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Abstract

Objective: Heat stress (HS) is known to induce delayed protection against myocardial infarction. We have previously shown that inducible nitric oxide synthase (iNOS), was involved in mediating this form of preconditioning. Since iNOS and cyclooxygenase-2 (COX-2) are co-induced in various cell types, the goal of this study was to investigate whether COX-2 could also participate to the HS-induced cardioprotection.

Methods and Results: A total of 78 male Wistar rats, subjected to either heat stress (42°C for 15 min) or sham anaesthesia were used for this study. Twenty-four hours later, they were treated or not with a selective COX-2 inhibitor, either celecoxib (3 mg kg^{-1}, ip) or NS-398 (5 mg kg^{-1}, ip), 30 min before being subjected to a 30-min occlusion of the left coronary artery followed by a 120-min reperfusion, *in vivo*. HS resulted in a marked increase in myocardial COX-2 protein expression at 24 h, associated with a significant protection against infarction (46.0 ± 1.4% in Sham *vs* 26.8 ± 3.8% in HS group) ($P \leq 0.05$). Administration of selective COX-2 inhibitor 24 h after HS, completely abrogated this delayed cardioprotection (46.4 ± 3.6% and 48.0 ± 2.8%, respectively in HS+celecoxib and HS+NS-398 groups).

Conclusion: This study provides the first evidence of an implication of COX-2 as a mediator of HS-induced cardioprotection. This suggests that prostaglandins are involved in this type of cardioprotective preconditioning.

List of abbreviations

COX-2	Cyclooxygenase-2
HR	Heart Rate
HS	Heat Stress
Hsp	Heat shock proteins
I	Infarct zone
IL-1β	Interleukin-1β
iNOS	inducible Nitric Oxide Synthase
IP	Ischaemic Preconditionning
LCA	Left Coronary Artery
LV	Left Ventricle
MAP	Mean Arterial Pressure
MAPK	Mitogen Activated Protein Kinase
NF-κB	Nuclear Factor-kappa B
PGs	Prostaglandins
PKC	Protein Kinase C
R	Risk zone
ROS	Reactive Oxygen Species
TNF-α	Tumor Necrosis Factor-α

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Introduction

Environmental stresses, such as heat stress (HS), are known to induce the synthesis of heat shock proteins (Hsp) which seem to play an important role in the cell ability to survive noxious stresses [1]. Heat stress is also known to increase myocardial tolerance to a subsequent period of ischaemia by preserving myocardial function [2] and reducing myocardial necrosis [3], [4]. The development of this late cardioprotection, which occurs 24 to 48 h after HS, implies an immediate activation of triggers followed by the synthesis of proteins which mediate the protection.

Recently, we have demonstrated that inducible nitric oxide synthase (iNOS) is implicated both as a trigger [5] and a mediator [6] of the HS-induced cardioprotection. Indeed, iNOS inhibitors, administered both during HS or 24h later, abolish the HS-induced reduction of infarct size, in the rat.

Stress-induced iNOS expression seems to be associated with simultaneous induction of cyclooxygenase-2 (COX-2) in various cell types including smooth muscle cells [7] and cardiomyocytes [8]. Cyclooxygenase, also named prostaglandin H_2 synthase, is the enzyme that catalyses the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid [9]. Exogenous PGs perfusion has been shown to preserve myocardial metabolism [10], decrease infarct size [11] and recent studies support a role for COX-2, the inducible isoform of cyclooxygenase, as an essential mediator of the delayed protection induced by ischaemic preconditioning (IP) [12], [13].

Since the HS-induced delayed cardioprotection is similar to that seen with IP, mediators of IP (for a review, see [14]) might also been responsible for HS-induced cardioprotection. Therefore, the goal of this study was to determine whether COX-2 could contribute to the HS-induced cardioprotection. We thus examined the effect of two selective COX-2 inhibitors, celecoxib and NS-398, on the HS-induced reduction of infarct size, *in vivo* in the rat.

Methods

Experimental protocol and treatment groups

Male Wistar rats (280-320 g) were used for these studies. 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).

