iNOS is a mediator of the heat stress-induced preconditioning against myocardial infarction in vivo in the rat

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Abstract

Objective: The inducible isoform of nitric oxide synthase (iNOS) is known to be a trigger of the heat stress (HS)-induced cardioprotection. Since iNOS also appears to mediate various forms of myocardial preconditioning, the goal of this study was to investigate its role as a mediator of the HS response. Methods and Results: Male Wistar rats were divided in six groups, subjected or not to HS (42 °C internal temperature, for 15 min). Twenty-four hours later, they were treated or not with either L-NAME, a non-selective inhibitor of NO synthase isoforms, or 1400W, a selective iNOS inhibitor, 10 min before being subjected to a 30-min left coronary artery occlusion followed by a 120-min reperfusion, in vivo. The infarct size (tetrazolium staining) reducing effect conferred by heat stress (from 46.0 ± 1.4% in sham to 26.8 ± 3.8% in HS groups) was completely abolished by both L-NAME (53.9 ± 3.1%) and 1400W (51.8 ± 3.3%). Additional studies using Western blot analysis demonstrated a 3.8-fold increase in myocardial iNOS protein expression 24 h after HS. Conclusion: These results suggest an involvement of iNOS as a mediator of the protection conferred by heat stress against myocardial ischaemia.

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Keywords: Infarction; Ischemia; Nitric oxide; Preconditioning

1. Introduction

Heat stress (HS) is known to induce a delayed myocardial protection against sustained ischaemia–reperfusion injury [1] similar to that observed during the second window of ischaemic preconditioning (IP) [2,3]. This late cardioprotection, which appears 24 to 48 h after the stress (HS or IP), is a two-step phenomenon. Firstly, an immediate activation of molecular species, called triggers, initiates the protective response. This leads to the synthesis of new proteins, which confer or mediate the protection. In particular, a direct correlation between the amount of Hsp72 induced and the degree of myocardial protection has been observed both in the rat [4] and in the rabbit [5].

Recently, NO has been shown to be a trigger of the delayed phase of IP, in vivo in the rabbit [6,7]. We have also shown that NO triggers the HS-induced cardioprotection, since l-NAME (a non-selective NOS inhibitor) and l-NIL (a selective iNOS inhibitor) both abolished the HS-induced infarct size reduction, in the isolated rat heart [8]. Recent studies have shown that NO is also a mediator of the second window of IP. NO formation mediates this

Abbreviations: eNOS, endothelial nitric oxide synthase; HS, heat stress; Hsp, heat stress protein; I, infarct zone; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IP, ischaemic preconditioning; \( K_{\text{ATP}} \) channel, ATP-dependent potassium channel; LCA, left coronary artery; l-NAME, nitro-l-arginine-methylster; LV, left ventricle; MAP, mean arterial blood pressure; NO, nitrite oxide; p38 MAP, p38 mitogen activated protein; PKC, protein kinase C; R, risk zone; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; 1400W, N-\{(3-amino-ethyl)benzyl\} acetamidine

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delayed preconditioning against both myocardial stunning [6] and infarction [9,10], in the rabbit. Indeed, NOS inhibitors administered either during IP or 24 h later, were able to abolish the delayed cardioprotection. Thus, NO seems to act both as a trigger and as a mediator of the late phase of ischaemic preconditioning.

The aim of the present study was to investigate the role of iNOS-derived NO as a mediator of the HS-induced cardioprotection. Thus, 24 h after HS, we tested the effects on infarct size development in vivo of the non-selective NOS inhibitor, L-NAME, and the selective iNOS inhibitor, 1400W [11], given just prior to the ischaemia–reperfusion protocol.

2. Methods

2.1. Experimental treatment groups

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Male Wistar rats (280–320 g) were used for these studies. Firstly, rats were submitted to either heat stress (HS groups) or anaesthesia alone (sham groups). Subsequently, all animals were allowed to recover for 24 h. Then, ischaemia–reperfusion was performed in vivo, in anaesthetised animals. Six experimental groups were studied: sham group—rats received saline (NaCl 0.9%, i.v.) 10 min before ischaemia; sham+L-NAME group—animals were treated with L-NAME (10 mg kg$^{-1}$, i.v.) 10 min before ischaemia [12]; sham+1400W group—animals were treated with 1400W (1 mg kg$^{-1}$, i.v.) 10 min before ischaemia [13]. In HS, HS+L-NAME and HS+1400W groups, rats were similarly treated before ischaemia, 24 h after heat stress.

The experimental protocol is summarised in Fig. 1.

