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Heat Shock Suppresses the Permeability Transition in Rat Liver Mitochondria*

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Heat shock proteins inhibit apoptotic and necrotic cell death in various cell types. However, the specific mechanism underlying protection by heat shock proteins remains unclear. To test the hypothesis that heat shock proteins inhibit cell death by blocking opening of mitochondrial permeability transition (MPT) pores, mitochondria from heat-preconditioned rat livers were isolated by differential centrifugation. Heat shock inhibited MPT pore opening induced by 50 μM $CaCl_2$ plus 5 μM $HgCl_2$ or 1 μM mastoparan and by 200 μM $CaCl_2$ alone. Half-maximal swelling was delayed 15 min or more after heat shock compared with control. Heat shock also increased the threshold of unregulated (Ca^{2+}-independent and cyclosporin A-insensitive) MPT pore opening induced by higher doses of $HgCl_2$ and mastoparan. Heat shock treatment decreased mitochondrial reactive oxygen species formation by 27% but did not change mitochondrial respiration, membrane potential, Ca^{2+} uptake, or total glutathione in mitochondrial and cytosolic extracts of liver. Western blot analysis showed that mitochondrial Hsp25 increased, whereas Hsp10, Hsp60, Hsp70, Hsp75, cyclophilin D, and voltage-dependent anion channel did not change after heat shock. These results indicate that heat shock causes resistance to opening of MPT pores, which may contribute to heat shock protection against cellular injury.

Heat shock proteins (HSPs)[1] are a family of chaperone proteins induced by hyperthermia, oxidative stress, ischemia/reperfusion, hypoxia, energy depletion, viral infection, UV radiation, proinflammatory cytokines like tumor necrosis factor-α, and other stress inducers (for review see Ref. 1). HSPs provide tolerance against both thermal and oxidative stress (1, 2) and inhibit cell death caused by H_2O_2 (3, 4), ATP depletion (5), Fas ligand (6), tumor necrosis factor-α (7), transforming growth factor-β (8), endotoxin lipopolysaccharide (9), ceramide (10), and ischemia-reperfusion injury (11, 12). In L929 cell lines, overexpression of human Hsp27, *Drosophila* Hsp27, or human αB-crystalline decreases endogenous reactive oxygen species (ROS) production and abolishes the burst of intracellular ROS induced by tumor necrosis factor-α (7). *In vitro*, Hsp70 and Hsp27 inhibit cytochrome *c*-mediated procaspase 9 processing either by blocking cytochrome *c* release from mitochondria or by binding the released cytochrome *c* in the cytosol, thus blocking the apoptotic pathway (13, 14).

The protection by HSPs against such varied stimuli suggests a common pathway of protection. A preferential target of HSPs protection may be mitochondria. Overexpression of the mitochondrial HSPs, Hsp60 and Hsp10, protects cardiac myocytes from ischemia/reperfusion injury through maintaining mitochondrial integrity and function (15). Reduced expression of Hsp60 by an antisense oligonucleotide precipitates apoptosis, which is accompanied by cytochrome *c* release from mitochondria and caspase activation (16). Heat shock treatment also prevents decreases of state 3 respiration in isolated myocardial mitochondria and mitochondrial membrane potential in the U937 human premonocytic cell line induced by H_2O_2 (4, 15).

Opening of mitochondrial permeability transition (MPT) pores plays an important role in regulating apoptotic and necrotic cell death (17). Increased Ca^{2+}, ROS, ADP, and atractyloside activate the MPT, whereas cyclosporin A (CsA), bongkrekic acid, Mg^{2+}, Ca^{2+} chelation, low pH, and ubiquinone analogues such as ubiquinone 0 and decylubiquinone inhibit the MPT (18, 19). Recently, we presented data that there are two open conductance modes for MPT pores: a regulated mode that is activated by Ca^{2+} and inhibited by CsA, and an unregulated open mode that does not require Ca^{2+} and is not inhibited by CsA (20–24). In general, low dose MPT induction opens regulated pores, whereas high dose induction opens unregulated pores (20).

Despite evidence that HSPs protect mitochondria against injury, no studies have investigated whether the MPT is involved in HSPs protection. Here we investigated the consequence of heat shock to rats on the MPT in isolated rat liver mitochondria. We show that heat shock treatment inhibits the induction of regulated MPT pore opening and increases the threshold of unregulated MPT pore opening, effects that correlate with increased expression of mitochondrial Hsp25. The results suggest that heat shock-inducible factors regulate against the onset of the MPT, which may explain the protection by HSPs against cell injury.

EXPERIMENTAL PROCEDURES

Tetramethylrhodamine methyl ester (TMRM), Fluo-5N, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Molecular Probes (Eugene, OR); antibodies to inducible Hsp70 (Hsp72), Hsp10, Hsp25, Hsp60, and mitochondrial Hsp70 (Grp75) were from StressGen Biotechnologies (Victoria, British Columbia, Canada); antibody to Hsp75 (tumor necrosis factor receptor-associated protein 1: TRAP-1) was from Lab Vision Corp. (Fremont, CA); antibody to voltage-dependent anion channel (VDAC) was from Calbiochem; and custom-

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[1] The abbreviations used are: HSPs, heat shock proteins; CsA, cyclosporin A; CypD, cyclophilin D; $\Delta\Psi_m$, mitochondrial membrane potential; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; VDAC, voltage-dependent anion channel; DCF, dichlorofluorescein.

made anti-cyclophilin D (CypD) antibody was from Bethyl Laboratory (Montgomery, TX). Other reagent grade chemicals were obtained from Sigma.

