Heat pretreatment differentially affects cardiac fatty acid accumulation during ischemia and postischemic reperfusion

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Cornelussen, Richard N. M., Ger J. Van Der Vusse, Theo H. M. Roemen, and Luc H. E. H. Snoeckx. Heat pretreatment differentially affects cardiac fatty acid accumulation during ischemia and postischemic reperfusion. Am J Physiol Heart Circ Physiol 280: H1736–H1743, 2001.—We investigated whether the cardioprotection induced by heat stress (HS) pretreatment is associated with mitigation of phospholipid degradation during the ischemic and/or postischemic period. The hearts, isolated from control rats and from heat-pretreated rats (42°C for 15 min) either 30 min (HS0.5-h) or 24 h (HS24-h) earlier, were subjected to 45 min of no-flow ischemia, followed by 45 min of reperfusion. Unesterified arachidonic acid (AA) accumulation was taken as a measure for phospholipid degradation. Significantly improved postischemic ventricular functional recovery was only found in the HS24-h group. During ischemia, AA accumulated comparably in control and both HS groups. During reperfusion in control and HS0.5-h hearts, AA further accumulated (control hearts from 82 ± 33 to 109 ± 51 nmol/g dry wt, not significant; HS-0.5h hearts from 52 ± 22 to 120 ± 53 nmol/g dry wt; P < 0.05). In contrast, AA was lower at the end of the reperfusion phase in HS24-h hearts than at the end of the preceding ischemic period (74 ± 18 vs. 46 ± 23 nmol/g dry wt; P < 0.05). Thus accelerated reperfusion-induced degradation of phospholipids in control hearts is completely absent in HS24-h hearts. Furthermore, the lack of functional improvement in HS0.5-h hearts is also associated with a lack of beneficial effect on lipid homeostasis. Therefore, it is proposed that enhanced membrane stability during reperfusion is a key mediator in the heat-induced cardioprotection.

STUDYING THE EFFECTS OF HEAT PRETREATMENT ON CARDIAC FUNCTION

SUBSTANTIAL EVIDENCE INDICATES that heat stress (HS) pretreatment is associated with a transient protection of the heart against subsequent noxious events like ischemia-reperfusion (reviewed in Refs. 2 and 19). These cardioprotective effects have been attributed to the stress-induced elevated synthesis of specific proteins, known as heat shock proteins (HSP) (14, 17, 25). Although the involvement of HSP and, in particular, the major inducible member of the HSP family, i.e., HSP70, is believed to be essential in this process (16, 23, 29, 31), it is basically unknown how these proteins protect the cardiac cell against ischemia-reperfusion-induced damage. In addition, it is unknown whether the cardioprotective effects of HS can already be observed during the ischemic period or only exist during reperfusion.

Because ischemia-reperfusion-induced damage is a multifactor event, many cellular processes have been suggested to be affected by HS. For instance, HS-induced cardioprotection has been attributed to a lesser calcium overload (24), increased free radical scavenging capacity (1, 26), and ion-channel blockade (21). All of these processes could be indicative for an increased sarcolemmal stability in the hearts from heat-pretreated rats. Indeed, one of the most consistent findings has been a lesser postischemic cytosolic enzyme release (7, 9, 43) and a decreased incidence of ventricular arrhythmias (34). More evidence for an improved sarcolemmal integrity during postischemic reperfusion stems from a recent study (39) in hearts from heat-pretreated rats, in which the tissue fatty acid (FA) content was measured. The study found that tissue levels of arachidonic acid (AA) were significantly lower in hearts from heat-pretreated than in nonpretreated rats at the end of the reperfusion phase.

