Attenuation of heat shock-induced cardioprotection by treatment with the opiate receptor antagonist naloxone

HEMAL H. PATEL, ANNA HSU, AND GARRETT J. GROSS
Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Received 20 September 2001; accepted in final form 15 January 2002

Patel, Hemal H., Anna Hsu, and Garrett J. Gross. Attenuation of heat shock-induced cardioprotection by treatment with the opiate receptor antagonist naloxone. Am J Physiol Heart Circ Physiol 282: H2011–H2017, 2002. First published January 17, 2002; 10.1152/ajpheart.00828.2001.—Whole body hyperthermia induces heat shock proteins (HSPs), which confer cardioprotection. Several opioid receptor subtypes are expressed in the heart and are linked to cardioprotection; however, no one has attempted to link the protection elicited by heat stress (HS) to opioids. Therefore, we investigated the effect of an opiate receptor antagonist, naloxone, on HS-induced cardioprotection. Anesthetized Sprague-Dawley rats were subjected to HS (42°C for 20 min) with and without naloxone pretreatment and were allowed to recover for 48 h. They then underwent 30 min of ischemia followed by 2 h of reperfusion. An acute HS group was given an intravenous bolus of naloxone (3 mg/kg) 10 min before index ischemia. Infarct size (IS), expressed as a percentage of the area at risk (IS/AAR), was determined. The right heart was excised for analysis of HSP content by Western blot.

Heat-shocked rats showed significant reductions in IS/AAR versus control (16 ± 3 vs. 58 ± 4%, P < 0.001). Pretreatment with naloxone before HS attenuated the protective effects in a dose-dependent fashion, with significant attenuation of protection occurring at 15 mg/kg naloxone versus heat shock (42 ± 6 vs. 16 ± 3%, P < 0.001). Acute treatment with naloxone (3 mg/kg) 24 h after recovery from HS also significantly attenuated the delayed protective effect (47 ± 4 vs. 16 ± 3%, P < 0.001). No difference was seen in the level of HSP70 induced in the different groups. We conclude that heat shock-induced cardioprotection can be attenuated by naloxone, an opiate receptor antagonist, without reducing heat shock-induced cardioprotection; whole body hyperthermia.

The opioid system promises to be a major target for pharmacological intervention in the treatment of myocardial infarction. Protection from stresses such as ischemia is elicited by activation of opioid receptors in numerous organs. Opioids are implicated in both early (26) and delayed cardioprotection (10), and it is well established that the δ1-opioid receptor is the dominant form leading to cardioprotection via ischemic preconditioning in rats (25). Treatment with agonists (3, 10, 26) and antagonists (10, 25) of δ1-opioid receptors results in cardioprotection and blockade of protection, respectively.

In addition to opioid receptor stimulation, other stimuli also result in cardioprotection. One such stimulus is whole body hyperthermia. This stress increases mRNA levels for heat shock protein (HSP)72 in dogs (8) and rats (35), and subsequent protein induction is protective to the heart (9). Induction of HSP90 (5), HSP70 (4, 5, 9), HSP60 (15), HSP25 (4, 16), and HSP10 (5) protects the whole heart or cells derived from the heart. Similarly, inhibition of the induction of HSP72 by use of 14-mer antisense results in an increased susceptibility to hypoxic injury in cardiac myocytes (19).

Hutter et al. (14) showed that not only are HSPs necessary, but also the amount of HSP72 produced by varying degrees of heat stress is directly related to the level of protection that is afforded against ischemia-reperfusion injury. Many groups have shown that whole body hyperthermia can elevate HSPs and afford protection against reperfusion arrhythmias (29) and ischemia-reperfusion injury (6, 9).

However, although detectable levels of HSPs can be seen as soon as 2 h after whole body hyperthermia with a peak at 12 h, no protection is associated with this initial rise in HSPs (24). Protection is observed at 24 h after heat stress; however, increased levels of HSPs continue to be detected in heat-shocked animals even after the 24-h time window of protection (24). There seems to be a disparity between protein induction and protection afforded because the time course of HSP induction does not correspond temporally with protection. It is possible that HSPs are an epiphenomenon being elevated in response to whole body hyperthermia without directly contributing to cardioprotection.

Some investigations (7, 29, 34) have suggested that the protection may be a response to the elevation of antioxidants, whereas other studies (1, 17) have shown that antioxidants may not be the mediators. It may be possible that HSPs modulate other end effectors of delayed cardioprotection that have been reported. It...
has been shown by different groups that opening the ATP-sensitive $K^+$ ($K_{ATP}$) channel may be important in heat shock-mediated protection and that blockade of this channel can attenuate heat shock-induced protection (12, 22).