Adult male Sprague-Dawley rats (250–300 g) that were fasted overnight were used in all the experiments. For heat shock treatment, rats were anesthetized (50 mg of pentobarbital/kg body weight) and immersed in a 42 °C water bath in plastic bags. Rectal temperature was monitored using a digital thermometer. After rectal temperature reached 42 °C after about 30 min, the rats were maintained at 42 °C for 15 min before removing them from the water bath. Sham-treated rats were subjected to anesthesia only. An additional control group received no treatment. In preliminary experiments, Hsp72 expression in liver homogenates was assessed by Western blotting from 0 to 40 h after heat shock. These experiments showed that peak expression of Hsp72 occurred at 20 h (data not shown). Accordingly, subsequent experiments were performed 20 h after heat shock and sham treatment.

Rat liver mitochondria were isolated by differential centrifugation and resuspended at 50 mg of protein/ml in 200 mM sucrose and 2 mM HEPES, pH 7.4 buffer, as described previously (25). Mitochondria were either used immediately or aliquoted, frozen in liquid nitrogen, and stored at −80 °C. In addition, supernatants of the first 10,000 × *g* spin were centrifuged at 100,000 × *g* for 30 min at 4 °C. The high speed supernatant representing the cytosolic fraction was frozen in liquid nitrogen and saved for total glutathione measurement and Western blot analysis. Protein concentrations in mitochondria and cytosol were determined by a Biuret procedure using bovine serum albumin as standard (26).

Mitochondrial oxygen consumption using succinate as a substrate was assessed using a Clark-type oxygen electrode (27). Mitochondrial swelling was monitored at 25 °C by absorbance at 540 nm with a ThermoMax 96-well plate reader (Molecular Devices, Sunnyvale, CA) in incubation buffer containing 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH_2PO_4, pH 7.2, and the MPT was induced by $CaCl_2$, $HgCl_2$, and mastoparan, as described previously (20). Mitochondrial membrane potential and Ca^{2+} uptake/release were monitored using 1 μM TMRM and 1 μM Fluo-5N, respectively, as described previously (27). ROS formation was monitored fluorometrically after ester loading mitochondria with 10 μM H_2DCFDA from the rate of increase of the green fluorescence of DCF, the oxidized product of H_2DCF. Total glutathione in frozen mitochondria and cytosolic fractions was quantified using the Bioxytech GSH-420 kit (Oxis Research, Portland, OR) according to the manufacturer's instructions.

To measure the heat shock proteins Hsp10, Hsp25 (rodent homologue of human Hsp27), Hsp60, Hsp70, Hsp75, and the putative MPT pore complex proteins CypD and VDAC in cytosolic and mitochondrial fractions, proteins were resolved by 8–15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was carried out and developed using the ECL Plus kit (Amersham Biosciences) according to the manufacturer's instructions. Signals were imaged using a Molecular Dynamics Storm Image System (Eugene, OR).

RESULTS

Heat Shock Inhibits the MPT Induced by Low and High Levels of MPT Inducers—To determine the possible role of heat-inducible factors in MPT regulation, liver mitochondria isolated from control, sham-control, and heat shock-treated rats were incubated with low and high doses of MPT inducers. MPT was monitored by swelling detected by absorbance at 540 nm. In control mitochondria, a low dose of $HgCl_2$ (5 μM) induced rapid mitochondrial swelling in the presence of 50 μM $CaCl_2$ (Fig. 1*A*, *trace a*). Swelling was half-maximum after about 5 min of addition of $HgCl_2$ plus $CaCl_2$ and was maximum after about 8 min. MPT induction in mitochondria isolated from control and sham control rats was not different, so only the results from the control group are shown. By contrast, in mitochondria isolated from rats subjected to heat shock, the Ca^{2+}-dependent MPT produced by low dose $HgCl_2$ induction was substantially delayed. In the heat shock group, half-maximum swelling was reached more than 15 min later than in the control group (Fig. 1*A*, *trace b*).

We also induced the unregulated MPT by exposure of mitochondria to higher doses of $HgCl_2$ in the absence of added Ca^{2+}. In control mitochondria, large amplitude mitochondrial swelling began immediately after the addition of 20 μM $HgCl_2$ and reached half-maximum within 5 min (Fig. 1*B*, *trace a*). Again, there was no difference between the control and sham control groups (data not shown). After heat shock, half-maximal swelling was delayed about 15 min (Fig. 1*B*, *trace b*). When the concentration of $HgCl_2$ was increased to 25 and 30 μM, heat shock treatment still delayed onset of the MPT (data not shown). By contrast, after addition of 40 μM $HgCl_2$, a still higher concentration of $HgCl_2$, swelling was virtually the same in the control and heat shock groups (Fig. 1*C*, *trace a* and *b*, respectively). Therefore, the threshold to induce onset of the unregulated MPT by $HgCl_2$ was increased from 20 to 40 μM by heat shock treatment.