Evidence is accumulating that the damage induced by ischemia is most likely to be of different nature than that induced by reperfusion (15, 28). To determine whether the effects of HS pretreatment are ischemia or reperfusion specific, we investigated ischemia-reperfusion-associated changes in FA homeostasis in hearts isolated from control and heat-pretreated rats. To further delineate the supposed coupling between HS-mediated effects on mechanical function after ischemia-reperfusion and increased sarcolemmal stability, the study was extended to hearts studied 30 min after HS (HS0.5-h). It is known that at this time point, HS renders the heart more susceptible to ischemia-reperfusion injury, whereas the earliest time point at which cardioprotection has been documented is 24 h after HS (HS24-h) (5).
MATERIALS AND METHODS

Animals

The experiments were performed on 14-wk male Lewis rats (~300 g body wt). The rats were kept under restful housing conditions (artificial 12:12-h light-dark cycle) and had free access to water and food (Diet SRM-A, Hope Farms; Woerden, The Netherlands). All of the procedures involving animals were performed in accordance with the institutional ethical guidelines for the care and use of experimental animals.

Heat-Stress Protocol

The rats were anesthetized subcutaneously with 10 \( \mu \)g of fentanyl/100 g body wt plus 0.5 mg fluanison/100 g body wt and placed on a temperature-controlled heating pad until rectal temperature, which was continuously monitored, reached 42°C (average time to reach 42°C was 50 min). The animals were kept at this temperature for 15 min (7). All of the animals survived the HS treatment (\( n = 55 \)). Control animals were subjected to the same protocol but were kept at 37°C (control, \( n = 38 \)).

Either 30 min or 24 h after HS pretreatment, the animals were slightly reanesthetized with 2 \( \mu \)g of Hypnorm (100 g body wt plus 0.2 mg fluanison/100 g body wt sc) and euthanized by cervical dislocation. The heart was immediately removed from the thorax and either prepared for perfusion in an isolated heart perfusion setup or for determination of HSP70 tissue content (see Fig. 1). Control animals were also euthanized 0.5 or 24 h after sham treatment and investigated in the same way. Because no statistically significant differences were detected in hemodynamic function and HSP70 content, the respective data of the latter two groups were pooled.

Isolated Heart Perfusion

The hearts were quickly removed from the thorax, immersed in an ice-cold Tyrode buffer, and connected via the aorta to a perfusion apparatus (33, 36). A second cannula was connected to the left atrium to allow antegrade perfusion. The specific characteristics of the perfusion system, composition of perfusion buffer, and measurements of hemodynamic variables were described earlier (7, 33, 36). During perfusion, diastolic aortic pressure and left atrial filling pressure were kept at 60 and 10 mmHg, respectively. No-flow ischemia was induced by clamping of both cannulas.

Experimental Protocols

The experimental protocol is shown in Fig. 1. After a stabilization period of 10 min, when the hearts were perfused in the retrograde way, the left atrial cannula was opened and the hearts were allowed to eject against a pressure head of 60 mmHg. In the first series of experiments, the isolated hearts were freeze-clamped after 30 min of antegrade perfusion (preischemic series, control group, \( n = 6 \); HS0.5-h group, \( n = 7 \); and HS24-h group, \( n = 8 \)). To this end, the ventricular tissue was separated from the atria and immediately freeze-clamped with aluminum tongs that were chilled in liquid nitrogen. In the second series, after 30 min of normoxic perfusion, hearts were subjected to 45 min of global ischemia and subsequently freeze-clamped (ischemic series, control group, \( n = 6 \); HS0.5-h group, \( n = 5 \); and HS24-h group, \( n = 6 \)). After ischemia, a third group of hearts was reperfused during 15 min in the retrograde method and 30 min in the antegrade method at an identical afterload pressure as applied during the preischemic period; i.e., 60 mmHg. At the end of this reperfusion period, the hearts were immediately freeze-clamped as described above (postischemic series, control group, \( n = 12 \); HS0.5-h group, \( n = 6 \); and HS 24-h group, \( n = 8 \)). In this group, the maximum steady-state hemodynamic values attained during the preischemic and postischemic antegrade perfusion periods were used for calculation of the percentage recovery.

Throughout the experiments, coronary effluent samples (enriched with 3% BSA for enzyme stability) were immediately frozen in liquid nitrogen. The frozen tissue and coronary effluent samples were stored at -80°C for biochemical analysis.

Creatine Kinase Activity

Coronary effluent samples were monitored for the presence of creatine kinase (CK) as marker of cell membrane damage (3).