Although it has been established that heat stress induces cardioprotection, much controversy surrounds the exact mechanism via which this protection is mediated. Therefore, we investigated the role of the opioid system in heat stress-mediated cardioprotection. Because there seems to be a lack of temporal correlation between HSP induction and cardioprotection, we believe that heat stress modulates the opioid system as well as HSPs; however, it is the opioid system that mediates cardioprotection. We administered the non-selective opioid antagonist naloxone either before the heat stress stimulus on day 1 and immediately before the index ischemic period on day 3 to determine at which time opioids produce cardioprotection in association with heat stress.

**METHODS**

**Study groups.** Male Sprague-Dawley (200–250 g) rats were used. The rats were divided into three groups (Fig. 1). All rats underwent the general surgical procedure 48 h after whole body hyperthermia. The rats were fed standard rodent food and had ad libitum access to water.

**Heat shock protocol and naloxone pretreatment.** Rats were anesthetized with 40 mg/kg pentobarbital sodium intraperitoneally. Anesthetic was supplemented as needed. Rats were pretreated with naloxone (5, 10, or 15 mg/kg ip) once they were anesthetized. The rats were then placed on a heating blanket and covered with terracotta cloth for insulation. Colonic temperature, measured using a rectal probe with a digital output, was elevated to 42°C. Colonic temperature was maintained for 20 min with a deviation of ±0.2°C. Once the desired temperature was obtained, the rats were removed or kept on the heating blanket as needed to maintain the proper temperature. After 20 min of hyperthermia, the rectal probe was removed, and the rats were returned to a cage where they had access to food and water. The posthypothermic status of the rats was monitored until the rats awoke from the anesthetic. Recovery periods lasted for 48 h, at which time the rats underwent the general surgical procedure.

**HSP measurement.** The right ventricle was placed in lysis buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.05 μl/mg protease inhibitor cocktail (Sigma), 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.3% [β-mercaptoethanol] on ice. The hearts were then homogenized using a Tekmar Tissumizer (model SDT-1810) and placed directly on ice. The homogenized slurry was transferred to a clean tube and centrifuged at 10,000 rpm for 10 min at 4°C (Eppendorf centriuge 5801 R). The supernatant was aliquoted to sterile microcentrifuge tubes. Protein concentrations were measured using the Bio-Rad Protein Assay with bovine serum albumin as the standard. The aliquoted samples were kept at −80°C until used.

Total protein (50 μg) was loaded onto a 10% SDS-PAGE gel and separated by electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride membrane by electroelution. The efficiency of the transfer was determined by looking at the transfer of pre-stained molecular markers (Kaleidoscope Prestained Standards, Bio-Rad). Heat-shocked HeLa Cell Extract (LYC-HL101F, StressGen) was run as a positive control on each membrane. The membranes were blocked in milk overnight and then probed with specific antibodies. The membranes were incubated with primary mouse monoclonal antibody for HSP70 (detects only inducible HSP72, not the constitutive form HSP73) at 1:5,000 dilution (SPA-810, StressGen). They were then incubated with an anti-mouse IgG-horseradish peroxidase conjugate secondary antibody. Specific antibody binding was detected using ECL (RPN2106, Amersham Pharmacia Biotech) and visualized by exposure to X-ray film.

**General surgical procedure and acute naloxone treatment.** Rats were anesthetized with 120–150 mg/kg Inactin intraperitoneally. The right jugular vein was cannulated for the...
delivery of saline. The right carotid artery was cannulated for the measurement of blood pressure and heart rate. Pressure and rate measurements were monitored using a Gould PE50 or PE23 pressure transducer connected to a Grass model 7 polygraph. A tracheotomy was then performed. The trachea was intubated with a cannula connected to a rodent artificial ventilator (model CIV-101, Columbus Instruments: Columbus, OH, or model 683, Harvard Apparatus; South Natick, MA). The rats were ventilated with room air at 38–45 breaths/min supplemented with \( O_2 \). Atelecisia was prevented by maintaining a positive end-expiratory pressure of 5–10 mHg. Arterial pH, \( P_{CO_2} \), and \( P_{O_2} \) were monitored at control, 15 min of occlusion, and at 60 and 120 min after reperfusion using a blood gas system (AVL 995 pH/blood gas analyzer). Normal values were maintained by adjusting the respiratory rate and/or the tidal volume. Body temperature was maintained at 35–37°C using a heating pad.

