Heat shock treatment results in increased recruitment of labeled PMN following myocardial infarction

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Heat shock treatment results in increased recruitment of labeled PMN following myocardial infarction. Am J Physiol Heart Circ Physiol 293: H3210–H3215, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00773.2007.—One of the proposed mechanisms for the myocardial protective effects of heat shock (HS) treatment has been a reduction in the inflammatory response. The objective of the present study was to evaluate the impact of HS treatment in an established model of polymorphonuclear cell (PMN) migration following myocardial infarction (MI). Isolated purified PMNs (10 × 10^6 cells) labeled with ^51Cr were injected into Lewis rats following a left thoracotomy and ligation of the left anterior descending coronary artery causing MI. Two experimental groups of animals were created: MI group (n = 11) and HS+MI group (n = 7). HS treatment consisted of an elevation in core temperature to 42°C for 15 min 24 h prior to MI. An additional group of control animals underwent sham thoracotomy (n = 5). All animals were euthanized at 24 h after MI, and gamma counts were obtained to estimate PMN migration. Myocardial injury was confirmed in all experimental animals (histology and echocardiography). The serum troponin I and infarct size (triphenyltetrazolium chloride) were similar in both groups. Labeled PMN migration was significantly higher in HS+MI animals (14.3 × 10^6 ± 3.7 × 10^5 PMN) compared with MI group (9.5 × 10^6 ± 3.6 × 10^5; P = 0.01), suggesting increased PMN migration as a result of HS treatment. HS treatment did not affect PMN migration to positive skin control sites (LPS). ICAM-1 myocardial expression was not significantly increased in HS+MI compared with MI group. In summary, HS treatment results in increased PMN migration into myocardium following MI independent of ICAM-1. These findings suggest that the proposed cardioprotective effect of HS may not be entirely due to a downregulation of myocardial inflammation as previously proposed.

heat shock (HS) proteins (HSP) are highly conserved molecular chaperones that are constitutively expressed under normal conditions (5–10% total protein). They have gained tremendous attention because of their perceived ability to protect cells against pathological stress. HSPs are believed to confer cytoprotective functions under physiological stress such as ischemia (23, 38, 39). In rats, whole body heat shock (42°C for 15 min) has been shown to result in increased HSP expression, which was associated with improved cardiac functional recovery following ischemia (9). Furthermore, HS treatment confers myocardial protection to subsequent ischemic exposure and has been shown to reduce infarct size (10, 14, 29, 35). Although there is little debate about the likely beneficial effects of HS treatment prior to ischemic stress, the mechanism for this protection remains to be determined. Some have suggested that the mechanism by which HS is protective to the myocardium is by downregulating the inflammatory response. In fact, HS treatment has been shown to suppress activation of the IKK/NF-κB proinflammatory pathway (5–8).

Much of the evidence to date suggests that a key component of cardiac remodelling after myocardial infarction (MI) is an inflammatory response that can modulate left ventricular (LV) tissue repair (17, 18, 37). As part of this inflammatory response, leukocytes have been shown to accumulate within the myocardium following MI (31, 33). In fact polymorphonuclear cells (PMN) that accumulate in the first 24 h have been suggested to be important effector cells responsible for some of the observed myocardial cell damage (11). In the present paper we sought to investigate the effects of HS treatment prior to MI and its effect on PMN migration.

MATERIALS AND METHODS

Animal model. Inbred Lewis rats (RT1.A) weighing 300–350 g were purchased from Charles River Canada (St. Constant, QC, Canada) and housed in the Carlton Animal Care Facility, Dalhousie University, with food and water ad libitum for 1 wk prior to experimentation in accordance with the guidelines of the Canadian Council of Animal Care. All animal experimentation was approved by the Dalhousie University Animal Care Committee. Rats were randomly assigned to the sham group, MI group, or HS+MI group.