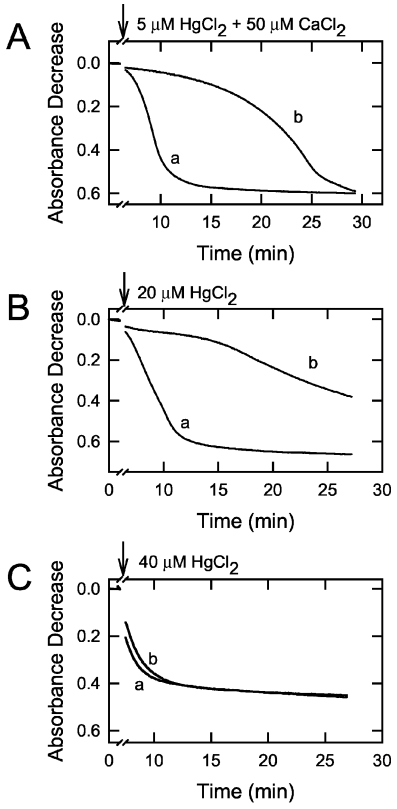

FIG. 1. **Heat shock treatment delays onset of the regulated MPT and increases the threshold of unregulated MPT induced by $HgCl_2$.** Mitochondria (0.5 mg of protein/ml) were added to incubation buffer, and the onset of the MPT was monitored by absorbance, as described under "Experimental Procedures." After a 2–3-min preincubation, 5 μM $HgCl_2$ plus 50 μM $CaCl_2$ (*A*), 20 μM $HgCl_2$ (*B*), and 40 μM $HgCl_2$ (*C*) were added. *Traces a* and *b* are control mitochondria and heat shock-treated mitochondria, respectively. The data shown are representative of four independent experiments.

We also examined the effect of heat shock on MPT induction by the amphipathic peptide mastoparan. As shown in Fig. 2*A*, in control mitochondria, a low dose of mastoparan (1 μM) plus 50 μM $CaCl_2$ induced mitochondrial swelling that reached half-maximum after about 15 min (*trace a*). This regulated MPT induction was completely blocked by heat shock pretreatment (*trace b*). When mitochondria were incubated with 3 μM mastoparan in the absence of $CaCl_2$, rapid swelling occurred in mitochondria from control rats (Fig. 2*B*, *trace a*) but not in mitochondria from heat shock-treated rats (*trace b*). By contrast, when mitochondria were exposed to 4 μM mastoparan, a still higher concentration of mastoparan, the onset and progression of the MPT were virtually same in the control and heat shock-treated groups (Fig. 2*C*, *trace a* and *b*, respectively), indicating that the threshold of unregulated MPT induction by mastoparan was increased by heat shock pretreatment.

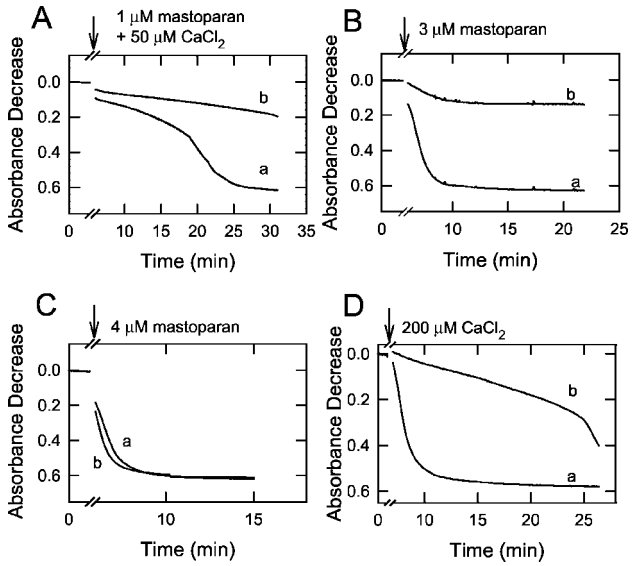

FIG. 2. **Heat shock treatment delays onset of the regulated MPT and increases the threshold of unregulated MPT induced by mastoparan.** Mitochondria (0.5 mg of protein/ml) were added to incubation buffer, and the onset of the MPT was monitored by absorbance, as described under "Experimental Procedures." After a 2–3-min preincubation, 1 μM mastoparan plus 50 μM $CaCl_2$ (*A*), 3 μM mastoparan (*B*), 4 μM mastoparan (*C*), and 200 μM $CaCl_2$ (*D*) were added. *Traces a* and *b* are control mitochondria and heat shock-treated mitochondria, respectively. The data shown are representative of four independent experiments.