Once heart rate and blood pressure had stabilized, a left thoracotomy was performed at the fifth intercostal space. A pericardiostomy was then performed, followed by adjustment of the left atrial appendage to locate the left coronary artery. A ligature (6-0 Prolene) was passed below the left descending coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Occlusion for a period of 30 min was elicited by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat. This resulted in left ventricular ischemia. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood pressure. Reperfusion, for a period of 2 h, was achieved by unclamping the hemostat and loosening the snare. Reperfusion was confirmed by a marked hyperemic response at reperfusion.

In the acute naloxone treatment group, naloxone was given as a 3 mg/kg iv bolus through the jugular vein 10 min before the 30-min occlusion period. Occlusion was elicited by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat. Reperfusion, for a period of 2 h, was achieved by unclamping the hemostat and loosening the snare.

**Determination of infarct size.** After the 2-h period of reperfusion, the coronary artery was again occluded using the snare. The area at risk (AAR) was determined by negative staining. Patent blue dye was administered via the jugular vein to stain the nonoccluded area of the left ventricle. The heart was excised, and the left ventricle was separated from the remaining tissue and cut into thin cross-sectional pieces. The normal areas were stained blue, whereas the AAR remained pink. The normal area and AAR were separated and placed in different vials containing 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 100 mM phosphate buffer (pH 7.4). These vials were incubated at 37°C for 15 min. TTC is an indicator of viable and nonviable tissue. Tissues were fixed overnight in 10% formaldehyde, and the infarcted tissue was dissected from the AAR using a dissecting microscope (Cambridge Instruments). Infarct size (IS) and AAR were determined by gravimetric analysis. IS was expressed as a percentage of the AAR (IS/AAR).

**Statistical analysis.** All values are expressed as means ± SE. For the infarct size, densitometry, hemodynamic data, left ventricular mass, and AAR, statistical significance was determined by performing a one-way ANOVA with Bonferroni's multiple comparison test as the post hoc test. Significance was attributed to those groups that had a \( P \) value <0.05.

**RESULTS**

**Hemodynamics.** The hemodynamic data, which include heart rate, mean arterial blood pressure (MAP), and rate-pressure product (RPP), are summarized in Table 1. Some differences in heart rate and RPP from the control were observed in the heat-shocked and naloxone-treated rats compared with the control group. The differences were mainly observed at baseline and during the 30-min occlusion period. No significant differences from control were observed among any of the groups at 2 h of reperfusion.

**Heat shock and acute naloxone treatment.** Naloxone (3 mg/kg), given as an intravenous bolus 10 min before the index ischemia-reperfusion protocol following the 48-h recovery period after heat stress (Fig. 2), significantly attenuated the cardioprotective effects of heat stress (46 ± 4 vs. 16 ± 3%, \( P < 0.001)\). However, the acute naloxone treatment group was not significantly different from the non-heat-shocked control group given naloxone 10 min before index ischemia (46 ± 4 vs. 51 ± 3%).

**Heat shock and pretreatment with naloxone.** Pretreatment with naloxone just before heat stress on day 1 also attenuated the cardioprotective effects of heat stress in a dose-dependent manner (Fig. 3). Significant attenuation of the cardioprotective effects of heat stress was observed in the naloxone group as compared with the control and 5 mg/kg pretreated groups.

**Table 1. Hemodynamics**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>15 min Ischemia</th>
<th>2 h Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart rate, beats/min</td>
<td>MAP, mmHg</td>
<td>RPP, mmHg/1,000</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>340 ± 28</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>48 h heat shock</td>
<td>12</td>
<td>280 ± 9*</td>
<td>84 ± 5</td>
</tr>
<tr>
<td>Acute naloxone (3 mg/kg)</td>
<td>9</td>
<td>309 ± 11</td>
<td>109 ± 3</td>
</tr>
<tr>
<td>48 h heat shock + naloxone</td>
<td>12</td>
<td>277 ± 14*</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>3 mg/kg acute</td>
<td>9</td>
<td>270 ± 15*</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>5 mg/kg pretreated</td>
<td>9</td>
<td>293 ± 15</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>10 mg/kg pretreated</td>
<td>11</td>
<td>286 ± 11</td>
<td>94 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), number of measurements. Rats were subjected to 30 min of ischemia followed by 2 h of reperfusion. Specific treatment group is listed. Measurements were taken at baseline and after 15 min of ischemia and 2 h of reperfusion. MAP, mean arterial pressure; RPP, rate-pressure product. *Significant difference from specific control group.

