

Evidence for a Novel Set of Small Heat-Shock Proteins That Associates with the Mitochondria of Murine PC12 Cells and Protects NADH:Ubiquinone Oxidoreductase from Heat and Oxidative Stress

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Received January 4, 1999, and in revised form February 22, 1999

Several previously unreported small heat-shock proteins (sHsps) were detected in mitochondria from heat-stressed rat PC12 cells, but not in unstressed controls. Functional inactivation of the mitochondrial sHsps with murine Hsp25 antibody indicated that these sHsps protect NADH:ubiquinone oxidoreductase and NADH dehydrogenase activity (i.e., complex I) in submitochondrial vesicles during heat and oxidative stress. These results (i) confirm the existence of multiple sHsps in mammals and indicate that several of these sHsps associate with the mitochondria, (ii) indicate a conserved function between plant and mammalian mitochondrial sHsps in protecting electron transport during stress, and (iii) suggest that these sHsps may play an important role in diseases whose etiology is based upon oxidative damage of complex I. © 1999

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Key Words: small heat-shock protein; mitochondria; heat stress; oxidative stress; oxidative phosphorylation.

The small heat-shock proteins (sHsps)² are one of the major classes of heat-shock proteins and are produced by virtually all organisms, including eubacteria and archaeobacteria (1, 2). sHsps are evolutionarily related via a conserved sequence domain in the carboxyl region

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² Abbreviations used: sHsp, small heat-shock protein; mitosHsp, mitochondrial small heat-shock protein; SMV, submitochondrial vesicle; MIB, mitochondrial isolation buffer; DTT, dithiothreitol; ANOVA, analysis of variance; BSA, bovine serum albumin; MPP, 1-methyl-4-phenylpyridinium.

of the protein called the “small heat-shock protein domain” or the α -crystallin domain (1, 2). The sHsps examined to date share a number of biochemical characteristics, such as formation of homogeneous oligomers and prevention of aggregation of other proteins under denaturing conditions *in vitro* (1). Overexpression of recombinant sHsps or repression of sHsps in plant, yeast, and mammal cells correlates with changes in levels of thermotolerance (1, 3–5). In *Escherichia coli*, a sHsp was demonstrated to stabilize stress-denatured proteins and actively participate in a cooperative multichaperone network involved in renaturation of stress-denatured proteins (6). Recently, it was demonstrated that during heat stress, the sHsps in the chloroplast and mitochondrion of plants protect photosynthetic and respiratory electron transport, respectively (7–9). These studies indicate that the sHsps are an important adaptation to stress in diverse organisms.

The physiological function and cellular localization of the sHsps in mammals remain enigmatic. A number of studies provide evidence that the major murine and human sHsp associates with the cytoskeleton *in vivo* and can affect the behavior of actin organization *in vitro* (10, 11). Evidence also exists for localization of the major mammalian sHsp (Hsp25) to the nucleus, as well as the cytosol, suggesting either that localization of the mammalian sHsp is similar to that of the Class II sHsps in plants or that there are two different gene subfamilies of mammalian sHsps (12). Because of the low number of sulfur-containing amino acid residues in mammalian sHsps and the low concentration of sHsps in cells, detection of sHsps in mammals using ³⁵S autoradiography is difficult. To date, only one sHsp is commonly recognized and studied in mammals (Hsp25

in murine systems and Hsp27 in human systems) (for review, see Ref. 13). However, there is some evidence for the induction of multiple low-molecular-weight heat-shock proteins in mammals using [^3H]leucine radiolabeling or immunoblotting with polyclonal antibodies raised against small heat-shock protein domain antigens (e.g., 13–16). In support of these observations, probing of stress-induced cDNA libraries using *shsp* DNA probes and purification and characterization of a number of unknown low-molecular-weight proteins have recently uncovered several low-molecular-weight proteins in mammals that have homology to the sHsp superfamily (e.g., 17–20).

Plants and fungi possess a specific subfamily of sHsp that has been demonstrated to localize to, or strongly associate with, the mitochondrion (mitosHsp). In *Neurospora crassa*, disruption of the gene for the mitochondrial-associating sHsp results in reduced thermotolerance and evidence for an unidentified role in protecting glucose metabolism during heat stress (21). Production of the mitosHsp in plants is strongly correlated with organismal thermotolerance and acquired thermotolerance of oxidative phosphorylation (22, 23). Furthermore, induction of the plant mitosHsp is correlated with the protection of cells against oxidative injury (24). Recent work from our lab has demonstrated that the plant mitosHsp protects respiratory electron transport, specifically, the stress-labile NADH:ubiquinone oxidoreductase (complex I) during heat stress (8).

