART

VC Patel, DM Yellon, KJ Singh, GH Neild*, and RG Woolfson*,+

The Hatter Institute for Cardiovascular Studies, Department of Academic Cardiology, and *Department of Nephrology, Institute of Urology and Nephrology, University College London Medical School, Gower Street, London WC1E 6AU, UK

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Recent data has suggested a dual role for nitric oxide (NO) so that it can both attenuate myocardial injury during ischaemia and reperfusion as well as mediate reperfusion injury. In this study in the *in situ* rabbit heart, we have shown that pretreatment with intravenous N^G-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthesis) significantly reduced infarct size following sustained coronary artery occlusion and reperfusion. L-NAME was also noted to increase myocardial lactate concentration. This study provides further evidence that protection against ischaemia-reperfusion injury can be derived from manipulation of the microcirculation.

The mechanism of myocardial injury following ischaemia and reperfusion is multi-factorial and depends on the duration of ischaemia, the presence of a collateral circulation, the adhesion, activation and extravasation of neutrophils together with the generation of free radicals. The basal release of low levels of NO by the endothelium maintains the coronary microvasculature in active vasodilatation and minor augmentation of NO levels during ischaemia and reperfusion, which are insufficient to affect blood pressure have been shown to mitigate against myocardial injury (1-3). Conversely, it has been suggested that NO may be cytotoxic when present in substantial excess (4). This deleterious role of NO may be important firstly, during early reactive hyperaemia which is in part a response to increased NO release (5,6) and, secondly, in the presence of activated leucocytes which express inducible NO synthase (7,8). Evidence to support a noxious role for NO comes from experimental models of myocardial injury following hypoxaemia (9), post-ischaemic cerebral reperfusion injury (10) and neutrophil-mediated tissue injury (11).

+To whom correspondence should be addressed at The Hatter Institute for Cardiovascular Studies, Department of Academic Cardiology, University College London Medical School, Gower Street, London WC1E 6AU, UK.

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In view of this biphasic role for NO, we have investigated the effects of inhibition and augmentation of endothelium-derived NO production during regional myocardial ischaemia and reperfusion in the *in situ* rabbit heart, using infarct size as the endpoint.

MATERIALS AND METHODS

Male New Zealand White rabbits (mean weight 2.59 ± 0.04 kg, n=54) were anaesthetised with pentobarbital (40 mg/kg i.v.), ventilated mechanically and the left carotid artery cannulated. Following a left thoracotomy, the heart was exposed and a snare permitting intermittent occlusion placed proximally around the left circumflex artery. Myocardial ischaemia was confirmed by regional cyanosis and akinesis, with reperfusion being confirmed by blushing of the previously cyanotic myocardium. Heparin (1000 units) was administered intravenously at the start and conclusion of each experiment. L-NAME (10mg/kg), L-arginine (300mg/kg) or normal saline was infused over 1 min and the animals then monitored for 10 min. Sustained ischaemia lasted either 30 or 50 min and was followed by either 2 or 3 h reperfusion.

GROUP 1: Control - 30 min ischaemia and 2h reperfusion.
GROUP 2: L-NAME - 30 min ischaemia and 2h reperfusion.
GROUP 3: Control - 50 min ischaemia and 3h reperfusion.
GROUP 4: L-NAME - 50 min ischaemia and 3h reperfusion.
GROUP 5: L-arginine - 50 min ischaemia and 3h reperfusion.

At the conclusion of the experiment, the heart was excised, mounted on a Langendorff apparatus and perfused with saline for 1 min. The snare was occluded and the heart perfused with 1-10 μm diameter fluorescent particles to define the borders of the dependent myocardium supplied by the occluded artery (i.e. risk area). The heart was frozen, sliced into 2 mm thick sections, thawed and incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C for 20 minutes to define infarcted tissue. Infarct (TTC-negative) and risk (lacking UV fluorescence) areas were planimetered for the entire heart with infarct area being expressed as a percentage of risk area. This experimental method is well established (12).

In a second series of experiments, *in situ* biopsy of apical myocardium was performed 10 minutes after the intravenous injection of either L-NAME (10mg/kg) or saline. Biopsies were taken with pre-cooled (-180°C) tissue forceps with the samples being stored in liquid nitrogen prior to being freeze dried before analysis. Tissue extraction was performed using 350μl of ice cold perchloric acid (6%) followed by neutralisation to pH 5.5-6.0. Quantitative lactate estimation was performed using the enzymatic reaction of lactate dehydrogenase linked to nicotinamide adenine dinucleotide in glycine-hydrazine buffer medium (13).

Statistics: Results are expressed as mean ± standard error. Statistical analysis between groups was performed using ANOVA followed by Scheffe's F-test.

RESULTS

L-NAME significantly reduced the ratios of infarct/risk area following either 30 min (Groups 1 *vs.* 2, $p<0.05$) or 50 min (Groups 3 *vs.* 4, $p<0.02$) of ischaemia and reperfusion

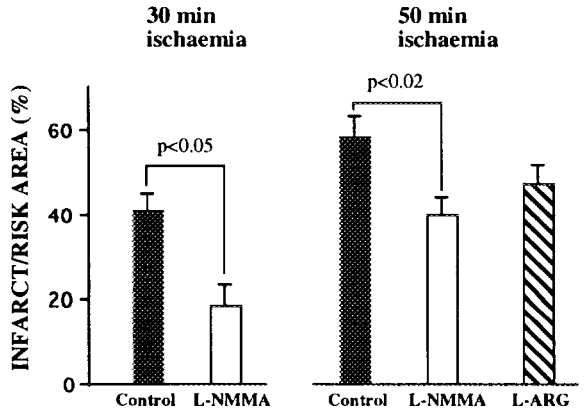

FIGURE 1. Infarct area expressed as percentage of risk area following either 30 or 50 min of coronary artery occlusion and reperfusion in the *in situ* rabbit heart.