Fig. 1. Experimental protocol. Rats were submitted to either heat stress (HS groups) or anaesthesia without hyperthermia (sham groups). Subsequently, all animals were allowed to recover for 24 h. Then, a 30-min ischaemia followed by a 120-min reperfusion was performed in vivo. Six experimental groups were studied: sham ($n=8$) and HS ($n=7$) groups—rats received saline (NaCl 0.9%, i.v.) 10 min before ischaemia; sham+L-NAME ($n=8$) and HS+L-NAME ($n=7$) groups—animals were treated with L-NAME (10 mg kg$^{-1}$, i.v.) 10 min before ischaemia; sham+1400W ($n=7$) and HS+1400W ($n=7$) groups—animals were treated with 1400W (1 mg kg$^{-1}$, i.v.) 10 min before ischaemia.

2.2. Heat stress protocol

Heat stress was achieved, as previously described [14], by placing anaesthetised (with 25 mg kg$^{-1}$ i.p. sodium pentobarbitone) rats in an environmental chamber under an infrared light. Their body temperature, recorded with a rectal probe (thermocouple type K), was increased to 42±0.2°C and maintained for 15 min. Sham control animals were anaesthetised only. All rats were allowed to recover for 24 h, with food and drink ad libitum.

2.3. Ischaemia–reperfusion protocol

Twenty-four hours after heat stress, rats were re-anaesthetised (60 mg kg$^{-1}$ i.p. sodium pentobarbitone), intubated and mechanically ventilated with a rodent ventilator (Harvard Apparatus) using a rate of 55 cycles min$^{-1}$ and a tidal volume of 1 ml 100 g$^{-1}$ body weight. Rectal temperature was carefully maintained with a heating pad at 37±0.3°C throughout the experiment.

Polyethylene tubes were inserted in the penile vein and in the left carotid artery for drug injection and systemic arterial blood pressure monitoring (8 channel, MacLab ADInstruments), respectively.

For temporary occlusion of the left coronary artery (LCA), a 3/0 silk suture (Mersilk W546, Ethicon) was placed around the artery a few millimetres distal to the aortic root. After 20 min of stabilisation, regional ischaemia was induced by tightening the snare around the LCA for 30 min. Thereafter, the heart was reperfused for 120 min, rapidly excised, rinsed in saline and the coronary artery ligature was retied. Infarct size determination was then performed. Unisperse blue dye (Ciba-Geigy) was slowly infused through the aorta to delineate the myocardial risk zone. After removal of the right ventricle and connective tissues, the heart was frozen and then sectioned into 2-mm transverse sections from apex to base (6–7 slices/heart). Following defrosting, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in
phosphate buffer (pH 7.4) for 10–20 min and fixed in 10% formaldehyde solution to distinguish clearly stained viable tissue and unstained necrotic tissue. Left ventricular infarct zone (I) was determined using a computerised planimetric technique (Minichromax, Biolab) and expressed as a percentage of the risk zone (R) and of the left ventricle (LV).

### 3. Results

#### 3.1. Mortality and exclusion

A total of 65 rats were used to study the infarct size development in vivo. Reasons for exclusion are summarised in Table 1.

#### 3.2. Haemodynamic data

Table 2 summarises haemodynamic data recorded in the experimental groups during the stabilisation period and the ischaemia–reperfusion protocol. The heart rate did not differ between groups. l-NAME pretreated groups had increased mean arterial pressure values at baseline compared to the sham group.

#### 3.3. Myocardial infarct size

Infarct-to-risk ratio (I/R) was reduced from 46.0 ± 1.4% in sham group to 26.8 ± 3.8% in HS group (P < 0.05, Fig. 2B). The infarct size reduction induced by heat stress was abolished by l-NAME and 1400W pretreatment (53.9 ± 3.1% after l-NAME and 51.8 ± 3.3% after 1400W) just prior to ischaemia. In non heat-stressed rats, treatment with l-NAME (50 ± 4%) and 1400W (44.6 ± 3.0%) had no effect on infarct size. Similar results were observed with the I/LV ratio of the six groups. Myocardial risk size expressed as the percentage of the left ventricle (R/LV) was similar for all six groups (Fig. 2A). Therefore, differences in infarct size did not result from variability in the risk zone.

#### 3.4. iNOS expression following heat stress

Western blot analysis of myocardial iNOS expression showed a marked increase of this protein, 24 h after HS. Optical density analysis of the protein bands (~130 kDa) revealed a 3.8-fold increase in iNOS concentration in HS compared with sham (P = 0.029) (Fig. 3).