In mammals, the mitochondria in general and complex I in particular are highly susceptible to damage incurred by treatments that produce active-oxygen species, resulting in both decreased complex I activity and increased complex I denaturation (25). This, in turn, depending on the cell and tissue type in which the oxidative stress occurs, can either cause or be etiologically related to a number of neurodegenerative and cardiovascular diseases (26, 27). Studies examining mammalian cell responses to heat and oxidative stress indicate that heat-shock proteins may play some role in mitochondrial acclimation and tolerance to these stresses (28, 29). In this study, we specifically examined whether sHsps associate with the mitochondria in murine PC12 cells and, if sHsps are associated with mitochondria, whether they possess a function homologous to the plant mitosHsp by specifically protecting complex I from heat and oxidative stress.

MATERIALS AND METHODS

Rat PC12 cells (adrenal pheochromocytoma) were obtained from American Type Culture Collection (Rockville, MD) and propagated at 36.5°C and 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% heat-denatured fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin. Cells were heat stressed by first ramping the incubator temperature over 1 h from 36.5 to 38.5°C, maintaining temperature for 1 h at 38.5°C, and then ramping the temperature

over 1 h from 38.5 to 41.5°C. The temperature was then decreased from 41.5 to 38.5°C over an hour and maintained at 38°C for 4 to 6 h, after which the temperature was decreased from 38 to 36.5°C over an hour. At this point, cells were harvested for mitochondrial isolation or whole-cell analysis. Preliminary evidence indicated that there is more than 50% mortality when cells are heat-stressed above 45°C.

Mitochondria were isolated from non-heat-stressed and heat-stressed cells using a method modified from Almeida and Medina (30). Cells were ruptured with a glass/Teflon homogenizer in a mitochondrial isolation buffer (MIB) consisting of 0.3 M sorbitol, 50 mM Hepes/KOH (pH 7.4), 2 mM EDTA, 2.5 mM DTT, 4 mM L-cysteine, and 20 units of trypsin. The homogenate was subjected to centrifugation at 1500g for 10 min, after which the pellet was discarded and the supernatant was centrifuged at 17,000g for 15 min. Following this second centrifugation, the supernatant was discarded and the pellet was then resuspended in MIB. This partially purified preparation was then subjected to the two differential centrifugation steps again. The final pellet was resuspended and layered on a discontinuous Percoll step gradient (8). The mitochondrial fraction was collected and washed with MIB lacking trypsin, but containing trypsin inhibitor, and then centrifuged at 10,000g for 10 min. The resulting pellet was resuspended in either a phosphate buffer, triethanolamine buffer, or a SDS-PAGE buffer, depending on the assay (see below). Mitochondria samples to be spectrophotometrically assayed were frozen at -80°C, thawed, and sonicated as in (8).

Complete complex I activity (electron transport from NADH to a quinone receptor) was measured using a modification of the method of Singer (31) and followed the oxidation of NADH at 340 nm using ubiquinone-1 as the electron acceptor. NADH dehydrogenase activity alone (partial complex I activity) was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm (8). Ferricyanide concentrations of 0.15, 0.35, 0.60, 0.85, and 1.25 mM were used to determine V_{\max} and K_m at each temperature treatment. Submitochondrial vesicles (SMVs) assayed under nonstress, oxidative, or heat-stress treatments were incubated with either no protein added or the addition of either anti-murine Hsp25 antibody (Ab₂₅) (1:300, v/v) (StressGen, Victoria, BC, Canada; Cat. No. SPA-801), bovine serum albumin (BSA) (0.2 mg/ml), or rabbit IgG (1:300, v/v) (Sigma, St. Louis, MO). For enzymatic analysis of complex I activity under heat-stress temperatures, SMVs were incubated at 48°C for 30 min in the presence of 1 mM KCN and 0.2 mM NADH to maximize heat stress effects on complex I (as in 26). For enzymatic analysis of complex I activity under oxidative stress conditions, SMVs were incubated at 28°C for 20 min in the presence of 1 mM KCN, 0.2 mM NADH, 0.1 mM NaN₃, and 0.15 mM H₂O₂ (as in 26). Protein concentration was determined as described (32). A concentration of 40 µg/mL of total SMV protein was used for all spectrophotometric assays.

Electron transport data were analyzed by three-way (control/preheat stress × assay temperature or H₂O₂ treatment × protein addition) analysis of variance (ANOVA). Differences among protein additions within each control/preheat stress × assay temperature or H₂O₂ treatment combination were analyzed by one-way ANOVA and Tukey's multiple comparison test, following significant results from the initial ANOVA.