First, 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, ischaemia-reperfusion was performed *in vivo*, in anaesthetised rats. Eight experimental groups were studied: *Sham group* - rats were only anaesthetised, *Sham+Vehicle group* − animals were given vehicle (20% DMSO in saline, ip) 30 min before ischaemia, *Sham+Celecoxib group* - animals were treated with the selective COX-2 inhibitor, celecoxib (3 mg kg^{-1}, ip) [13], 30 min before ischaemia, *Sham+NS-398 group* - animals were given the selective COX-2 inhibitor, NS-398 (5 mg kg^{-1}, ip) [12], [13], 30 min before ischaemia. In *HS, HS+Vehicle, HS+Celecoxib* and *HS+NS-398 groups*, rats were similarly treated 24h after heat stress. Celecoxib and NS-398 were dissolved in 20% DMSO in saline.

The experimental protocol is summarised in Figure 1.

Mortality and exclusion

A total of 78 rats were used for this study. Reasons for exclusion are summarised in Table 1.

Heat stress protocol

Heat stress was achieved, as previously described [6], by placing lightly anaesthetised rats (25 mg kg^{-1} ip sodium pentobarbitone) in an environmental chamber under an infrared light. Their body temperature, recorded with a rectal probe, was increased to 42 ± 0.2°C for 15 min. Sham control animals were anaesthetised only. All rats were allowed to recover for 24 h.

Ischaemia-reperfusion protocol

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

Polyethylene tubes were inserted in the penile vein and in the left carotid artery for drug administration and mean arterial pressure (MAP) monitoring (MacLab, 8 channels, ADInstrument), 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 as described below. 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 clearly distinguish 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 COX-2

To determine COX-2 expression, additional animals were submitted to either HS or sham anaesthesia (n = 4 in each group). Twenty-four hours later, the animals were re-anaesthetised (60 mg kg^{-1} ip sodium pentobarbitone) and treated with heparin (500 U kg^{-1}, iv) before their heart was quickly harvested. Total cellular protein was extracted and samples were loaded at 100 µg/lane. Proteins were separated by 10 % SDS/PAGE and electrotransferred to a nitrocellulose membrane. COX-2 was detected using a rabbit antibody (Upstate Biotechnology, Euromedex) at a dilution of 1/250 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 COX-2 signal was normalised to the corresponding Ponceau signal and presented as arbitrary units (a.u).

Products

Celecoxib, NS-398 and 2,3,5-triphenyltetrazolium chloride were obtained from Sigma Chemical.

Statistical analysis

All data are presented as mean ± s.e.mean. Comparisons in heart rate and mean arterial pressure were performed using repeated measure ANOVA with *post-hoc* multiple comparison Tuckey tests. Infarct size was analysed by a one-way ANOVA. COX-2 protein expression was analysed using a non parametric Mann-Withney rank sum test. *P* values ≤ 0.05 were considered significant.

Results

Haemodynamic data

Table 2 summarises haemodynamic data recorded in the eight experimental groups during the stabilisation period and the ischaemia-reperfusion protocol. Mean arterial pressure and heart rate did not significantly differ among the various groups.

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 2).This effect of heat stress was abolished in celecoxib ($46.4 \pm 3.6\%$) and NS-398 ($48.0 \pm 2.8\%$) treated groups.

In non heat-stressed rats, treatment with celecoxib ($49.7 \pm 3.5\%$) and NS-398 ($52.9 \pm 3.2\%$) had no effect on infarct size. Similar results were observed with the I/LV ratio (Table 3). Myocardial risk size expressed as the percentage of the left ventricle (R/LV) was similar in the eight experimental groups (Table 3). Therefore, differences in infarct size did not result from variability in the risk zone.

Myocardial COX-2 protein expression

Western blot analysis of myocardial COX-2 (72 kDa) expression showed a marked increase of this protein, 24 h after HS (1534 ± 193 a.u in HS group *vs* 97 ± 72 a.u in Sham group, $P = 0.029$) (Figure 3).

Discussion

Although the protective effect conferred by prior heat stress against subsequent cardiac ischaemic injury is a well known phenomenon (for a review, see [15]), the exact signalling pathway of this delayed protection remains largely unknown. The pertinent finding of this work is the implication of COX-2 as a mediator of this form of cardioprotection. Indeed, the HS-induced reduction in infarct size was abolished by the administration of selective COX-2 inhibitors, celecoxib and NS-398, just prior to ischaemia. Furthermore, we have also shown a marked increase in myocardial COX-2 protein expression 24 h after HS. These findings imply that COX-2 activity is necessary during ischaemia to mediate the reduction in infarct size, *in vivo*, in the heat stressed rat.