Lipid Content

Myocardial lipid content was measured in freeze-clamped tissue as described earlier (41). Briefly, the ventricles (~250 mg) were pulverized at -21°C and wetted with 2 ml of ice-cold methanol. Thereafter, at room temperature, 4 ml of chloroform was added. At this point, heptadecanoic acid was added to correct for losses during extraction and assay procedure. Lipid extracts were subjected to one-dimensional TLC on plates coated with silica gel 60 (Merck). The lipid spots were predeveloped with chloroform-methanol-water-acetic acid (10:10:1:1 vol/vol/vol/vol) and developed with a mixture of petroleum ether-diethyl ether-acetic acid (24:5:0.3 vol/vol/vol). The spots corresponding to unesterified FA, triacylglycerols, and phospholipids were made visible with rhodamine B 6G and fluorescein in methanol. Spots were scraped and transmethlated according to Morrison and Smith (27). Subsequently, the methyl esters were extracted with pentane and after evaporation of pentane and dissolved in 2,2,4-trimethylpentane-ethyl-acetate-acetic acid-H2O (con-

<table>
<thead>
<tr>
<th>Heat Shock</th>
<th>Creatine kinase</th>
<th>Creatine kinase</th>
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<tr>
<td>42°C for 15 min</td>
<td>Recovery 30 min or 24 hrs Pre-ischemia for 30 min</td>
<td>Ischemia for 45 min Reperfusion for 45 min</td>
</tr>
<tr>
<td>OR</td>
<td>Control 37°C for 15 min</td>
<td>HSP70</td>
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Fig. 1. Schematic representation of the experimental protocol (see MATERIALS AND METHODS). HSP, heat shock protein.
Heat shock proteins (HSPs) play a critical role in cellular homeostasis, particularly in response to stress. The paper discusses the effects of heat shock on cardiac fatty acid content and hemodynamic variables in rats. It introduces a method for determining myocardial HSP70 content and evaluates the impact of heat stress (HS) on this protein in the context of preischemic and reperfusion hemodynamic variables.

**Immunoblotting**

Rats were subjected to heat stress for 0.5 or 24 hours, followed by sham treatment, and their hearts were analyzed for HSP70 content. The expression of HSP70 was significantly higher in the hearts of rats treated with HS24-h compared to the control group, indicating a heat-induced increase in HSP70 expression.

**Statistical Analysis**

All values are presented as means ± SD. The results were analyzed using a two-tailed Student’s t-test, and differences were considered statistically significant at P < 0.05.

**RESULTS**

**Myocardial HSP70 Content**

The myocardial HSP70 tissue content was assessed in rats subjected to various conditions. The HSP70 content reached a peak 24 hours after the stress treatment.

**Functional Recovery**

Within the three series of control groups, i.e., the preischemic, ischemic, and postischemic groups, comparison of all preischemic hemodynamic variables revealed no significant differences. The myocardial HSP70 tissue content accumulated to 0.41 ± 0.19 mg/g total protein. The HSP70 concentration increased two-fold (P < 0.05) in hearts investigated 30 min after HS.

The paper also discusses the effects of HS pretreatment on functional recovery. In hearts treated with 24 h of HS pretreatment, the postischemic recovery of all cardiac output (summation of coronary flow and aortic flow) recovered up to 77% of the preischemic value, whereas it only recovered to 54% in the control hearts (P < 0.05). Left ventricular developed pressure recovered up to 92% in heat-pre treated hearts compared with 79% in the control hearts (P < 0.05).