To determine if the mitochondrial samples were free of intracellular contamination, mitochondrial samples were solubilized in a buffer containing 1% SDS, 0.05 M Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, boiled, and then subjected to reducing SDS-PAGE (15.5% polyacrylamide gels). Proteins were subjected to Western blotting and assayed with (i) anti-mitochondrial Hsp60 antibody (StressGen, Cat. Nos. SPA-804 and SPA-805) to ensure that mitochondrial preparations were enriched in intact mitochondria, (ii) anti- α B-crystallin antibody (StressGen, Cat. No. SPA-223) to determine if cytosolic elements were copurifying with the mitochondria, or (iii) antibody to acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY; Cat. No. 06-761) to ascertain the extent of nuclei con-

tamination. To determine if murine Hsp25 or a homologue of Hsp25 associated with the mitochondria, replicate blots were assayed with antibody against recombinant murine Hsp25 (same antibody as above). Replicate gels were assayed by Coomassie blue staining and silver staining to ensure equal soluble protein loads per lane. Mitochondrial isolation and Western immunoblot assays were independently replicated four times.

RESULTS

To determine if sHsps associated with the mitochondria of PC12 cells, mitochondria from unstressed and heat-stressed cells were assayed with a polyclonal antibody to murine recombinant Hsp25. Because this antibody is polyclonal and was raised against the entire Hsp25 protein, it has the potential of detecting homologous domains of other members of the sHsp family (as demonstrated in Ref. 16). In mitochondria from heat-stressed cells, Ab₂₅ exhibited a cross-reaction with at least four major bands (Fig. 1A, lane 4). The lowest-molecular-weight band, designated HspA25, was the same apparent molecular weight as the only band detected in heat-stressed whole-cell samples. Three bands detected above the 29-kDa marker, but below the 36.6-kDa marker, designated sHspD1, sHspD2, and sHspD3, could only be detected in heat-stressed mitochondria. A very light band could be detected with Ab₂₅ in both unstressed whole-cell and mitochondrial samples when 60 μ g of protein were subjected to SDS-PAGE (not shown), but not with 40 μ g of protein as in Fig. 1. This protein had the same migration rate as sHspA25, indicating that sHspA25 was being constitutively expressed at very low levels, as in (13, 14). Disruption of mitochondrial integrity with a polytron homogenizer, followed by trypsin treatment of both non-heat-stressed and heat-stressed mitochondria indicated that all members of these sHsps were susceptible to trypsin degradation (data not shown).

Replicate Western blots of non-heat-stressed and heat-stressed whole-cell and mitochondrial samples were assayed with α B-crystallin antibody, mitochondrial Hsp60 antibody, and acetylated-Histone H4 antibody. Anti- α B-crystallin antibody reacted with a single band in whole-cell extracts, but did not react with comparable mitochondrial proteins, demonstrating that mitochondria were not contaminated with major cytosolic components (e.g., cytoskeletal elements) and that α B-crystallin antibody did not cross-react with either sHspA25 or the sHspDs (Fig. 1B). Anti-mitochondrial Hsp60 antibody reacted with single bands in both whole-cell and mitochondrial samples, and mitochondrial samples contained more Hsp60 than whole-cell samples, showing that mitochondria were purified and remained structurally intact in the presence of trypsin during isolation (Fig. 1C). Antibody against histone H4 reacted with single bands, but only in whole-cell samples, and not mitochondrial samples,