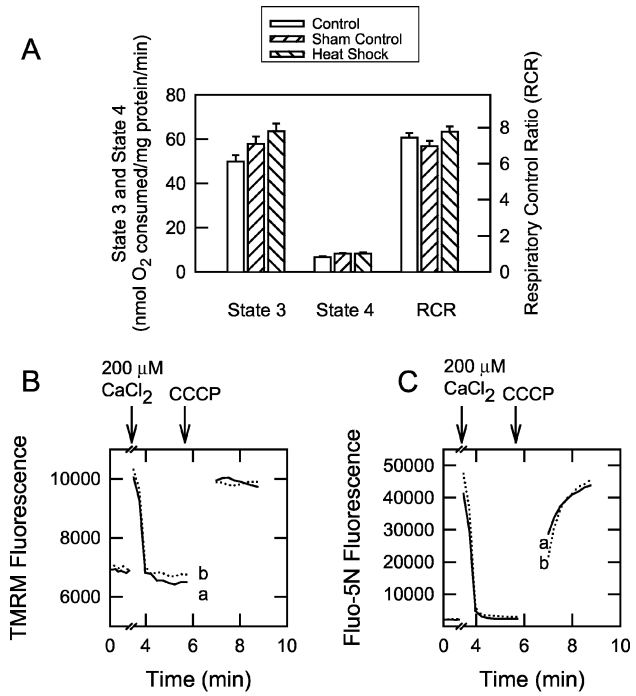

FIG. 3. **Heat shock treatment has no effect on mitochondrial respiration, membrane potential, and Ca^{2+} uptake.** *A*, oxygen consumption was assessed using a Clark-type oxygen electrode in a reaction medium containing 1 mg of protein/ml mitochondria, 150 mM sucrose, 5 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone, and 10 mM NaP_i buffer, pH 7.4. State 3 respiration rate was measured after the addition of 200 μM ADP. *B* and *C*, mitochondria (0.5 mg of protein/ml) were incubated in buffer containing 1 μM CsA plus 1 μM TMRM and 1 μM Fluo-5N. Red TMRM fluorescence (*B*) and green Fluo-5N fluorescence (*C*) were measured in a fluorescence plate reader, as described under "Experimental Procedures." After 2–3 min of preincubation, 200 μM $CaCl_2$ was added to monitor Ca^{2+} uptake. After complete uptake of Ca^{2+}, 1 μM carbonyl cyanide *p*-chlorophenylhydrazone (*CCCP*) was added to depolarize mitochondria. *Traces a* (*solid lines*) and *b* (*dotted lines*) are control mitochondria and heat shock-treated mitochondria, respectively. The data shown are representative of three independent experiments.

Mitochondria were also treated with high (200 μM) $CaCl_2$ alone. In mitochondria from control rats, 200 μM $CaCl_2$ produced rapid large amplitude swelling, which reached half-maximum within 3 min (Fig. 2*D*, *trace a*). Heat shock treatment delayed MPT induction by high $CaCl_2$, and half-maximum swelling after 200 μM $CaCl_2$ required more than 20 min in mitochondria isolated from heat shock-treated rats (Fig. 2*D*, *trace b*).

Mitochondrial Respiration, Membrane Potential, and Ca^{2+} Uptake after Heat Shock Treatment—To determine whether delay of onset of the MPT induced by heat shock was related to changes of mitochondrial respiration, we measured mitochondrial state 3 and state 4 respiration and the respiratory control ratio using succinate plus rotenone as substrate in control, sham control, and heat shock mitochondria. As shown in Fig. 3*A*, state 3 and state 4 respiratory rates and respiratory control ratios were not significantly different between groups. These findings indicated that the protective effect of heat shock on MPT induction was not mediated by changes of mitochondrial respiration.

To ascertain whether changes of mitochondrial membrane potential and Ca^{2+} uptake were related to resistance to the MPT after heat shock, we measured $\Delta\Psi_m$ and Ca^{2+} uptake using the fluorescence dyes, TMRM and Fluo-5N, respectively. Mitochondria were preincubated with 1 μM TMRM, 1 μM Fluo-5N, and 1 μM CsA. CsA was added to prevent MPT onset and consequent Ca^{2+} release. Under these conditions, $\Delta\Psi_m$-dependent TMRM accumulation into mitochondria causes self-quenching of TMRM fluorescence. As shown in Fig. 3*B*, during the 2–3-min preincubation, TMRM fluorescence in both control (*trace a*) and heat shock (*trace b*) groups was relatively low. After the addition of 200 μM $CaCl_2$, mitochondria transiently depolarized as Ca^{2+} uptake occurred, as shown by an increase of TMRM fluorescence. Fluo-5N fluorescence increased immediately after adding $CaCl_2$ and then progressively declined as uptake of Ca^{2+} into mitochondria occurred. Once all the Ca^{2+} had accumulated, $\Delta\Psi_m$ recovered as shown by the return of TMRM fluorescence to previous levels. Subsequent addition of 1 μM CCCP to uncouple mitochondria caused rapid mitochondrial depolarization and release of accumulated Ca^{2+}, as indicated by a rapid increase of TMRM and Fluo-5N fluorescence. As shown by the representative experiments in Fig. 3, *B* and *C*, control and heat shock mitochondria were not different with respect to changes of $\Delta\Psi_m$ and rates of Ca^{2+} uptake. Taken together, the results of Fig. 3 indicate that the inhibition of MPT induction after heat shock was not due to alterations of mitochondrial energetic status or Ca^{2+} accumulation.

