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

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## 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.

**Discipline:** experimental; **Object of study:** heart; **Level:** organism; **Field of study:** pharmacology.

**Keywords:** Heat Stress, Preconditioning, Myocardial Ischaemia, Infarction, iNOS.

**List of abbreviations**

eNOS: endothelial nitric oxide synthase

HS: heat stress

Hsp: heat stress protein

I: infarct zone

IL-1 $\beta$ : interleukin-1 $\beta$

iNOS: inducible nitric oxide synthase

IP: ischaemic preconditioning

K<sub>ATP</sub> channel : ATP-dependant potassium channel

LCA: left coronary artery

L-NAME: nitro-L-arginine-methylester

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- $\alpha$ : tumor necrosis factor- $\alpha$

1400W: N-[(3-Aminoethyl)benzyl] acétamidine

## 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 48h after the stress (HS or IP), is a two-step phenomenon. First, 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 delayed preconditioning against both myocardial stunning [6] and infarction [9,10], in the rabbit. Indeed, NOS inhibitors administered either during IP or 24h 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.

## Methods

### ***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 n° 85-23, revised 1996).

Male Wistar rats (280-320 g) were used for these studies. First, 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%, iv) 10 min before ischaemia; *Sham+L-NAME group* - animals were treated with L-NAME (10 mg kg<sup>-1</sup>, iv) 10 min before ischaemia [12]; *Sham+1400W group* - animals were treated with 1400W (1 mg kg<sup>-1</sup>, iv) 10 min before ischaemia [13]. In *HS*, *HS+L-NAME* and *HS+1400W groups*, rats were similarly treated before ischaemia, 24h after heat stress.

The experimental protocol is summarised in Figure 1.

### ***Heat stress protocol***

Heat stress was achieved, as previously described [14], by placing anaesthetised (with 25 mg kg<sup>-1</sup> ip 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 15 min. Sham control animals were anaesthetised only. All rats were allowed to recover for 24 h, with food and drink *ad libitum*.

### ***Ischaemia-reperfusion protocol***

Twenty-four hours after heat stress, rats were re-anaesthetised (60 mg kg<sup>-1</sup> ip sodium pentobarbitone), intubated and mechanically ventilated with a rodent ventilator (Harvard

Apparatus) using a rate of 55 cycles min<sup>-1</sup> and a tidal volume of 1 ml 100 g<sup>-1</sup> 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).

#### ***Western blot analysis of myocardial iNOS***

To determine iNOS expression, additional animals (n = 4 in each group) were submitted to either HS or sham anaesthesia. Twenty-four hours later, animals were re-anaesthetised and treated with heparin as described above, before their heart was quickly harvested. Total

cellular protein was extracted and samples were loaded at 50 µg/lane. Proteins were separated by 8% SDS/PAGE and electrotransferred to nitrocellulose membrane. iNOS was detected using a rabbit polyclonal antibody (Chemicon International) and visualised with an enhanced chemiluminescence system (Pierce). After scanning, the blots were analysed for optical density using an image analysis program (NIH Image for Windows). Each iNOS signal was normalised to the corresponding Ponceau signal and presented as a percentage of Sham iNOS content (100% in arbitrary units).

### ***Statistical analysis***

All data are presented as mean  $\pm$  s.e.mean. Comparisons in heart rate and systemic arterial blood pressure were performed by repeated measures ANOVA with *post-hoc* multiple comparison Tukey tests. Infarct size was analysed by a one-way ANOVA. iNOS protein expression was analysed using non parametric tests (Mann-Withney rank sum test). *P* values  $\leq 0.05$  were considered significant.

### ***Materials***

L-NAME (nitro-L-arginine-methylester) was obtained from Sigma Chemical (France) and 1400W (N-[*(3*-Aminoethyl)benzyl] acétamidine) was from Acros Organics (France); Unisperse blue dye was from Ciba-Geigy (France); 2,3,5-triphenyltetrazolium chloride was from Sigma (France).

## Results

### ***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.

### ***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 pre-treated groups had increased mean arterial pressure values at baseline compared to the Sham group.