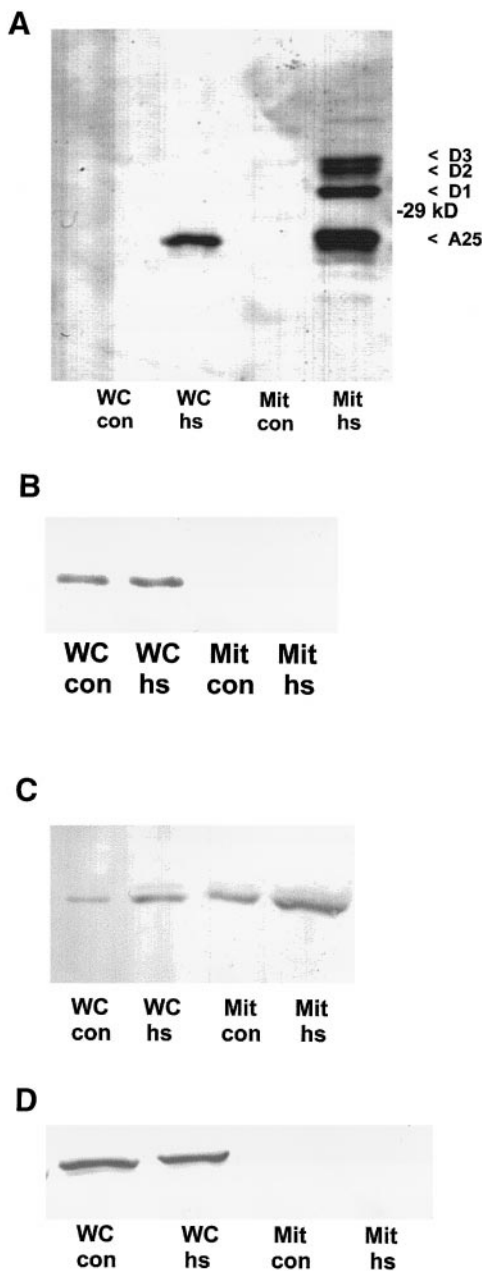


FIG. 1. Mitochondria were isolated from unstressed and pre-heat-stressed PC12 cells, subjected to SDS-PAGE (40 μ g per lane), and Western (immuno) blotting. (A) Western blot assayed with antibody specific to murine Hsp25 (Ab₂₅). (B) Western blot assayed with an antibody specific to α B-crystallin, a protein that associates with cytoskeletal elements. (C) Western blot assayed with antibody specific to mitochondrial Hsp60. (D) Western blot assayed with an antibody specific to histone H4, a protein found in the nucleus. WC, whole cell; Mit, mitochondria; con, control; hs, heat stress.

indicating that there was no nuclei contamination of mitochondrial samples (Fig. 1D).

To determine if the mitosHsps could protect complex I activity from heat stress, we used the polyclonal anti-Hsp25 antibody as a noncompetitive inhibitor to

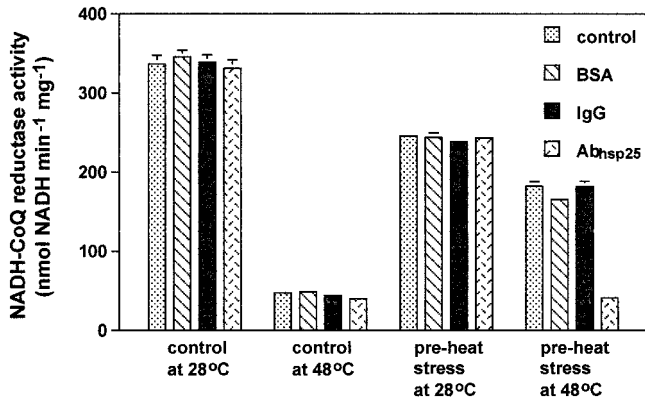


FIG. 2. The effect of antibodies to recombinant murine Hsp25 on complex I electron transport from NADH oxidation to CoQ, reduction determined spectrophotometrically at 340 nm. Submitochondrial vesicles purified from either unstressed (control) or heat-stressed PC12 cells were assayed at either 28 or 48°C. Either no protein was added, or BSA (0.2 mg/mL), rabbit IgG (1:300, v/v), or antibody specific to recombinant murine Hsp25 (Ab₂₅) (1:300, v/v). Results are means \pm 1 SE; $n = 6$.

disrupt the function of the mitochondrial sHsps, as described previously for plant mitochondria and chloroplasts (7, 8). The rate of complex I activity assayed at 28°C was higher in non-heat-stressed mitochondria than in pre-heat-stressed mitochondria ($P < 0.0001$), indicating that complex I was damaged by the cell culture heat-stress treatment (Fig. 2). The rate of complex I activity when assayed at 48°C was almost three-fold higher in pre-heat-stressed mitochondria than in control mitochondria ($P < 0.0001$), indicating that acclimation to high temperatures occurred in pre-heat-stressed samples (Fig. 2). This acclimation appeared to be due entirely to the production of the mitosHsps, because addition of Ab₂₅, which was used to disrupt mitosHsp function, decreased complex I activity in pre-heat-stressed mitochondria assayed at 48°C by about 75% ($P < 0.05$), to levels comparable to controls at 48°C. Addition of IgG or BSA had no effect upon complex I activity on control or pre-heat-stressed samples assayed at either 28 or 48°C ($P > 0.05$).