AJP-Heart Circ Physiol • VOL. 282 • JUNE 2002 • www.ajpheart.org
stress was seen at 15 mg/kg of naloxone (40 ± 5 vs. 16 ± 3%, P < 0.001).

HSP70 expression and naloxone treatment. HSP70 expression was seen in each of the groups (Fig. 4A). The densitometry results that compare the levels of protein expression of the different groups to the HeLa heat-shocked cell lysate sample show that naloxone treatment did not significantly alter HSP70 expression from that of the 48-h heat-shocked rats (Fig. 4B).

DISCUSSION

Our results demonstrate that naloxone, a nonselective opioid antagonist, abrogated the protective effects of whole body hyperthermia without altering the expression of HSP70. This is the first time opioids and heat stress have been linked mechanistically in providing delayed cardioprotection in an intact rat model. Others have previously shown a link between hyperthermia and opioids in the rat brain (27); however, the elevation of endogenous opioids in response to hyperthermia was deleterious to normal brain function. The important implication though is that heat stress elicits a response in inducing not only HSPs but also endogenous opioids, the function of which may be different based on tissue localization and signaling machinery associated with the opioid receptors. We show that acute treatment with naloxone (3 mg/kg) just before the index ischemia period on day 3 completely attenuates the protective effects of heat stress, and following naloxone pretreatment on day 1 there is a dose-dependent attenuation of the protective effects of heat stress.
although this inhibition occurred at a higher dose of naloxone (15 mg/kg) than that observed on day 3. This is most likely the result of giving naloxone by intraperitoneal injection on day 1 versus intravenous injection on day 3. This suggests that there is a link between whole body hyperthermia and opioid receptor stimulation that leads to cardioprotection. It is likely that whole body hyperthermia, in addition to increasing the expression of heat shock proteins, alters the expression of other factors most likely including endogenous opioids. Furthermore, based on our observations, it appears that it may be the latter of the two that mediates heat stress-induced cardioprotection.

We recently showed (21) that heat shock-induced delayed cardioprotection was dependent on the strain of rat; however, there appeared to be little difference in the induction of HSP70 and HSP90 between the different strains. We concluded that HSP induction could be an epiphenomenon, and it may be some factor that is coinduced with HSPs that mediates the delayed cardioprotective effect. Other studies have shown that HSPs can be induced as early as 2 h and peak at 12 h without having any protective effect at these time points (24). This is puzzling because it seems that optimal levels of proteins thought to mediate protection are present, yet cardioprotection is lacking at these early time points.

Qian et al. (23) have shown that in rats ischemic preconditioning does not induce delayed cardioprotection, even though levels of HSP70 are comparable with those observed after whole body hyperthermia. In addition, Taylor et al. (31) show that exercise alone without HSP induction confers protection. This suggests that the stimuli rather than the end product of the stimuli are more important in determining cardioprotection. However, it is likely that a common factor exists between the stimuli that mediates delayed cardioprotection.

Opioids have long been characterized by neuroanatomical localization and have been primarily related to brain function. The opioid families are composed of enkephalins, dynorphins, and endorphins that interact with δ-, κ-, and μ-opiate receptors, respectively. Opioid peptides are not limited to neuronal tissue but can also be localized in nonneuronal tissue. Enkephalins and their precursors have been found in the heart (13, 28, 33) as well as other tissues that include the spleen, vas deferens, stomach, lung, pancreas, and liver (2, 30). These enkephalins appear to be localized in such a way as to be able to modulate cardiovascular activity.

Levels of preproenkephalin (ppENK) mRNA are highest in the heart compared with tissues such as the brain (13); however, the interesting point is that although the mRNA levels are high, the corresponding peptide levels are low. This suggests that there is a large pool of precursor present in the heart that can either be primed for more production or converted to active peptide by an appropriate signal. In rats, it was found that the level of ppENK increases in the ventricles following myocardial ischemia (20) and cardiac hypertrophy (11). These examples illustrate a possible stress-induced regulation of the endogenous opioid system. It has been suggested that the large pool of ppENK mRNA localized in the heart could serve an

---

**Fig. 5.** Schematic representing possible signaling pathway. We propose in the study that HS causes the induction or possible release of factors such as HSPs or endogenous opioids. We believe for the data presented that it is the endogenous opioids that then contribute to downstream signaling through the δ-opioid receptor to manifest delayed cardioprotection possibly through stimulation of mitochondrial ATP-dependent K channels (mitoK$_{ATP}$), as has been proposed in numerous models.
endocrine or autocrine function (32, 33). It seems likely that these opioid precursors may be the common mediator that is induced by various stimuli including whole body hyperthermia.