### 4. Discussion

This study provides the first demonstration of an implication of iNOS-derived NO as a mediator of HS-induced...
Cardioprotection. The pertinent findings of our study can be summarised as follows. (1) Heat stress can induce an adaptive response in the myocardium, resulting in a delayed resistance to infarction 24 h later. This is in accordance with previous studies [14,15]. (2) The HS-induced cardioprotection was abolished by the administration of NOS inhibitors, L-NAME and 1400W, when given before ischaemia. This finding implies that NOS activity is necessary during ischaemia to mediate the reduction in infarct size in vivo in the heat stressed rat. (3) We observed a significant increase in myocardial iNOS protein expression 24 h after heat stress. Taken together, the present results demonstrate that iNOS-derived NO is a mediator of the late protection conferred by heat stress against myocardial infarction in vivo in the rat.

4.1. Pharmacological treatments: L-NAME and 1400W

L-NAME is a non-selective NOS inhibitor, acting on the three isoforms. The fact that L-NAME completely abrogated the HS-induced reduction in infarct size demonstrates that this protective effect is mediated by the activation of NOS, with no indication of the isoform involved. The increase in baseline mean arterial blood pressure (MAP) in the L-NAME treated groups is consistent with the inhibition of NOS and is in accordance with previous results [16]. However, this effect of L-NAME on MAP cannot explain the abrogation of the cardioprotection in the HS+L-NAME group, since 1400W was able to exert the same effect without affecting baseline MAP levels.

1400W, a selective iNOS inhibitor, was used to determine whether this isozyme was involved in the cardioprotective mechanism. 1400W was chosen because, amongst all the NOS inhibitors available, it was shown to be the most selective for iNOS both in vitro and in vivo [11]. Thus, 1400W was chosen instead of aminoguanidine because of its higher selectivity for iNOS (5000 times

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### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Stabilisation</th>
<th>Ischaemia 5 min</th>
<th>Ischaemia 30 min</th>
<th>Reperfusion 15 min</th>
<th>Reperfusion 60 min</th>
<th>Reperfusion 120 min</th>
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<tr>
<td>Mean arterial pressure</td>
<td>Sham</td>
<td>103 ± 7</td>
<td>84 ± 6</td>
<td>77 ± 4</td>
<td>80 ± 6</td>
<td>53 ± 5</td>
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<tr>
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<td>72 ± 7</td>
<td>74 ± 7</td>
<td>72 ± 8</td>
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<td></td>
<td>Sham+L-NAME</td>
<td>137 ± 7*</td>
<td>93 ± 5</td>
<td>88 ± 10</td>
<td>85 ± 10</td>
<td>79 ± 10</td>
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<td></td>
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<td>147 ± 7*</td>
<td>107 ± 11</td>
<td>105 ± 10</td>
<td>91 ± 11</td>
<td>83 ± 6</td>
</tr>
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<td>92 ± 5</td>
<td>75 ± 7</td>
<td>82 ± 7</td>
<td>79 ± 8</td>
<td>69 ± 8</td>
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<tr>
<td></td>
<td>HS+1400W</td>
<td>114 ± 9</td>
<td>97 ± 11</td>
<td>92 ± 12</td>
<td>85 ± 12</td>
<td>76 ± 10</td>
</tr>
<tr>
<td>Heart rate (beats min⁻¹)</td>
<td>Sham</td>
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<td>377 ± 14</td>
<td>366 ± 14</td>
<td>375 ± 8</td>
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<tr>
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<td>364 ± 21</td>
<td>370 ± 13</td>
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<td>HS+1400W</td>
<td>382 ± 8</td>
<td>372 ± 8</td>
<td>369 ± 9</td>
<td>380 ± 8</td>
<td>359 ± 13</td>
</tr>
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</table>

Data are mean±S.E.M. *P<0.05 vs. sham. HS, heat-stress; Sham, sham-anaesthesia; L-NAME, treatment with nitro-l-arginine-methylester; 1400W, treatment with N-[(3-aminoethyl)benzyl] acetamidine.

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![Fig. 2. (A) Myocardial risk size expressed as a percentage of the left ventricle (R/LV). (B) Infarct size is expressed as a percentage of the myocardial risk zone (I/R). Open circles represent individual values whereas solid circles represent mean±S.E.M. *P<0.001 (one-way ANOVA).](image)
more selective for iNOS than for eNOS). Indeed, amino-
guanidine, which has been abundantly used as the highest
selective iNOS inhibitor, also binds other enzymes such as
catalase [17] or iron-containing enzymes (for a review see
Ref. [18]) and possesses antioxidant properties per se [19].
The dose of 1400W used in this study had no effect on
MAP, suggesting that it did not inhibit NO production by
vascular eNOS. Our results suggest that the HS-induced
cardioprotection is likely to be due to iNOS, rather than
eNOS.