For heat shock treatment, animals were anesthetized with ketamine HCl 100 mg/kg ip (Parke Davis) and xylazine 5 mg/kg ip (Lloyd Laboratories) and then placed on a heating (50°C) until their rectal temperature reached 42°C for 15 min (9). Twenty-four hours after HS all animals were once again anesthetized with ketamine and xylazine followed by endotracheal intubation and ventilation. A left thoracotomy was performed, and the left coronary artery was ligated with 6-0 silk suture ~4 mm from its origin as previously described (34). The chest was closed (4-0 nonabsorbable suture), and the animals were allowed to recover in sternal position, warmed, and provided with 100% oxygen by nose cone until fully awake. Analgesia was extended by local xylocaine injection at the end of the procedure and the animal recovered overnight in warmed housing. There were three groups of animals: two experimental groups with coronary ligation and one negative control. Negative controls were sham-operated animals in which a left thoracotomy was performed without left coronary artery ligation. In all experimental animals the kinetics of PMN infiltration was evaluated by using labeled purified PMNs (see Leukocyte isolation below). Labeled cells were injected (intravenously) via the dorsal vein of the penis at the time of surgery, i.e., at the onset of ischemia. In all animals LPS (100 ng) was injected intradermally (2 skin sites/animal) at the time of surgery to serve as a positive control for PMN migration and compared with diluent (RPMI medium). Animals

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in all groups were killed 24 h after the onset of ischemia. The heart was removed and perfused with saline, and the radioactivity was determined by gamma counting as a measure of PMN accumulation. The distribution of PMN was also monitored by measurement of the radioactivity in the blood, liver, spleen, and the inflamed skin site at the time of death.

**Echocardiography.** In an additional group of animals (n = 5 MI and n = 5 HS + MI), transthoracic echocardiography was performed in a blinded fashion (to the echocardiographer) by using the Vivid 7 (GE) with a 7.5-MHz transducer on day 0 (prior to MI) and day 1. LV dimensions at the end of systole and end diastole (LVEDD and LVEDD, respectively) were measured digitally in the parasternal short-axis view at the level of the papillary muscles and averaged from three cardiac cycles (22). LV fractional shortening (FS, in %) was calculated as [(LVEDD − LVEDD)/LVEDD]×100. The ejection fraction (EF) calculations were volume based on the M-mode. End diastole was timed to the Q wave and end systole on the narrowest LV cavity size. Additional end-systolic and end-diastolic volumes were measured in single-plane apical four-chamber views.

**Leukocyte isolation.** Rat blood PMNs were isolated as previously described by Williams et al. (42) with some modifications. Briefly, a 21-gauge butterfly needle was inserted into the inferior vena cava of an anesthetized donor rat immunized with killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) 7–10 days earlier to induce leukocytosis. The animal was heparinized and the blood volume was gradually exchanged with 50 ml of 6% dextran (Sigma-Aldrich Canada, Oakville, ON, Canada) and collected into acid-citrate-dextrose (Sigma-Aldrich Canada). The red blood cells were sedimented at 1 g and the leukocyte-rich plasma was harvested. The leukocytes were pelleted by centrifugation and resuspended in calcium and magnesium-free Tyrode’s solution containing 10% platelet-poor plasma (PPP). The PMNs were purified on discontinuous Percoll gradients of 63% layered above 74% Percoll as previously described (25). The recovered PMNs had a purity of >92% and a viability of >99%. After being washed in Tyrode’s-5% PPP, the cells were resuspended at 50 × 10^6 PMNs per milliliter and incubated (37°C for 30 min) with 1 μCi 51Cr/10^6 neutrophils (MP Biomedicals, Irvine, CA). The labeled PMNs were then washed in Tyrode’s-20% PPP and 10 × 10^6 cells were injected intravenously into three groups of animals.

**Histological processing.** Tissue was fixed in 4% formalin for 24 h, paraffin embedded, serially sectioned (5 μm), and stained with hematoxylin and eosin. Immunocytochemical techniques on paraffin-embedded tissue were used to localize expression of specific proteins such as cell adhesion molecules. Briefly, slides were deparaffinized and antigens retrieved with a sodium citrate solution. Endogenous peroxidases were quenched (HOOH/PBS), and nonspecific staining was blocked (DAKO Biotin Blocking System, DakoCytomation, Mississauga, ON, Canada). Sections were incubated with goat anti-ICAM-1 (CD54) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by biotinylated anti-goat IgG and by streptavidin-horseradish peroxidase (LSAB +; DakoCytomation) and developed using 3,3’-diaminobenzidine as the chromogen (DakoCytomation).