### ***Myocardial infarct size***

Infarct-to-risk ratio (I/R) was reduced from  $46.0 \pm 1.4\%$  in Sham group to  $26.8 \pm 3.8\%$  in HS group ( $P \leq 0.05$ , Figure 2B). The infarct size reduction induced by heat stress was abolished by L-NAME and 1400W pre-treatment ( $53.9 \pm 3.1\%$  after L-NAME and  $51.8 \pm 3.3\%$  after 1400W) just prior to ischaemia. In non heat-stressed rats, treatment with L-NAME ( $50 \pm 4\%$ ) and 1400W ( $44.6 \pm 3.0\%$ ) 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 (Figure 2A). Therefore, differences in infarct size did not result from variability in the risk zone.

### ***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 ( $\approx 130$  kDa) revealed a 3.8-fold increase in iNOS concentration in HS compared with Sham ( $P=0.029$ ) (Figure 3).

## **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 24h 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.

### ***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 more selective for iNOS than for eNOS). Indeed, aminoguanidine, 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 [18]) and possesses antioxydant 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.

### ***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 ischaemic 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 arrhythmias, 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. Furthermore, 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 adenosine A1 receptor agonist-induced cardioprotection occurs independently of either early generation of NO or induction

of iNOS [26,27], an implication of iNOS has also been shown in this form of pharmacological preconditioning [28].

### ***iNOS protein upregulation by heat stress***

In support for the role of iNOS in HS-induced preconditioning, 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] preconditioning. Although mechanisms involved in this HS-induced upregulation of iNOS protein expression remain unknown, a potential signalling pathway is proposed in the following paragraph.

### ***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 [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, an hypothetical signalling pathway can be proposed (Figure 4). Following HS, NO itself can activate iNOS gene transcription [34]. Furthermore, the production of

tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), via the generation of ROS during hyperthermia [35], can activate nuclear factor- $\kappa$ B (NF- $\kappa$ B) [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.

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**Table 1. Exclusion criteria**

	Sham	Sham + L-NAME	Sham + 1400W	HS	HS + L-NAME	HS + 1400W	Total
Bleeding	0	1	0	1	2	0	4
Irreversible VF during ischaemia	0	5	6	0	4	2	17
Total number of rats excluded	0/8	6/14	6/13	1/8	6/13	2/9	21/65

HS = heat-stress, Sham = sham-anaesthesia, L-NAME = treatment with nitro-L-arginine-methylester, 1400W = treatment with N-[(3-Aminoethyl)benzyl] acétamidine, VF = ventricular fibrillation.

**Table 2. Haemodynamic data**

	Group	Stabilisation	Ischaemia		Reperfusion		
			5 min	30 min	15 min	60 min	120 min
<b>Mean arterial pressure (mmHg)</b>	Sham	103 ± 7	84 ± 6	79 ± 7	77 ± 4	80 ± 6	53 ± 5
	HS	119 ± 6	100 ± 9	86 ± 11	72 ± 7	74 ± 7	72 ± 8
	Sham+L-NAME	137 ± 7*	93 ± 5	96 ± 8	88 ± 10	85 ± 10	79 ± 10
	HS+L-NAME	147 ± 7*	107 ± 11	115 ± 12*	106 ± 10	91 ± 11	83 ± 6
	Sham+1400W	92 ± 5	75 ± 7	80 ± 7	82 ± 7	79 ± 8	69 ± 8
	HS+1400W	114 ± 9	97 ± 13	97 ± 11	92 ± 12	85 ± 12	76 ± 9
<b>Heart rate (beats min<sup>-1</sup>)</b>	Sham	380 ± 13	377 ± 14	355 ± 17	366 ± 14	375 ± 8	352 ± 20
	HS	384 ± 7	390 ± 6	360 ± 20	364 ± 21	370 ± 13	370 ± 15
	Sham+L-NAME	384 ± 13	372 ± 21	352 ± 25	351 ± 24	347 ± 21	368 ± 16
	HS+L-NAME	387 ± 9	393 ± 12	392 ± 18	380 ± 20	395 ± 13	397 ± 15
	Sham+1400W	402 ± 14	374 ± 17	347 ± 16	357 ± 12	338 ± 23	356 ± 24
	HS+1400W	382 ± 8	372 ± 8	369 ± 9	369 ± 11	380 ± 8	359 ± 13

Data are mean ± s.e. mean. \*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.