Heat Shock Treatment Decreases ROS Formation but Has No Effect on Glutathione—ROS are important inducers of MPT (28, 29), and HSP expression decreases cellular ROS formation after cytokine stimulation (7, 8). Because mitochondria are a major source of ROS production, we measured ROS formation in isolated mitochondria loaded with 10 μM H_2DCFDA. Compared with control mitochondria, heat shock treatment decreased DCF formation rate by 27% measured by the rate of formation of green fluorescent DCF (Fig. 4*A*). In control mitochondria, 1 μM CsA suppressed DCF formation by 28% to levels nearly identical to heat shock mitochondria (Fig. 4*A*). By contrast, CsA had virtually no effect on ROS formation by heat shock mitochondria (Fig. 4*A*).

Glutathione levels also modulate MPT induction, and heat shock proteins may protect against oxidative stress by increasing the intracellular glutathione levels (7). Accordingly, we measured mitochondrial and cytosolic glutathione with and

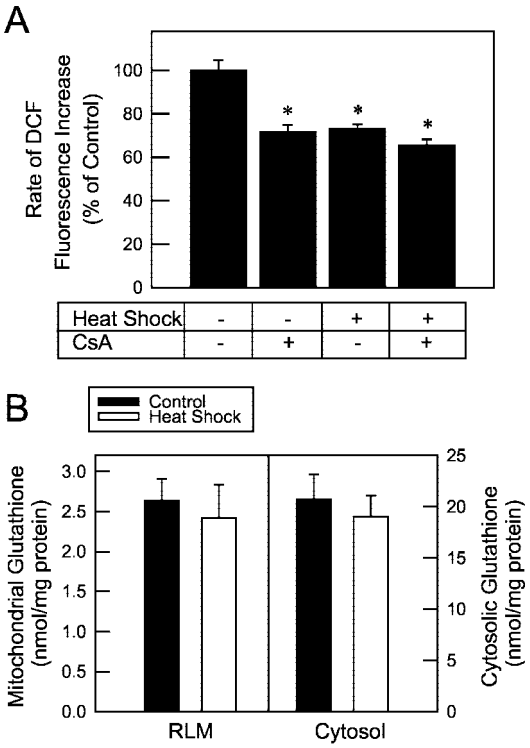

FIG. 4. **Heat shock treatment decreases mitochondria formation of reactive oxygen species but does not affect glutathione levels.** *A*, mitochondria (0.5 mg of protein/ml) were incubated in a buffer containing 10 μM H_2DCFDA. Green fluorescence was monitored with a fluorescence plate reader. After 20–30 min of incubation, the rate of fluorescence increase became linear, and this rate was used to represent the rate of ROS formation. Data shown are means ± S.E. from three independent experiments (*, $p < 0.01$ by Student's t test, compared with heat shock (−) and CsA (−) mitochondria). *B*, glutathione was determined in mitochondria (5 mg of protein) and cytosolic fractions (100 μl) using a BIOXYTECH GSH-420 kit. Data shown are means ± S.E. from four to five independent experiments.

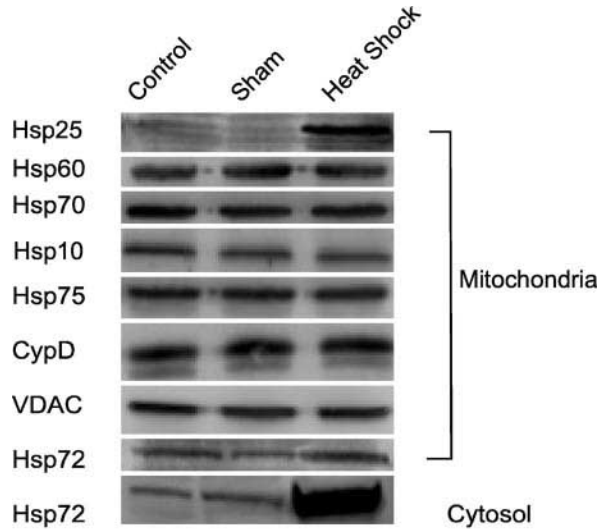

FIG. 5. **Heat shock treatment increases Hsp25 expression but not Hsp60, Hsp70, Hsp10, Hsp75, CypD, or VDAC in mitochondria.** Proteins (10 μg) of liver mitochondria and cytosol from control, sham control, and heat shock treatment groups were resolved by 8 (Hsp75, Hsp72, Hsp60, and Hsp70), 12 (Hsp25 and VDAC), or 15% (Hsp10 and CypD) SDS-PAGE. All blots were mitochondrial proteins, except the last blot, which were cytosolic proteins as indicated. Western blotting was carried as described under "Experimental Procedures." Data shown are representative of three independent experiments.

without heat shock. In control rats, glutathione levels were 2.6 and 20.7 nmol/mg protein, respectively, in mitochondria and the 100,000 × *g* cytosolic supernatant. However, heat shock treatment did not alter either mitochondrial or cytosolic glutathione (Fig. 4*B*).