Pharmacological treatments: celecoxib and NS-398

To minimise the possibility that the loss of HS-induced protective effect could be due to non specific effects, we chose to evaluate the effect of two very selective COX-2 inhibitors, celecoxib and NS-398. These drugs have been reported to be highly selective for COX-2 (IC_{50}: 0.04 and 0.1 μM, respectively) *vs* COX-1 (IC_{50}: 15 and 16.8 μM, respectively) [9]. Furthermore, since haemodynamic variables and risk zones were similar among all experimental groups (Tables 2 and 3), the loss of late protection in celecoxib and NS-398-treated rats cannot be explained by changes in these parameters.

COX-2 and myocardium: deleterious or salutary effects?

COX-2 is generally thought to be detrimental. Indeed, numerous works have shown an essential role of COX-2 in inflammation, cancer or apoptosis (for a review, see [9]). A recent study demonstrates that induction of COX-2 increases the production of proinflammatory prostanoids and contributes to dysfunction in ischaemic myocardium [16]. Furthermore, an

induction of COX-2 has been shown in myocardium of patients with congestive heart failure [17].

However, the present study demonstrates the implication of COX-2 as a mediator of the HS-induced cardioprotection. This is in agreement with recent studies also suggesting a beneficial role of this protein in the myocardium. Indeed, a specific role for COX-2 as a mediator of delayed cardioprotection conferred by other types of preconditioning has been reported. Bolli's group showed that COX-2 mediates the ischaemic preconditioning (IP)-induced protection against both myocardial stunning and infarction [13], in conscious rabbits [13] or in mice [12]. The role of COX-2 in mediating late cardioprotection has also been described in a pharmacological model of late preconditioning induced by the activation of δ-opioid receptors [18].

COX-2 upregulation and late preconditioning

In support for the role of COX-2 in the HS-induced preconditioning, we observed a marked induction of COX-2 protein expression in rat myocardium 24 h after hyperthermia. An increase in myocardial COX-2 protein level has also been reported 24 h after IP in rabbit myocardium [13]. Other preconditioning, such as hypoxia [19], oxidative stress [20] or cytokine administration [21] have also been shown to induce COX-2 in different cell types, including cardiomyocytes.

COX-2 and the HS response

The signalling pathways whereby HS leads to COX-2 expression in the heart are unknown. An interesting observation is that COX-2 is co-induced with iNOS in response to various stresses such as ischaemia, hypoxia or cytokines (for a review, see [9]). It has recently been demonstrated that the enzymatic activity of newly synthesized COX-2 following IP requires

iNOS-derived NO, which implies that COX-2 is located downstream of iNOS in the protective pathway of this late preconditioning [22]. Moreover, similarities appear between the signalling pathways that control stress-induced COX-2 and iNOS expression, both involving reactive oxygen species (ROS) [23], [24], [25], protein kinase C (PKC) [26], [27], and nuclear factor kappa B (NF-κB) [19], [28].

We have shown that ROS [6], PKC [29], p38 mitogen activated kinase (MAPK) [30] and iNOS [5] can trigger the HS-induced cardioprotection. More recently, we have also shown that iNOS, which is upregulated following HS, is also a mediator of the HS-induced cardioprotection [6].

It has been proposed that cytokine release (in particular IL-1β and TNF-α) upon ROS generation during hyperthermia could activate nuclear factor-kappaB (NF-κB) [31], [32]. NF-κB promotes iNOS [33] and COX-2 [34] expression and could thus, along with PKC [26], [27], [35] and other MAPK [8], [34], [35], be responsible for the gene transcription leading to this protein co-induction and their cardioprotective effect upon HS.

COX-2 mediated cardioprotection

COX-2 catalyses the first two steps in the biosynthesis of PGs from arachidonic acid [9]. PGs, in particular PGI$_2$ and PGE$_2$, are known to be involved in the antiarrhythmic effect [36] and the preservation of endothelial function [37] induced by IP. Moreover, a recent study [13] demonstrated that PGE$_2$ and/or PGI$_2$ are the likely effectors of COX-2-dependent cardioprotection in a rabbit model of IP. An increased production of PGI$_2$ also appears to mediate the opioid-induced late phase of preconditioning [18].