4.2. iNOS and preconditioning

The fact that NO could be a mediator of the HS-
response is in accordance with a previous study, where we
have shown that the HS-induced reduction in infarct size
was abolished by l-NAME perfusion before and during
ischaemia, in the isolated rat heart [20].

A specific role of iNOS-derived NO as a mediator of
delayed cardioprotection has also been reported for is-
chaemic preconditioning (IP). Thus, iNOS induction was
shown to be necessary for the development of delayed
protection conferred by IP in anaesthetised rabbit models
of myocardial infarction [9,10] and stunning [6]. Vegh et al.
[21] have also demonstrated that iNOS inhibition prevents
the development of delayed preconditioning against arrhythmas, in the dog. Using iNOS knockout mice, Guo et al. [22] have shown that targeted disruption of the iNOS
gene completely abrogates the infarct-sparing effect of late
IP, demonstrating that the activity of iNOS is indispensable
for this cardioprotective phenomenon to occur. Further-
more, iNOS appears to be a final mediator of several other
forms of delayed myocardial preconditioning, such as that
induced by NO donors [23], endotoxin derivatives [24] and
exercise [25]. Although recent studies suggest that adeno-
sine A1 receptor agonist-induced cardioprotection occurs
independently of either early generation of NO or induc-
tion of iNOS [26,27], an implication of iNOS has also been
shown in this form of pharmacological preconditioning
[28].

4.3. iNOS protein upregulation by heat stress

In support for the role of iNOS in HS-induced precondi-
tioning, we observed an upregulation of iNOS protein
expression in rat myocardium 24 h after hyperthermia. An
increase in iNOS protein level has been reported with
ischaemic [29,30] as well as pharmacological [27] pre-
conditioning. Although mechanisms involved in this HS-
induced upregulation of iNOS protein expression remain
unknown, a potential signalling pathway is proposed in the
following section.

4.4. HS-induced cardioprotection and NO

Taken together, the results of this study and those from a
previous study from our group show that NO can act both as a trigger and as a mediator of the cardioprotection conferred by heat stress. However, the exact signalling pathways leading to this protection are still under investigation (for a review see Ref. [1]). We have previously shown that catecholamines [31], protein kinase C (PKC) [14] and p38 MAP kinase (p38MAPK) [32] are involved in triggering the HS-induced cardioprotection. More recently, NO [8] and reactive oxygen species (ROS) [33] have also been shown to act as triggers of the HS response.

The fact that HS-induced cardioprotection requires 24 h to occur suggests that this phenomenon is related to a de novo synthesis of proteins, which might mediate the response. Indeed, the present study shows an increase in iNOS protein expression induced by HS, that is related to the cardioprotective effect. Although the mechanism of NOS induction in this study remains unknown, a hypothetical signalling pathway can be proposed (Fig. 4). Following HS, NO itself can activate iNOS gene transcription [34]. Furthermore, the production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), via the generation of ROS during hyperthermia [35], can activate nuclear factor-κB (NF-κB) [36], which in turn can lead to the transcription of the iNOS gene [37]. The other actors of the triggering response, such as PKC and p38 MAPK, can also activate gene transcription [38]. Indeed, other proteins have been shown to be induced by HS, such as endogenous cardiac antioxidant enzymes [39,40] and heat stress proteins (Hsp) [4].

The present study shows that the final step in the cardioprotective effect of HS also appears to be mediated by NO which could act via an activation of mitochondrial K<sub>ATP</sub> channels. Indeed, Sasaki et al. [41] have shown that NO can directly activate mitochondrial K<sub>ATP</sub> channels, which have been shown to be necessary in the HS-induced cardioprotection, in the rat [42] and in the rabbit [43,44]. Further experiments are needed to confirm the link between NO and mitochondrial K<sub>ATP</sub> channels in our model and to assess other potential NO targets.

In conclusion, this study provides new information regarding the signalling pathway leading to the development of the late phase of cardioprotection induced by heat stress. In this study, we demonstrate for the first time the implication of NO as a mediator of the HS-induced cardioprotection in vivo in the rat, since L-NAME and 1400W pretreatments abolished the infarct-sparing effect. Furthermore, we show a robust increase in iNOS protein expression induced by HS.

References