**Table 1. Preischemic and reperfusion hemodynamic variables in hearts of control and heat-shocked rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Preischemia</th>
<th>Reperfusion</th>
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<tr>
<td></td>
<td>CF (ml/min/g)</td>
<td>AoF (ml/min/g)</td>
</tr>
<tr>
<td>Control</td>
<td>12.1 ± 1.4</td>
<td>57 ± 6</td>
</tr>
<tr>
<td>HS0.5-h</td>
<td>13.3 ± 1.2</td>
<td>53 ± 11</td>
</tr>
<tr>
<td>HS24-h</td>
<td>14.2 ± 0.9*</td>
<td>64 ± 7</td>
</tr>
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</table>

Values are means ± SD for rat hearts. CF, coronary flow (ml/min/g); AoF, aortic flow (ml/min/g); LVDP, left ventricular developed pressure (mmHg). *Significantly different from the values in the control group.
Table 2. Total preischemic and postischemic reperfusion loss of creatine kinase from isolated hearts of control and heat-shocked rats

<table>
<thead>
<tr>
<th></th>
<th>30 Min Preischemia, U/g wet wt</th>
<th>45 Min Reperfusion, U/g wet wt</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>4.0 ± 2.2</td>
<td>33.9 ± 18.9</td>
</tr>
<tr>
<td>HS0.5-h</td>
<td>4.5 ± 2.2</td>
<td>23.6 ± 1.0</td>
</tr>
<tr>
<td>HS24-h</td>
<td>6.0 ± 3.2</td>
<td>16.6 ± 5.0†</td>
</tr>
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</table>

Values are means ± SD. *Significantly different from the values in the control group. †Significantly different from the values in the HS0.5-h group.

Cardiac FA Content

**Preischemia.** As shown in Table 3, at the end of the preischemic period, no significant differences were found between the total (unesterified) FA tissue content in the control and the two heat-pretreated groups of hearts. Also, no significant differences could be detected between the absolute and relative contents of the individual FA in the three groups except in oleic (18:1) and linoleic (18:2) acid, which were significantly higher in the HS0.5-h than in the HS24-h group (data not shown).

**End ischemia.** During the course of 45 min of ischemia, total FA accumulated to comparable levels in all three experimental groups (Table 3). Also, the individual FA increased similarly in the three groups (Figs. 2 and 3).

**End reperfusion.** When the tissue content of FA was measured in hearts freeze-clamped 45 min after the restoration of flow, it was found that the FA content had further increased in the control and HS0.5-h groups. In control hearts, the levels increased from 882 ± 299 at the end of the ischemic period to 1,290 ± 745 nmol/g dry wt ($P = 0.12$). Only stearic acid was significantly increased compared with the end-ischemic values (Fig. 2). In the group of HS0.5-h hearts, the FA increased significantly from end-ischemic values of 626 ± 224 to end-reperfusion levels of 1,489 ± 471 nmol/g dry wt ($P < 0.05$). In contrast, at the end of the reperfusion period in the HS24-h hearts, total FA tended to be lower than at the end-ischemic phase. The levels found were 756 ± 227 nmol/g dry wt (end ischemic) and 494 ± 239 (end reperfusion) nmol/g dry wt ($P = 0.11$), respectively. Although in this group all individual FA were lower, only AA and linoleic acid were significantly lower at the end of the reperfusion episode than at the end of the ischemic episode (see Figs. 2 and 3). Moreover, at the end of the reperfusion period, HS24-h hearts displayed a significantly lower total and individual tissue FA content than control or HS0.5-h hearts.

As far as the phospholipid tissue content is concerned, comparable values were found in the three groups of hearts at the end of the preischemic period. The levels amounted to 206 ± 53 in control hearts and to 179 ± 42 and 200 ± 59 μmol/g dry wt in hearts investigated 30 min and 24 h after HS, respectively. The total phospholipid tissue content did not change either during ischemia or reperfusion in all three groups. Compared with control hearts, the relative FA composition in the phospholipid pool was not affected by HS throughout the entire experimental protocol (data not shown).