Heat Shock Treatment Increases the Expression of Hsp25 but Not Other Heat Shock Proteins in Mitochondria—To assess the expression of heat shock proteins, we evaluated a panel of proteins by Western blot analysis in mitochondrial fractions from control, sham control, and heat shock groups. As shown in Fig. 5, Hsp25 was minimal in control and sham control mitochondria but increased substantially after heat shock. The expression of Hsp25 was also increased in cytosol (data not shown). In contrast, the levels of the constitutively expressed mitochondrial heat shock proteins Hsp60, Hsp70, Hsp75, and Hsp10 were not affected after heat shock. We also assessed protein levels of putative MPT pore proteins, CypD and VDAC, before and after heat shock. As shown in Fig. 5, CypD and VDAC expression was not altered after heat shock. To confirm that the observed increase of Hsp25 in mitochondria was not due to cytosolic contamination, samples were subjected to Western blot using Hsp72 antibody. As shown in Fig. 5, Hsp72 expression was not changed in mitochondria after heat shock but was increased in the cytosol, indicating that the observed increase of Hsp25 expression in mitochondria was not due to cytosolic contamination.

DISCUSSION

Numerous studies have shown that heat shock treatment prevents cell death in various cell types after different stimuli (1–12). The specific mechanisms underlying heat shock protection remain unclear (13–16, 30). Because onset of the MPT plays an important role in regulating both necrotic and apoptotic cell death (for review see Ref. 17), this study evaluated the effect of heat shock treatment on the MPT. We show that heat shock treatment inhibits onset of the regulated MPT induced by low doses of $HgCl_2$ (Fig. 1*A*), mastoparan (Fig. 2*A*), and $CaCl_2$ (Fig. 2*D*). As a result, half-maximal mitochondrial swelling occurred more than 4 times later in the heat shock group than in the control and sham control groups. Heat shock also increased the threshold of the unregulated MPT induced by $HgCl_2$ from 20 to 40 μM (Fig. 1, *B* and *C*) and by mastoparan from 3 to 4 μM (Fig. 2, *B* and *C*). Thus, heat shock pretreatment both delays onset of the regulated MPT and increases the threshold of the unregulated MPT. These findings suggest that the protection by heat shock against cellular injury may be mediated, at least in part, by preventing MPT induction.

The precise mechanisms of heat shock-induced resistance against MPT induction remain unclear. The MPT pore is a voltage-dependent channel (31, 32). Mitochondrial respiration creates and maintains the mitochondrial membrane potential. High mitochondrial membrane potential favors a closed conductance state, whereas low membrane potential promotes pore opening. Accordingly, we determined whether mitochondrial state 3 and state 4 respiratory rates and membrane potential were affected by heat shock treatment. Our results showed no difference of respiratory rates, respiratory control ratio, and membrane potential between control and heat shock mitochondria (Fig. 3), indicating that the protection against the MPT by heat shock was not through an alteration of mitochondrial bioenergetic status. Ca^{2+} uptake into mitochondria is also critical for induction of the regulated MPT (33), but mitochondrial Ca^{2+} uptake was also unaffected by heat shock treatment (Fig. 3*C*). These results are consistent with previous studies (4, 15) showing that heat shock treatment itself does not change mitochondrial respiration or membrane potential.

In previous studies, heat shock protection against apoptotic stimuli was associated with decreased ROS production and increased intracellular glutathione (7, 34). By using H_2DCFDA

as a probe of ROS formation, we found that ROS production by mitochondria was decreased by 27% in heat shock mitochondria (Fig. 4*A*), although both mitochondrial and cytosolic levels of glutathione were unaffected (Fig. 4*B*). The mechanism by which heat shock treatment causes this modest decrease of ROS production is unclear. One possibility is that heat shock treatment blocks transient MPT pore openings (35, 36) or sustained pore openings in a small subpopulation of mitochondria. Transient pore openings or the occurrence of the MPT in a small mitochondrial subpopulation may contribute to ROS formation in control mitochondria. This hypothesis is consistent with the finding that incubation of control mitochondria with 1 μM CsA decreased ROS formation to the levels observed in heat shock mitochondria (Fig. 4*A*). Inhibition of the MPT may also be the basis by which heat shock prevents H_2O_2-induced decreases in mitochondrial state 3 respiration and membrane potential reported by others (4).

By using Western blot analysis, we found that among the mitochondrial HSPs and putative MPT complex proteins evaluated, only Hsp25 increased after heat shock treatment. Other mitochondrial HSPs (Hsp10, Hsp60, Hsp70, and Hsp75) and the putative MPT complex proteins CypD and VDAC were not affected (Fig. 5). Hsp25 belongs to a family of small heat shock proteins, including Hsp27 (human homologue of Hsp25) and αB-crystalline (37). Hsp25 was reported previously (38) to be in the cytosol and nucleus. Recently, Hsp25 was identified in the mitochondria of mouse PC12 cells (39) and heat-shocked Jurkat T-lymphocytes (40). In our experiments, Hsp25 was barely detectable in liver mitochondria from control rats but increased greatly in both mitochondrial (Fig. 5) and cytosolic fractions (data not shown) after heat shock.