However, one likely possibility that explains the presence of HSP70 even when protection is blocked by naloxone may be that HSP70 is a trigger of protection. Then it would be likely that the trigger, HSP70, is elevated in response to a conditioning stimulus such as heat stress. Thus the reason we could block the protection by administration of naloxone on day 1 and day 3 could be that opioids are downstream of HSP70 generation in the signaling scheme involved in heat stress-induced delayed cardioprotection. The dose response associated with naloxone pretreatment suggests that the more naloxone that is injected the longer time it takes to be eliminated from the rat. This would result in higher plasma concentrations of naloxone for a longer time period and possibly modulate the effects of HSP70 expression that occur hours after heat stress. Lower doses would be eliminated from the rat before HSP70 could be expressed and act as the trigger for delayed protection. In addition, treatment with naloxone on day 3 would allow HSP70 protein expression to occur and the triggering steps to be put into motion. However, if opioid receptors are modulated by HSPs in some way and are downstream effectors of heat stress, then blockade of the receptors before ischemia would inhibit protection. It still appears that there is some connection between heat stress and opioid receptors that mediates delayed cardioprotection.

Although we show a link between opioids and hyperthermia in producing cardioprotection in the intact rat, a recent study by Zhou et al. (36) showed a link between opioids and HSPs in mediating delayed cardioprotection in rat ventricular myocytes. Their observations argue against HSPs being triggers of protection. They suggested that opioids are upstream in the signaling cascade as κ-opioid agonist administration induced HSP70. However, our observations (personal observations) in the intact rat suggest that δ-opioid receptor stimulation does not induce HSP production. It is possible that the concentration of δ-opioid receptor agonist used was too low; however, the concentration used was associated with a marked cardioprotective effect. In our studies, we observed that naloxone does not lower the induction of HSP70, suggesting that opioid receptor stimulation may not necessarily lead to HSP induction; however, it is also possible that hyperthermia may overwhelm the contribution of opioids to HSP induction. Sharma et al. (27) show in the rat brain that not only does hyperthermia increase HSP70 induction but also that this induction is abolished by pretreatment with naloxone. This suggests similar to Zhou et al. (36) that opioids are directly involved in HSP induction. Differences may arise from specific tissues studied, levels of endogenous opioids, or the concentration of opioid agonist used as a stimulus.

The link between stress, protein induction, opioids, and cardioprotection appears to be very complex (Fig. 5). Interestingly, a study by Mayfield et al. (18) suggests that hypoxic preconditioning is mediated by an opioid-dependent mechanism. They hypothesized that hypoxic preconditioning results in the release of endogenous opioids, which function to lower the set point of the animal by reducing body temperature and potentially reducing oxygen demand during hypoxia. They observed that the decrease in set point was abolished by naloxone treatment. Although the stress of hypoxia is different from that of hyperthermia, it appears that endogenous opioids may be released in response to both stimuli, and, in terms of heat stress, the response of endogenous opioid release may be a decrease in set point, as suggested by Mayfield et al. (18), producing a decrease in metabolism and potentially resulting in protection of the animal during severe stress. If this were the case, the administration of naloxone would be expected to eliminate the afforded protection as is suggested by our results.

In conclusion, heat stress-induced delayed cardioprotection partially involves the activation of opioid receptors. It appears that the modulation of opioid receptor activity is independent of HSP70 expression. Clinically, this work is relevant because it implicates opioids, which are already clinically used, and possibly mobilizers of endogenous opioids as a therapeutic basis for providing delayed protection to the myocardium. The implication of these findings is that different triggers such as opioids and heat stress appear to be connected in some broad network of pathways whose end effect is delayed cardioprotection.

This work was supported by an American Heart Association-Northland Affiliate Predoctoral Grant (to H. H. Patel) and National Heart, Lung, and Blood Institute Grant HL-08311 (to G. J. Gross).

REFERENCES


8. Dillmann WH, Mehta HB, Barriex A, Guth BD, Neeley WE, and Ross J Jr. Ischemia of the dog heart induces the appearance of cardiac mRNA coding for a protein with migration...