HS stimulates the accumulation of PGE$_2$ [38], [39]. Furthermore, PGE$_2$ and PGI$_2$ activate ATP-sensitive potassium (K$_{ATP}$) channels [11], [40], [41], [42]. These channels are known to

mediate the HS-induced cardioprotection [43], [44], [45], potentially by protecting cardiac mitochondria at reperfusion [46].

We can thus hypothesise that the COX-2-mediated cardioprotection following HS could, in part, be due to the activation of K_{ATP} channels, via PGE_2 and/or PGI_2 release. Further studies are needed to confirm this hypothesis.

In conclusion, this study provides the first evidence of an implication of COX-2 as a mediator of HS-induced cardioprotection. This suggests that PGs are involved in this protective mechanism, even if their exact nature and their location in the signalling pathway of HS preconditioning remain to be determined.

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Table 1. Exclusion criteria

	Sham	Sham+ Vehicle	Sham+ Celecoxib	Sham+ NS-398	HS	HS+ Vehicle	HS+ Celecoxib	HS+ NS-398	Total
Death during heat stress	-	-	-	-	-	-	1	-	1
Death during anaesthesia	-	-	-	-	-	-	1	-	1
Bleeding	-	-	-	-	1	-	-	1	2
Sustained VF during ischaemia	-	1	-	2	-	4	2	2	11
Total number of rats excluded	-	1	-	2	1	4	4	3	15
Total number of rats included in the study (in vivo+WB)	(8+4)	6	7	7	(7+4)	6	7	7	63

Sham - sham anaesthetised, HS - heat-stressed, Vehicle - 20% DMSO in saline, VF - ventricular fibrillation, WB - Western blot.

Table 2. Haemodynamic data.

	Group	Stabilisation	Ischaemia		Reperfusion		
			5 min	29 min	15 min	60 min	120 min
HR	Sham	380 ± 13	377 ± 14	355 ± 16	366 ± 14	375 ± 8	352 ± 14
(beats min^{-1})	Sham+Vehicle	384 ± 19	374 ± 25	347 ± 21	366 ± 14	362 ± 13	347 ± 13
	Sham+Celecoxib	397 ± 14	387 ± 15	369 ± 13	371 ± 12	344 ± 13	339 ± 13
	Sham+NS-398	389 ± 9	388 ± 16	380 ± 3	372 ± 6	382 ± 12	367 ± 17
	HS	413 ± 12	407 ± 12	386 ± 17	379 ± 14	386 ± 20	374 ± 17
	HS+Vehicle	378 ± 9	385 ± 13	363 ± 16	353 ± 20	364 ± 12	372 ± 14
	HS+Celecoxib	399 ± 17	395 ± 14	394 ± 13	386 ± 14	368 ± 12	363 ± 16
	HS+NS-398	402 ± 15	391 ± 18	361 ± 18	373 ± 17	382 ± 14	359 ± 11
MAP	Sham	104 ± 7	84 ± 6	79 ± 7	77 ± 4	80 ± 6	53 ± 4
(mmHg)	Sham+Vehicle	100 ± 4	78 ± 5	77 ± 5	75 ± 6	83 ± 6	67 ± 7
	Sham+Celecoxib	94 ± 3	79 ± 4	82 ± 7	87 ± 7	84 ± 4	70 ± 5
	Sham+NS-398	103 ± 5	91 ± 6	91 ± 6	76 ± 8	92 ± 6	72 ± 7
	HS	119 ± 6	100 ± 9	86 ± 11	72 ± 7	82 ± 8	72 ± 8
	HS+Vehicle	116 ± 6	102 ± 8	68 ± 6	76 ± 11	77 ± 9	68 ± 8
	HS+Celecoxib	107 ± 6	89 ± 10	85 ± 8	85 ± 7	77 ± 7	64 ± 6
	HS+NS-398	110 ± 6	94 ± 7	81 ± 4	87 ± 9	88 ± 8	72 ± 6

HR - heart rate, MAP - mean arterial pressure. Sham - sham-anaesthetised, HS - heat-stressed, Vehicle - 20% DMSO in saline.