## Legends to figures

**Figure 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%, iv) 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<sup>-1</sup>, iv) 10 min before ischaemia; *Sham+1400W* (n=7) and *HS+1400W* (n=7) groups - animals were treated with 1400W (1 mg kg<sup>-1</sup>, iv) 10 min before ischaemia.

**Figure 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.mean. \*P < 0.001 (one way ANOVA).

**Figure 3.** Western blot analysis of iNOS protein expression in myocardium.

A) Representative Western blots showing expression patterns of iNOS protein following heat stress (HS).  
B) Quantification by densitometric analysis of iNOS protein expression in Sham and heat-stressed rats (24h after HS). Data (n=4) are mean ± s.e.mean and are expressed as percent changes compared to Sham group values.

**Figure 4.** Hypothetical signalling pathway thought to occur following heat stress.

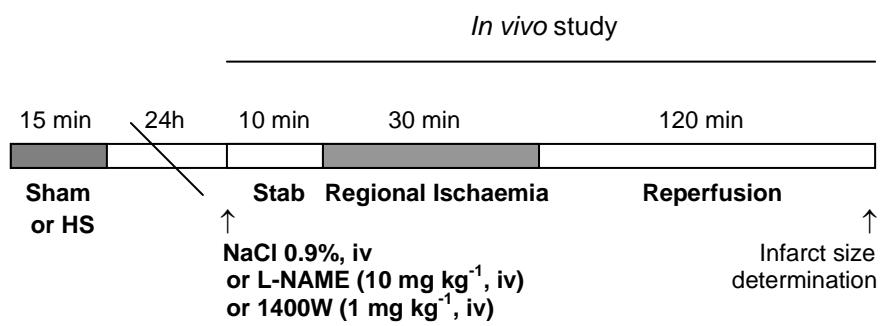


Figure 1.