**Infarct size measurement (TTC).** In a separate group of experimental animals, the heart was harvested, wrapped in plastic wrap, and placed at −20°C for 2 h. Heart tissue was cut into cross sections ~3 mm thick and placed in triphenyltetrazolium chloride (TTC) stain solution (1 g/100 ml in sodium phosphate buffer, pH 7.4). The slices were incubated in the stain for 20 min at 37°C with constant agitation. The tissue sections were then transferred to a 10% formalin solution for 20 min to further differentiate the staining and fix the tissue. The slices were compressed between two thin glass plates and photographed with a dissecting microscope and an AxioVision camera. Total heart and infarct surface areas were measured with ImageJ software by using known pixel-to-centimeter conversion. The surface areas were multiplied by the estimated 3-mm thickness to determine total heart and infarct volume of each slice. Composite pictures of TTC-stained hearts were compiled using Adobe Photoshop.

**Tissue processing for ELISA.** At the time that animals were euthanized (24 h), blood was collected and the serum cardiac troponin I was measured by ELISA (2010-2-HS, Life Diagnostics, West Chester, PA, RIC-100).

**Tissue processing for Western blot.** Heart tissue was also harvested 0, 2, 4, and 24 h after the onset of ischemia, frozen immediately, and stored at −70°C. To prepare protein extracts from myocardium, hearts were thawed and homogenized on ice in 200 mg/ml RIPA (RadioImmunoPrecipitation Assay: Triton X, sodium dodecyl sulfate, pepstatin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, iodacetamide) buffer. Protein samples (25 μg) were boiled for 2 min in sample buffer (250 mM Tris-HCl, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.003% bromophenol blue), separated on denaturing 10% SDS-polyacrylamide gels, and then transferred onto polyvinylidene fluoride membranes (Millipore; Billerica, MA). Membranes were blocked with 5% milk solution at room temperature for 2 h. After three washes in TBS-T (Tris-buffered saline with 0.1% Tween 20), immunoblots were then incubated overnight at 4°C with primary antibody [mouse anti-rat ICAM-1 1:2,000 (BD Pharmingen) or mouse biotin monoclonal anti-HSP-27 1:1,500 (StressGen)]. Blots were washed with TBS-T three times before secondary antibody (mouse-anti IgG peroxidase conjugate; 1:2,500, Sigma). After another three washes with TBS-T, membranes were reacted with the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer’s protocol and then exposed to film. Protein levels were quantified by scanning densitometry using image-analysis systems (Image J software).

**Statistical methods.** Data are reported as means and SE. Continuous variables were analyzed with an unpaired t-test. Statistical significance was defined as a P value of <0.05.

**RESULTS**

**Effect of coronary ligation on the myocardium.** The mean weight of all experimental animals was 346 ± 41 g, and no significant differences were evident between groups. In vivo coronary ligation was performed in all experimental animals followed by a 24-h recovery with 100% survival (n = 18). Sham operated-animals (+HS treated) underwent thoracotomy without coronary ligation (n = 5). The serum troponin I, a marker of myocardial cell injury and infarct size, measured at 24 h after the onset of ischemia was significantly higher in MI and HS+MI group compared with sham controls (Fig. 1). However, troponin I levels between MI and HS+MI groups...
were not different, suggesting similar degrees of myocardial injury (infarct size).

Transthoracic echocardiogram was performed in an additional group of experimental animals: MI group (n = 5) and HS+MI group (n = 5). Ligation of the left anterior descending artery was associated with a significant reduction in EF and FS in both experimental groups compared with baseline (P < 0.01). However, there was no significant difference in EF or FS pre or postinfarction between the MI vs. HS+MI groups further confirming similarities between groups in terms of infarct size (Fig. 2). In a separate group of animals infarct size were assessed using a standard TTC showing no significant infarct size differences between the MI and HS+MI group following coronary ligation (Fig. 3). A single outlier animal (1/10) in the MI group had a small infarct (<10%).