Data are mean ± s.e.mean.

Table 3. Risk (R) and infarct (I) sizes expressed as a percentage of the left ventricle (LV).

Group	N	R/LV	I/LV
Sham	8	58.6 ± 2.5	27.2 ± 1.7
Sham+Vehicle	6	53.8 ± 1.7	24.7 ± 1.0
Sham+Celecoxib	7	53.2 ± 2.9	26.9 ± 3.1
Sham+NS-398	7	59.5 ± 6.1	31.7 ± 2.6
HS	7	53.2 ± 2.9	14.4 ± 2.5*
HS+Vehicle	6	53.7 ± 1.3	10.5 ± 0.8*
HS+Celecoxib	7	57.1 ± 0.8	27.1 ± 2.3
HS+NS-398	7	50.1 ± 1.1	24.5 ± 1.9

Sham - sham-anaesthetised, HS - heat-stressed, vehicle - 20% DMSO in saline. Data are mean

± s.e.mean. *$P < 0.001$ *vs* all the other groups (one-way ANOVA).

Legends to figures

Figure 1. Experimental protocol.

HS - heat stress; LCA - left coronary artery.

Figure 2. Effect of selective COX-2 inhibitors, celecoxib and NS-398, on myocardial infarct size assessed after a 30-min left coronary artery occlusion followed by a 120-min reperfusion *in vivo* in rats subjected to sham-anaesthesia (Sham) or heat stress (HS). Infarct (I) size is expressed as a percentage of the risk zone (R). Individual values and mean ± s.e.mean are presented. *$P < 0.001$ *vs* all the other groups (one-way ANOVA).

Figure 3. a) Western immunoblots showing COX-2 ($\approx$72kDa) protein expression in myocardial samples harvested 24 h after sham-anaesthesia (Sham) or heat stress (HS). (+) - positive control of COX-2 from Raw 264.7 cells treated with LPS for 24 h.