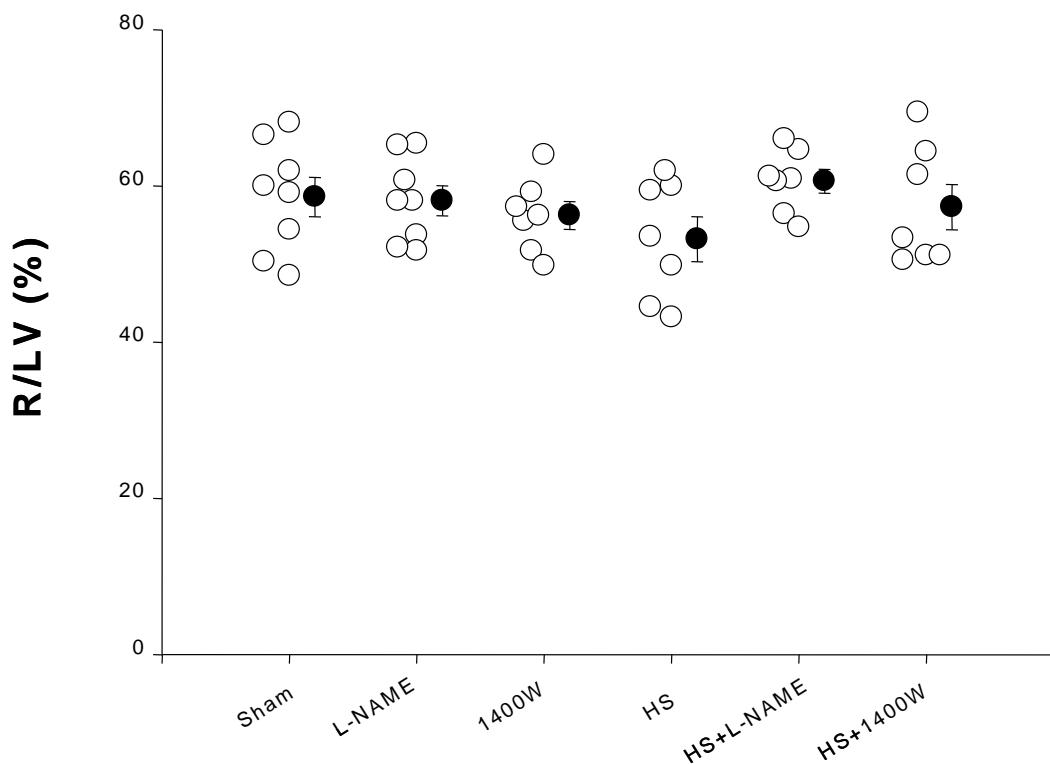
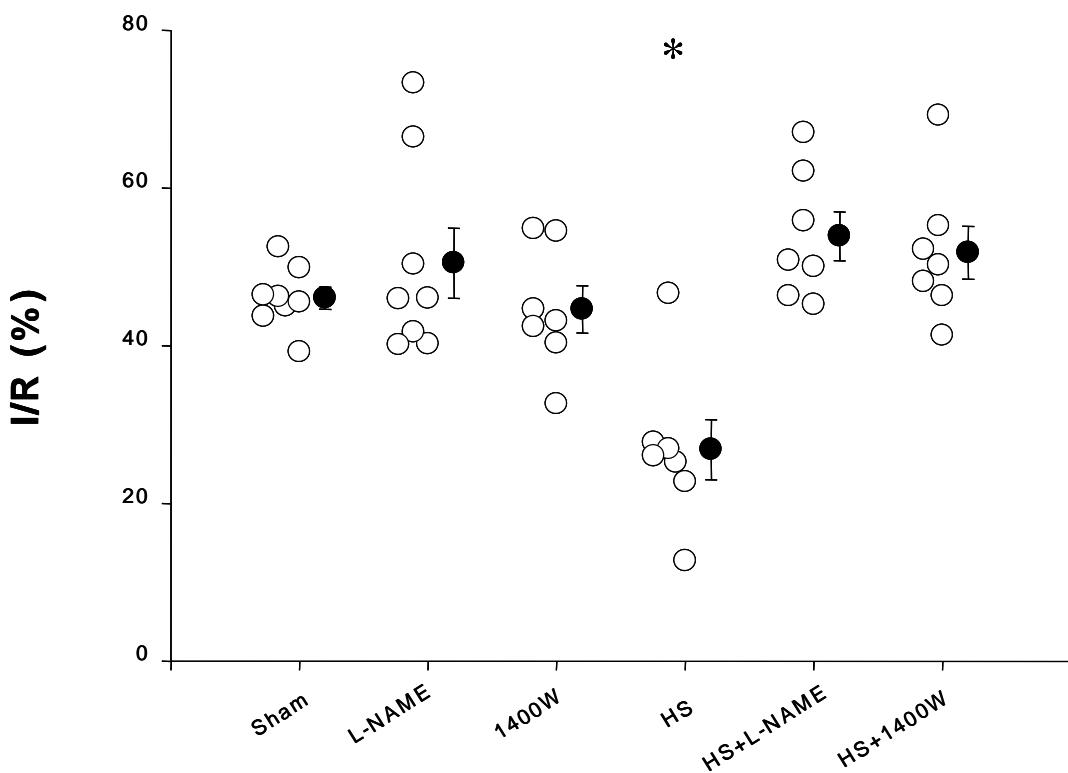
**A)****B)**

Figure 2.

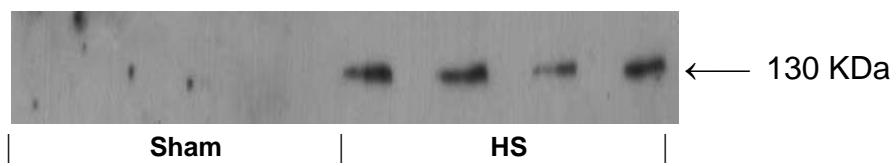
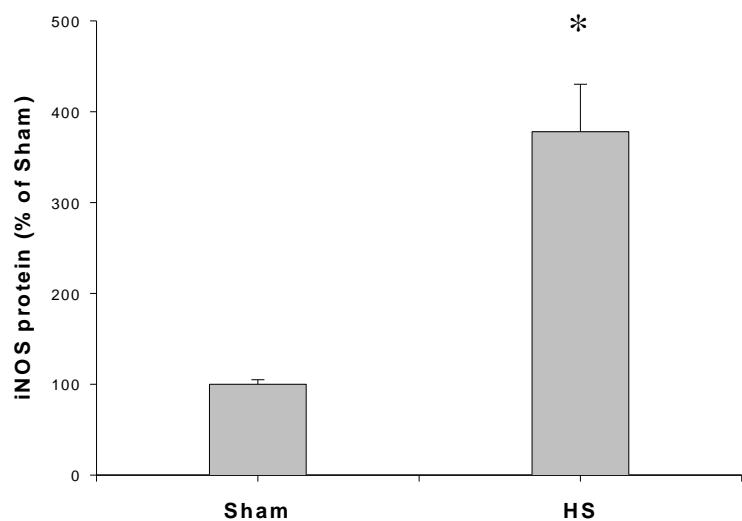
**A)****B)**