PMN accumulation in the posts ischemic myocardium. To understand better PMN migration associated with myocardial ischemia, tracer-type experiments were carried out using purified blood PMNs labeled with $^{51}$Cr and injected intravenously at the onset of ischemia (Fig. 4). Migration of labeled PMNs into the myocardium at 24 h was significantly higher in both experimental groups (MI and HS+MI) compared with sham control (Fig. 4). In addition, labeled PMN migration was significantly higher in the HS+MI group compared with the MI group (P = 0.01), suggesting significantly higher PMN migration in HS-treated animals.

Positive control migration of PMN was evaluated by using LPS intradermally compared with diluent injection sites. LPS caused a significant (P < 0.001) increase in labeled cell accumulation in all groups compared with diluent-injected sites (Fig. 5), indicating that the labeled PMNs were recruited to inflamed skin with rapid kinetics (migration to skin sites previously described) (26). In addition, HS treatment did not result in increased dermal migration by PMNs compared with non-HS-treated animals. There was no difference in the liver

![Fig. 2. Transthoracic echocardiography performed pre (day 0) and post (day 1) MI between MI (n = 5) and HS+MI groups (n = 5). Left ventricular (LV) dimensions at the end of systole and end diastole (LVESD and LVEDD, respectively) were measured digitally in the parasternal short-axis view at the level of the papillary muscles and averaged from 3 cardiac cycles (blinded echocardiographer). LV fractional shortening (FS, in %; B) was calculated as $[(LVEDD−LVESD)/LVEDD]×100$. The ejection fraction (EF; A) calculations were volume based on the M-mode. End diastole was timed to the Q wave and end systole on the narrowest LV cavity size.](http://ajpheart.physiology.org/)

![Fig. 3. Infarct size was measured 24 h after coronary ligation between the MI and HS+MI group by a standard triphenyltetrazolium chloride technique, which outlines dead tissue as white and live as bright red (A). The mean infarct sizes were expressed comparing the MI (n = 4) and HS+MI groups (n = 6) (B). P = 0.28.](http://ajpheart.physiology.org/)

![Fig. 4. Polymorphonuclear cell (PMN) infiltration in the ischemic myocardium. $^{51}$Cr-labeled blood neutrophils were injected intravenously at the time of coronary artery ligation in the MI group (n = 11) and the HS+MI group (n = 7). Sham-operated control animals were injected with labeled cells at the time of surgery (n = 10). At 24 h after surgery, the accumulation of labeled PMN was determined by gamma counting. Each bar shows mean ± SE. *P < 0.01 comparing MI and HS+MI.](http://ajpheart.physiology.org/)

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and spleen accumulation of the labeled cells, confirming that there distribution was not affected by the ischemia (data not shown).

Expression of ICAM-1 in postischemic myocardium. The ICAM-1/CD18 integrin interaction is believed to be important in recruiting PMNs to sites of inflammation (20, 21). As such, the expression of ICAM-1 in whole heart homogenates was evaluated by Western blot. ICAM-1 myocardial expression was significantly higher in the MI and HS+MI groups compared with control hearts (Fig. 6). However, there was no evidence that HS itself resulted in increased myocardial expression in ICAM-1. These findings suggest that the mechanism responsible for the increased migration by labeled PMNs seen in HS-treated animals may not be ICAM-1 dependent.

Expression of HSP27 in postischemic myocardium. The expression of myocardial HSP-27 increased with HS alone and was significantly higher in the MI and HS+MI groups compared with sham controls (Fig. 7). These findings suggest that the degree of upregulation in the expression of HSP27 was comparable between the MI and the HS-MI groups.

DISCUSSION

One of the key components of the myocardial response after infarction is the development of an inflammatory response within the myocardium (17, 18, 37). The inflammatory response following MI is characterized by inflammatory cell infiltration, activation of matrix metalloproteinases (MMP), and the generation of oxygen free radicals within the myocardium (41). One of the first inflammatory cells believed to infiltrate postischemic myocardium are PMNs (15, 16, 31, 33). PMNs have also been shown to be a major source of oxidants necessary for free radical generation and a major source of MMPs (11, 41). In fact, activated PMNs release proinflammatory cytokines, cytotoxic enzymes, and enzymes that produce oxygen-derived free radicals (11). Although the role for this inflammatory response is likely part of the myocardial healing process, the evidence to date suggests that PMNs may have deleterious effects resulting in further damage (17).