(see Figure 1 and Table 1). Pre-treatment with L-arginine reduced the ratio of infarct/risk area (Group 3 *vs.* 5, $p<0.08$) but this failed to meet our criterion of significance. Mean risk areas were not significantly different between comparable groups (see Table 1).

L-NAME but not L-arginine lead to a rapid hypertensive response together with a reflex bradycardia. However, there was no significant differences between rate pressure products of comparable groups at any time. See Table 1.

L-NAME significantly increased myocardial lactate concentration from 5.35 ± 0.85 μmol/g dry weight (control, n=4) to 16.36 ± 2.41 μmol/g dry weight (L-NAME, n=4), $p<0.03$.

TABLE 1

Haemodynamic data are shown at start (T=0 min), 10 min after saline, L-NAME or L-arginine (L-ARG) infusion (T=10 min) and at conclusion of experiment. (RPP, rate pressure product; ΔBP, change in blood pressure from time = 0 min, mmHg; ΔHR, change in heart rate from time = 0 min, bpm.)

GROUPS	**1**	**2**	**3**	**4**	**5**
n	10	10	7	10	8
Ischaemic period (min)	30	30	50	50	50
	CONTROL	**L-NAME**	**CONTROL**	**L-NAME**	**L-ARG**
Infarct/Risk (%)	40.6 ± 4.4	18.7 ± 4.6	60.7 ± 5.9	39.9 ± 4.3	47.3 ± 4.3
Risk area (cm^2)	5.2 ± 0.5	5.7 ± 0.7	3.5 ± 0.4	4.2 ± 0.4	5.1 ± 0.7
T = 0 min					
RPP	23793 ± 2035	22755 ± 1940	26983 ± 1103	24352 ± 1092	23332 ± 1362
T = 10 min					
ΔBP	- 9 ± 2	21 ± 3	- 1 ± 3	23 ± 5	0 ± 4
ΔHR	- 9 ± 4	- 19 ± 3	- 1 ± 6	- 27 ± 6	-1 ± 9
RPP	21899 ± 1560	21973 ± 1543	27085 ± 1064	26982 ± 1636	23297 ± 1473
T = conclusion of experiment					
RPP	16880 ± 958	16885 ± 2540	18296 ± 824	16973 ± 1398	15502 ± 2256

DISCUSSION

In this study, pretreatment with L-NAME reduced the ratio of infarct/risk area after both 30 and 50 minutes of regional myocardial ischaemia and reperfusion in the *in situ* rabbit heart. NO has been implicated in tissue injury, firstly, as a result of its capacity to interact with oxygen-derived free radicals to produce toxic intermediates (such as the peroxynitrite anion) (4) and, secondly, because NO avidly binds and thereby inactivates iron-sulphur-centred enzymes which are required for essential cellular metabolic activity including mitochondrial respiration (14). NO-induced injury is likely to be important in conditions of relative NO excess, for example in the presence of activated neutrophils (8,15) or during reactive hyperaemia (5,6). In this study the protection following pretreatment with L-NAME was not likely to have been mediated by an effect on neutrophil accumulation because inhibition of NO increases neutrophil adhesion to endothelial cells and this is associated with increased myocardial injury (16). In addition, L-NAME would not have inhibited formation of neutrophil-derived NO because it is not an effective inhibitor of the inducible NO synthase expressed by activated neutrophils (15). Therefore the observed protection following NO inhibition may have resulted from another mechanism which is likely to have involved the inhibition of endothelium-derived NO which is known to be released in substantially greater quantities during reactive hyperaemia (5,6).

Paradoxically, pre-treatment with L-arginine also reduced myocardial injury following 50 minutes of regional ischaemia and reperfusion in the *in situ* rabbit heart although this was not significantly different from control. An earlier study reported that pretreatment with L-arginine protected the *in situ* feline heart from regional ischaemia (3) and therefore we consider our findings to demonstrate a Type 2 statistical error which reflects inadequate group size. The mechanism for L-arginine protection, and for the protection conferred by low concentrations of NO donors (1,2), seems to involve the preservation of physiological endothelial cell layer integrity and enhancement of NO release leading to a reduction in the accumulation of neutrophils in the ischaemic zone.

An alternative and novel mechanism to explain our findings follows the now well-established observation that a brief period of ischaemia and reperfusion protects the myocardium against subsequent sustained ischaemia and reperfusion (17). This phenomenon, termed "ischaemic preconditioning", is mediated by the release of adenosine during the brief ischaemic period from ischaemic cells which fail to cycle ATP (12). During the brief period of reperfusion and prior to the sustained ischaemia, the increased ambient concentrations of adenosine stimulate cardiac myocytes (*via* A_1 receptors) which protects them against the subsequent ischaemic challenge by an unidentified mechanism (ref. 18 for review). In our

study, pre-treatment with L-NAME significantly increased myocardial lactate levels which may indicate mild tissue ischaemia. This evidence that L-NAME causes myocardial ischaemia is consistent with a previous study which noted that L-NAME increases the concentration of adenosine in the coronary perfusate (5). Thus these observations give rise to the novel possibility that L-NAME caused myocardial ischaemia with a resultant increase in adenosine release; this increase in ambient adenosine levels effectively "preconditioned" the myocardium against the subsequent sustained ischaemic insult.

In summary, our study provides further evidence that protection against myocardial injury following ischaemia and reperfusion can be derived from manipulation of the NO system. Furthermore, we have provided preliminary evidence of a novel mechanism to explain the observed protection against myocardial ischaemia-reperfusion injury following inhibition of endothelium-derived NO.

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