**DISCUSSION**

Irreversible damage inflicted on the heart by a prolonged ischemic period is effectuated by the loss of cellular integrity, influx of calcium ions, loss of intracellular proteins, and disruption of cell membranes. In this multifactor process, either elicited by ischemia alone or in combination with reperfusion, the cardiac cell membrane plays a key role and therefore is an important target when measures have to be taken to decrease the vulnerability to ischemia-reperfusion damage. Chien et al. (4) and Van der Vusse et al. (42) found that AA accumulates during ischemia-reperfusion (35). Because under normoxic conditions AA is predominantly esterified in the phospholipid pool, accumulation of (unesterified) AA points to degradation of membrane phospholipids during ischemia and especially reperfusion. Studies (12, 30, 38) using pharmacological inhibitors and antibodies of phospholipases in ischemia-reperfusion experiments show that postsischemic functional outcome is significantly better than in control hearts, which underscores the pivotal role of phospholipid-degrading enzymes in ischemia-reperfusion-induced damage of the heart. Other studies (7, 9, 43) have shown that a powerful endogenous measure to decrease loss of cardiac function is HS pretreatment. The first goal of the present study was to investigate whether heat-induced protection can be attributed to mitigated degradation of membrane phospholipids, whereas the second goal was to determine whether protection was exerted either during the ischemic or reperfusion phase or both.

In the present study, hearts from heat-pretreated rats only displayed a significant improvement of postsischemic left ventricular functional recovery when the ischemic insult was applied 24 h after the pretreatment. In agreement with other studies (7, 9, 43), this phenomenon was associated with reduced postsischemic release of intracellular enzymes and increased HSP70 native tissue content. The question whether HS pretreatment stabilizes cardiac cell membranes either during the ischemic phase or during postsischemic

Table 3. Myocardial total unesterified fatty acid content in control and heat-treated animals before, during, and after ischemia

<table>
<thead>
<tr>
<th></th>
<th>Preischemia</th>
<th>Ischemia</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td>Control</td>
<td>144 ± 51 (6)</td>
<td>882 ± 299 (6)</td>
<td>1,290 ± 745 (12)</td>
</tr>
<tr>
<td>HS0.5-h</td>
<td>183 ± 83 (7)</td>
<td>626 ± 229 (5)</td>
<td>1,489 ± 471 (6)</td>
</tr>
<tr>
<td>HS24-h</td>
<td>111 ± 42 (8)</td>
<td>756 ± 239 (6)</td>
<td>494 ± 239*† (8)</td>
</tr>
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</table>

Values are means ± SD; number in parentheses, no. of rat hearts.

Total fatty acids are expressed as nanomoles per gram dry weight.

*Significant difference with corresponding control. †Significant difference with corresponding HS0.5-h group. ‡Significant difference with corresponding ischemic value.
reperfusion was investigated indirectly through leakage of cytosolic enzymes into the coronary effluent after restoration of flow and more directly through detailed investigation of FA accumulation in cardiac tissue collected at the end of ischemia or at the end of reperfusion. In particular, the tissue content of polyunsaturated FA such as AA was used as an index for membrane phospholipid homeostasis during and after an ischemic episode (40). Whereas control and HS0.5-h hearts showed considerable accumulation of unesterified FA during ischemia-reperfusion, hearts from rats that were heat-pretreated 24 h earlier showed a decrease in their content of unesterified FA, including AA, at the end of the reperfusion but not at the end of ischemia. This phenomenon indicates that the protective properties of HS pretreatment are reperfusion associated.

Any measure to reduce the postischemic high tissue concentrations of unesterified FA could be beneficial to the recovery of cardiac function. For instance, high unesterified FA concentrations have been suggested to affect negatively mitochondrial respiration and calcium homeostasis (20, 37). More specifically, AA may induce disturbances in ion homeostasis, the protein kinase C-related signaling pathway, and in regulating GLUT-4 (reviewed in Ref. 37). In this respect, HS-induced reduction in FA accumulation seems to have a dual beneficial effect. On one hand, phospholipid degradation is limited leading to an improved membrane stability and, on the other hand, unesterified FA are kept low, thereby protecting essential intracellular processes.