In the present study we examined specifically the migration of PMN to the myocardium following MI comparing animals treated with or without HS. We have shown that HS treatment results in a significant increase in labeled PMN migration to the myocardium (24 h) post-MI something that has not been described before. In addition, we have shown that the increase PMN migration was myocardial specific since there was no
increased PMN migration in control dermal migration sites of animals treated with HS.

The rationale for this experimental approach was based on experimental models of myocardial ischemia in which HS treatment has been shown to confer significant myocardial protection (9, 23, 38, 39). This myocardial protection is manifested by enhanced myocardial recovery and reduced infarct size (10, 29, 35). This has led many to hypothesize that HS is protective to the myocardium by downregulating the inflammatory response. In support of this hypothesis there has been evidence that HS treatment suppresses activation of the IKK/NF-κB proinflammatory pathway and results in decreased IL-6 and ICAM-1 expression (5–8). In addition, HS has been demonstrated to block angiotensin II-induced inflammation including ICAM and IL-6 expression (5, 6). However, HS may also be involved in activation of the innate immune response by increasing TLR signaling potentially through increased NF-κB nuclear localization (2, 12, 13, 19, 32, 40). Taken together, our findings do not support the hypothesis that HS treatment results in reduced early myocardial inflammation as a mechanism for myocardial protection but suggest that its effect is likely more complex and may include increased migration by specific inflammatory cells.

There is a large body of literature suggesting that the mechanism responsible for PMN migration is dependent on firm adhesion to the endothelium to allow extravasation via CD18 and ICAM-1 integrin molecule interactions (4, 27, 28). Supporting a key role for CD18/ICAM-1 interaction in PMN infiltration has been the cardioprotective effects of monoclonal antibody therapy against CD18, or monoclonal antibody therapy against ICAM-1 in models of myocardial ischemia (1, 24, 43). More recently, data from knockout mice deficient in either CD18 or ICAM-1 have shown reduction in neutrophil infiltration ranging from 32 to 54% following myocardial ischemia (3). In the present study we were unable to detect over the first 24 h postinfarction any increased expression of ICAM-1 by HS treatment. Taken together with the increased migration by labeled PMN seen in HS-treated animals, our findings suggest that the mechanism responsible for the increased migration appears to be ICAM-1 independent.

Similarly to others we have shown that MI is a strong stimulus for HSP27 production by myocardial cells under stress, reaching levels produced by HS treatment alone. We have used an in vivo biomarker of MI used clinically that has been shown to correlate temporally with the degree of myocardial cell death (30). Troponin release measured in the serum 24 h post MI by ELISA was shown to be no different between groups, suggesting similar infarct size and therefore likely not accounting for the increased labeled PMN migration in the HS+MI group. Additional in vivo echocardiographic data performed in a subset of animals supports the similarity in terms of myocardial damage between the MI and HS+MI groups with significant reductions in EF and FS, which were not different between HS and HS+MI groups. Finally, we were unable to demonstrate any significant differences in infarct size between the MI and HS+MI group using TTC staining technique, confirming the validity of our MI model.

Part of the novelty of the present study is that few investigators have looked at the effect of HS treatment in an MI model. To date most of the published literature has focused on a short period of ischemia followed by return of blood flow, described as a reperfusion model. One limitation of our MI model is that HS may only be protective to the myocardium following short periods of ischemia. In fact, longer periods of ischemia may not result in myocardial functional recovery, suggesting that there is a limit in ischemic damage beyond which HS is no longer protective (10, 44). We argue that if one of the proposed mechanisms by which HS is protective to the myocardium is an anti-inflammatory effect, the MI model we have used should provide a strong inflammatory stimulus to study PMN migration. The present study was not designed to test myocardial function between treatment groups but instead to evaluate the effect of HS on labeled PMN migration. In spite of the above limitations HS resulted in a robust myocardial specific increase in labeled PMN migration that appears to be myocardial specific and CD18/ICAM-1 independent. We provide evidence suggesting that HS does not result in a reduction in early inflammatory cell migration but may in fact promote this migration by a yet-to-be-described mechanism.

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REFERENCES

HEAT SHOCK AND PMN RECRUITMENT TO THE MYOCARDIUM