Hsp25 may regulate MPT pore opening in two ways. First, Hsp25 expression may suppress ROS production and prevent adverse consequences such as mitochondrial depolarization, cytochrome *c* release, and apoptosis. Overexpression of human Hsp27 and mouse Hsp25 protects mouse L929 fibroblasts against oxidative stress induced by H_2O_2 and increases the activity of glucose-6-phosphate dehydrogenase, a regulator of antioxidant pathways (34). Inhibition of Hsp27 expression with *hsp27* antisense also potentiates mitochondrial depolarization and cytochrome *c* release after apoptotic stimuli in Jurkat cells (40). In mice lacking heat shock transcription factor 1, cardiac Hsp25 expression is decreased; superoxide production is increased; mitochondrial proteins including adenine nucleotide translocator 1 are more oxidized, and isolated mitochondria are more sensitive to MPT induction by $CaCl_2$ (41). In our experiments, however, ROS formation declined only 27% after heat shock, which was disproportionate with the suppression of MPT induction. In addition, this decrease of ROS formation appeared to be the consequence of MPT suppression rather than its cause, because CsA treatment of control mitochondria decreased ROS production to that observed in heat shock mitochondria (Fig. 4*A*).

As a second mechanism, Hsp25 may exert direct chaperone-related regulation of pore conductance, as proposed by a recent model of pore formation and gating (20). In this model, stress induces misfolding of mitochondrial membrane proteins, which expose their hydrophilic residues to the bilayer phase and then cluster to form aqueous pores that are permeable to ions and molecules up to a molecular mass of about 1,500 Da. Chaperone proteins and CypD bind to the pore complex to shut off conductance. However, increased Ca^{2+} causes CypD to perturb the MPT pore complex to an open conductance state, an effect that is antagonized by CsA. As the number of misfolded protein clusters exceeds the number of chaperones available to block MPT pore conductance, unregulated pores accumulate. This model explains observations that low levels of various MPT inducers cause Ca^{2+}-dependent and CsA-sensitive permeabilization of the inner membrane, whereas high levels cause Ca^{2+}-independent and CsA-insensitive onset of the MPT (20–24). The association of increased Hsp25 expression with inhibition of the regulated MPT and an increased threshold for the unregulated MPT implicates Hsp25 as a candidate chaperone involved in MPT regulation as predicted by the model. The new model is also consistent with the recent finding that overexpression of mitochondrially targeted CypD desensitizes cells against apoptotic stimuli (42).

Our findings in the present study that heat shock treatment inhibits onset of the regulated MPT and increases the threshold for onset of the unregulated MPT (Figs. 1 and 2) can be explained by the model of chaperone regulation of the MPT pore. First, increased concentration of heat-inducible chaperones (*e.g.* Hsp25) might be expected to retard onset of the MPT by a mass action effect, because the physiological role of the chaperones is to prevent mitochondrial permeabilization and uncoupling after formation of clusters of misfolded mitochondrial membrane proteins. Second, increased availability of heat-induced chaperones would be expected to block a greater number of the misfolded protein clusters, which explains the increased threshold for unregulated MPT induction by both $HgCl_2$ and mastoparan. In this respect we found that the concentration of inductor needed for onset of the unregulated MPT increased from 20 μM $HgCl_2$ and 3 μM mastoparan for control mitochondria to 40 μM $HgCl_2$ and 4 μM mastoparan in heat shock mitochondria (Figs. 1*C* and 2*C*). The increased threshold of unregulated MPT induction is thus explained by the proposed model.

In summary, this study shows for the first time that heat shock pretreatment delays onset of the regulated MPT and increases the threshold of unregulated MPT induction. Heat shock treatment does not alter mitochondrial respiration, membrane potential, Ca^{2+} uptake, or mitochondrial and cytosolic glutathione levels but does cause a modest decrease of ROS production. Of the several HSPs examined, only Hsp25 expression increased after heat shock. Thus, the effect of heat shock on MPT inhibition may be partially through the up-regulation of mitochondrial Hsp25 expression. The effects of heat shock treatment on MPT induction may underlie the mechanism of heat shock protection against various different cell injuries.