Accumulation of unesterified FA during ischemia and especially during the subsequent reperfusion...
Phase is a well-known phenomenon (4, 37, 42) and is believed to be the consequence of the activation of phospholipases, such as phospholipase A$_2$ (PLA$_2$). This enzyme preferentially degrades phospholipids and can be activated by enhanced calcium ion concentrations in the cell. It is tempting to believe that PLA$_2$ is less activated in the heat-pretreated heart because experimental findings (8) show that calcium homeostasis is better preserved after HS. At present, evidence for a lesser activation of PLA$_2$ by HS is circumstantial and scarce. Only in HSP70-overexpressing WEH1-S cells has it been found that the PLA$_2$ activity was inhibited after tumor necrosis factor-α stimulation (18). If mitigation of PLA$_2$ activity is playing an important role in HS pretreatment of the heart, this mechanism is obviously inconsequential for the alterations occurring in myocardial cells during the ischemic episode, because the present study clearly shows that unesterified FA accumulate to comparable extents in hearts from HS-pretreated and control rats. In contrast, it may be of importance after reinstallation of flow, because the AA content in hearts subjected to ischemia–reperfusion 24 h after HS pretreatment is significantly lower compared with the corresponding control hearts. This notion implies that the underlying mechanism of release of AA from the cellular phospholipid pool differs between ischemia and reperfusion. In addition, apart from PLA$_2$ activation, other mechanisms leading to net membrane phospholipid degradation may be involved. One of the factors is most likely oxygen free radicals. A burst of oxygen free radicals is generated during the early phase of reperfusion (11). Oxygen free radicals interact with polyunsaturated FA in the phospholipids of cell membranes leading to the formation of lipid peroxides (11). The consequences are alterations in permeability of membranes, destroyed transmembrane ion gradients, or malfunctioning of membrane-bound enzymes. HS pretreatment leads to an increase in the capacity to detoxify these free radicals through enhanced activities of both cardiac catalase and superoxide dismutase (9, 43). Although the precise role of these scavengers in the obtained cardioprotection by hyperthermia is disputed, it cannot be ruled out in this setting (10). In addition, hyperthermic pretreatment is known to enhance the expression of heme oxygenase (HO-1 or HSP32), the products of which are powerful antioxidants (32). In addition to the before-mentioned effects of lipid peroxides, oxygen free radicals also may inhibit the reacylation of phospholipids through inactivation of enzymes, required for phospholipid resynthesis, and thus impair repair processes (44). Our experimental approach did not allow us to directly measure whether active resynthesis of phospholipids occurs during reperfusion in the hearts from HS-pretreated rats. Nevertheless, circumstantial evidence indicates that this indeed occurs, because the tissue contents of AA were found to be decreased significantly during the postischemic phase. It is presently unknown whether HS pretreatment affects the activity of enzymes involved in phospholipid resynthesis. An alternative explanation for the decline in tissue FA levels during reperfusion in hearts 24 h after HS pretreatment might be the increase in mitochondrial oxidation of fatty acyl moieties. This hypothetical situation is, however, less likely for AA because this particular polyunsaturated FA is not a preferred substrate for oxidative consumption.

In addition to changes in membrane phospholipid homeostasis caused by ischemia–reperfusion, HS pretreatment itself may affect the physicochemical properties of lipids in (cardiac) cells (13). Our findings in the HS0.5-h pretreatment group of hearts suggest that transient membrane lipid perturbations occur in the heart. It is possible that in the early phase the temporary labilization of cardiac membranes by a mild HS may render cardiac cells more susceptible to ischemia–reperfusion damage. This could explain the lack of cardioprotection in the HS0.5-h group, despite a twofold higher HSP70 content. However, the lack of protection may also be attributed to HSP70 itself, because both the cellular localization (from nucleolar immediate after stress to cytoplasmic a few hours after stress) and posttranscriptional activation might hamper its ability to exert cardioprotective effects. Although attention has been focused mainly on HSP70, other studies have shown that overexpression of HSP90 (14) and HSP27 (25) in myogenic cells are protective against oxidative stress, whereas overexpression of HSP27 confers resistance against heat through actin filament stabilization (22).

In conclusion, the present study provides evidence that the significant improvement of postischemic functional recovery evoked by HS pretreatment is selectively associated with reduced membrane phospholipid degradation after reinstallation of flow through the previously ischemic heart, as indicated by a significant
decrease of the postischemic accumulation of AA. This decline may be caused by depressed PLA\(_2\) activity or active resynthesis of phospholipids or a combination of both, which leads to an early recovery of sarcolemmal stability from which general intracellular homeostasis could benefit.

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