b) Densitometric analysis of COX-2 protein expression 24 h after heat stress. Data are mean ± s.e.mean (n=4). *$P = 0.029$ (Mann-Withney rank sum test).
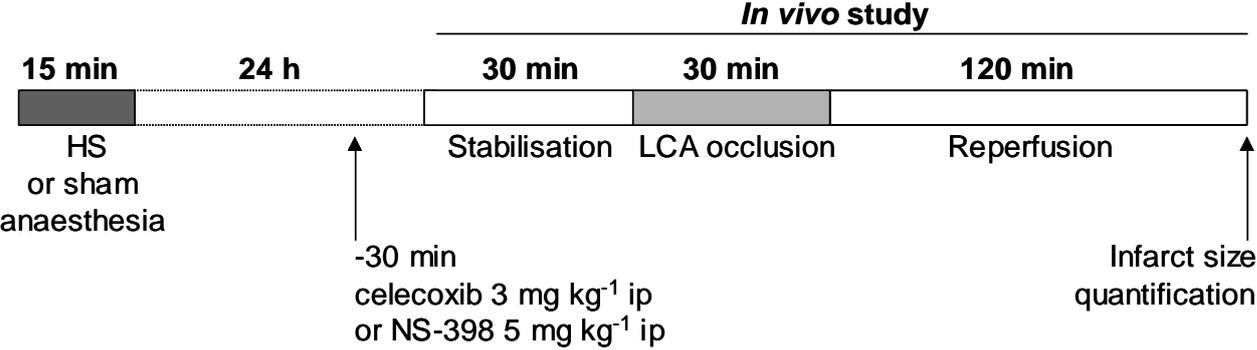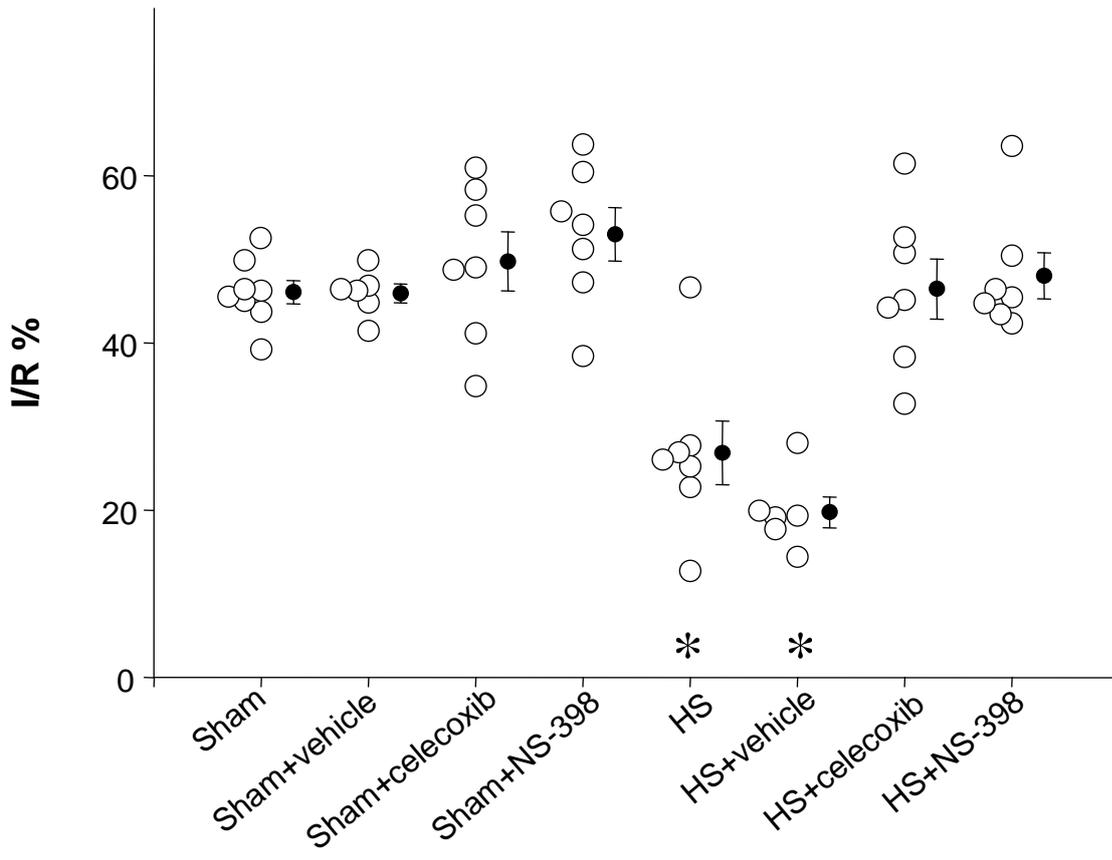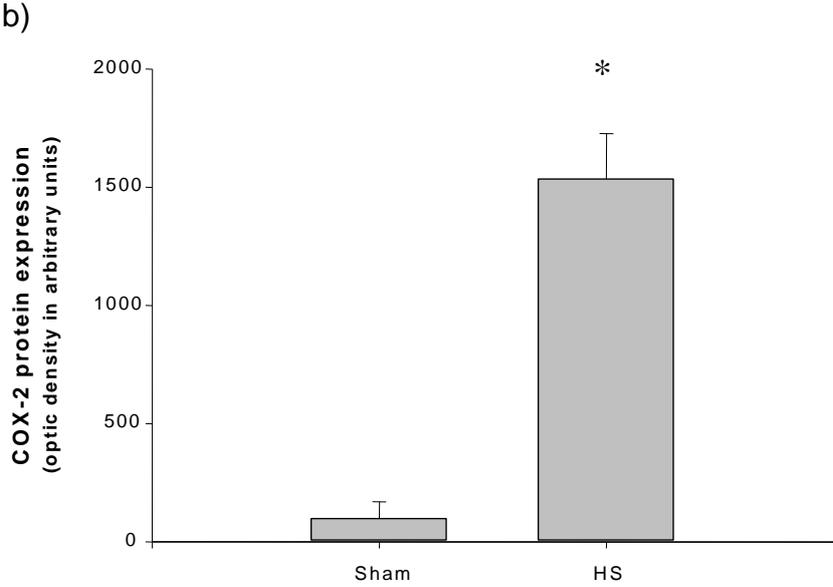
Figure 1.

Figure 2.

a)

≈ 72 kDa

+ | Sham | HS |

b)

Figure 3.