REFERENCES

1. Kregel, K. C. (2002) *J. Appl. Physiol.* **92,** 2177–2186
2. Arrigo, A. P. (1998) *Biol. Chem.* **379,** 19–26
3. Takuma, K., Mori, K., Lee, E., Enomoto, R., Baba, A., and Matsuda, T. (2002) *Brain Res.* **946,** 232–238
4. Bornman, L., Steinmann, C. M., Gericke, G. S., and Polla, B. S. (1998) *Biochem. Biophys. Res. Commun.* **246,** 836–840
5. Li, F., Mao, H. P., Ruchalski, K. L., Wang, Y. H., Choy, W., Schwartz, J. H., and Borkan, S. C. (2002) *Am. J. Physiol.* **283,** C917–C926
6. Mehlen, P., Schulze-Osthoff, K., and Arrigo, A. P. (1996) *J. Biol. Chem.* **271,** 16510–16514
7. Mehlen, P., Kretz-Remy, C., Preville, X., and Arrigo, A. P. (1996) *EMBO J.* **15,** 2695–2706
8. Diez-Fernandez, C., Andres, D., and Cascales, M. (2002) *Free Radic. Biol. Med.* **33,** 835
9. Dokladny, K., Kozak, A., Wachulec, M., Wallen, E. S., Menache, M. G., Kozak, W., Kluger, M. J., and Moseley, P. L. (2001) *Am. J. Physiol.* **280,** R338–R344
10. Ahn, J. H., Ko, Y. G., Park, W. Y., Kang, Y. S., Chung, H. Y., and Seo, J. S. (1999) *Mol. Cell* **9,** 200–206
11. Plumier, J. C., and Currie, R. W. (1996) *Cell Stress Chaperones* **1,** 13–17
12. Uchinami, H., Yamamoto, Y., Kume, M., Yonezawa, K., Ishikawa, Y., Taura, K., Nakajima, A., Hata, K., and Yamaoka, Y. (2002) *Am. J. Physiol.* **282,** G962–G971
13. Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., and Massie, B. (2000) *Mol. Cell. Biol.* **20,** 7146–7159
14. Bruey, J. M., Ducasse, C., Bonniaud, P., Ravagnan, L., Susin, S. A., Diaz-Latoud, C., Gurbuxani, S., Arrigo, A. P., Kroemer, G., Solary, E., and Garrido, C. (2000) *Nat. Cell Biol.* **2,** 645–652
15. Polla, B. S., Kantengwa, S., Francois, D., Salvioli, S., Franceschi, C., Marsac, C., and Cossarizza, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6458–6463

16. Kirchhoff, S. R., Gupta, S., and Knowlton, A. A. (2002) *Circulation* **105,** 2899–2904
17. Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. (1998) *Biochim. Biophys. Acta* **1366,** 177–196
18. Bernardi, P. (1999) *Physiol. Rev.* **79,** 1127–1155
19. Fontaine, E., and Bernardi, P. (1999) *J. Bioenerg. Biomembr.* **31,** 335–345
20. He, L., and Lemasters, J. J. (2002) *FEBS Lett.* **512,** 1–7
21. Pfeiffer, D. R., Gudz, T. I., Novgorodov, S. A., and Erdahl, W. L. (1995) *J. Biol. Chem.* **270,** 4923–4932
22. Broekemeier, K. M., and Pfeiffer, D. R. (1989) *Biochem. Biophys. Res. Commun.* **163,** 561–566
23. Kristal, B. S., and Brown, A. M. (1999) *J. Biol. Chem.* **274,** 23169–23175
24. Lenartowicz, E., Bernardi, P., and Azzone, G. F. (1991) *J. Bioenerg. Biomembr.* **23,** 679–688
25. Lemasters, J. J., Grunwald, R., and Emaus, R. K. (1984) *J. Biol. Chem.* **259,** 3058–3063
26. Gornall, A. G., Bardawill, D. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766
27. Blattner, J. R., He, L., and Lemasters, J. J. (2001) *Anal. Biochem.* **295,** 220–226
28. Crompton, M., Costi, A., and Hayat, L. (1987) *Biochem. J.* **245,** 915–918
29. Kowaltowski, A. J., Castilho, R. F., and Vercesi, A. E. (2001) *FEBS Lett.* **495,** 12–15
30. Wyttenbach, A., Sauvageot, O., Carmichael, J., Diaz-Latoud, C., Arrigo, A. P., and Rubinsztein, D. C. (2002) *Hum. Mol. Genet.* **11,** 1137–1151
31. Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S., and Bernardi, P. (1994) *J. Biol. Chem.* **269,** 16638–16642
32. Petronilli, V., Nicolli, A., Costantini, P., Colonna, R., and Bernardi, P. (1994) *Biochim. Biophys. Acta* **1187,** 255–259
33. Petronilli, V., Cola, C., and Bernardi, P. (1993) *J. Biol. Chem.* **268,** 1011–1016
34. Preville, X., Salvemini, F., Giraud, S., Chaufour, S., Paul, C., Stepien, G., Ursini, M. V., and Arrigo, A. P. (1999) *Exp. Cell Res.* **247,** 61–78
35. Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P., and Di Lisa, F. (1999) *Biophys. J.* **76,** 725–734
36. Huser, J., Rechenmacher, C. E., and Blatter, L. A. (1998) *Biophys. J.* **74,** 2129–2137
37. Caspers, G. J., Leunissen, J. A., and de Jong, W. W. (1995) *J. Mol. Evol.* **40,** 238–248
38. van de Klundert, F. A., Gijsen, M. L., van den IJssel, P. R., Snoeckx, L. H., and de Jong, W. W. (1998) *Eur. J. Cell Biol.* **75,** 38–45
39. Downs, C. A., Jones, L. R., and Heckathorn, S. A. (1999) *Arch. Biochem. Biophys.* **365,** 344–350
40. Samali, A., Robertson, J. D., Peterson, E., Manero, F., van Zeijl, L., Paul, C., Cotgreave, I. A., Arrigo, A. P., and Orrenius, S. (2001) *Cell Stress Chaperones* **6,** 49–58
41. Yan, L. J., Christians, E. S., Liu, L., Xiao, X., Sohal, R. S., and Benjamin, I. J. (2002) *EMBO J.* **21,** 5164–5172
42. Lin, D. T., and Lechleiter, J. D. (2002) *J. Biol. Chem.* **277,** 31134–31141