Figure 3.

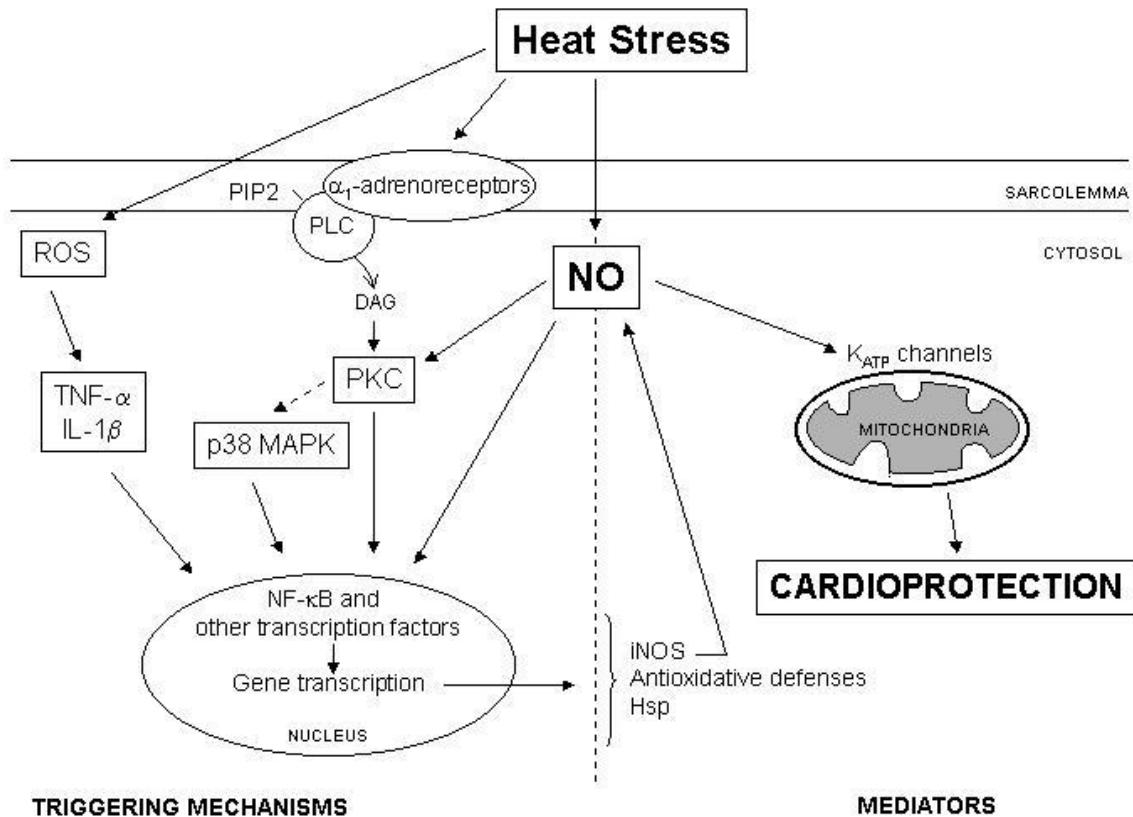


Figure 4.

**Figure 1.** ↑  
iNOS is a mediator...  
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**Figure 2A.** ↑  
iNOS is a mediator...  
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**Figure 2B.** ↑  
iNOS is a mediator...  
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**Figure 3.** ↑  
iNOS is a mediator...  
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**Figure 3B.** ↑  
iNOS is a mediator...  
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**Figure 4.** ↑  
iNOS is a mediator...  
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