To confirm if the mitosHsps protect complex I from heat stress, we monitored the activity of NADH dehydrogenase of complex I at 28 and 48°C by measuring the reduction of ferricyanide spectrophotometrically (Fig. 3). As before, activity rates were lower at 48°C than at 28°C ($P < 0.0001$), and pre-heat-stressed mitochondrial samples exhibited lower rates than unstressed controls at 28°C ($P < 0.0001$). However, also as before, activity rates were about 15% higher in SMVs from pre-heat-stressed cells assayed at 48°C than SMVs from unstressed cells assayed at 48°C ($P < 0.002$), indicating that high-temperature acclimation had occurred in pre-heat-stressed mitochondria. Again, this acclimation seemed to be due entirely to the

production of the mitosHsps, since addition of Ab₂₅ decreased NADH dehydrogenase activity in SMVs from pre-heat-stressed cells ($P < 0.05$) to rates ca. equal to that in SMVs from unstressed cells assayed at 48°C. Addition of IgG (Fig. 3) or BSA (data not shown) had no effect on dehydrogenase activity.

To determine if the mitosHsps could protect complex I activity from oxidative stress, we used the same techniques as above, after incubation of mitochondrial samples with 150 μ M H₂O₂, a concentration physiologically relevant to oxidative stress in mitochondria (25, 26). As before, complex I activity was higher in non-heat-stressed mitochondria than in pre-heat-stressed mitochondria ($P < 0.0001$; Fig. 4). Interestingly, complex I activity of control mitochondria incubated in the presence of H₂O₂ showed either no rate or a slightly negative rate (i.e., increase in the absorbance at 340 nm). When complex I activity was assayed in pre-heat-stressed mitochondria incubated in the presence of H₂O₂, rates were at least 30-fold higher than those in control mitochondria incubated with H₂O₂ ($P < 0.0001$). This acclimation appeared to be due entirely to the production of the mitosHsps, because addition of Ab₂₅ decreased complex I activity in pre-heat-stressed mitochondria ($P < 0.05$) to rates similar to control + H₂O₂ rates. Addition of IgG or BSA had no effect upon complex I activity in control or pre-heat-stressed samples incubated in the absence or presence of H₂O₂.

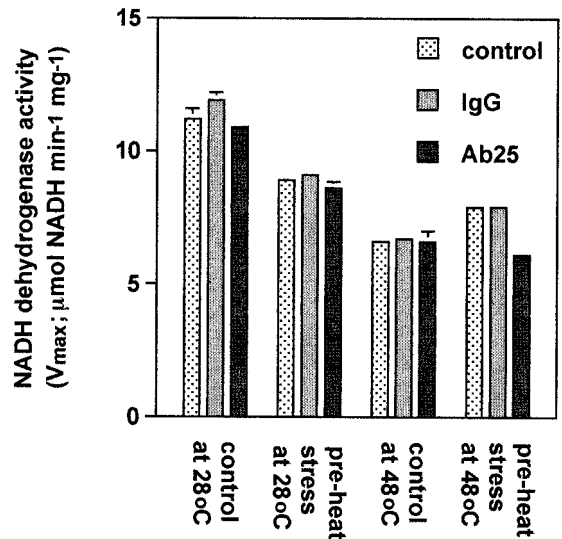


FIG. 3. The effect of antibodies to recombinant murine Hsp25 on NADH dehydrogenase activity. NADH dehydrogenase activity was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm. Submitochondrial vesicles purified from either unstressed (control) or heat-stressed PC12 cells were assayed at either 28 or 48°C. Either no protein was added, or BSA (0.2 mg/mL), rabbit IgG (1:300 v/v), or antibody specific to recombinant murine Hsp25 (Ab₂₅) (1:300, v/v). Results are means \pm 1 SE; $n = 3$.

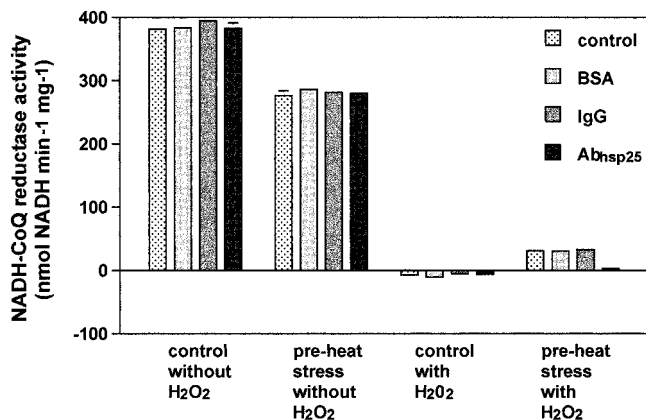


FIG. 4. The effect of antibodies to recombinant murine Hsp25 on complex I electron transport from NADH oxidation to CoQ₁ reduction determined spectrophotometrically at 340 nm. Submitochondrial vesicles purified from either unstressed (control) or heat-stressed PC12 cells were assayed in the absence or presence of 150 μ M H₂O₂. Either no protein was added, or BSA (0.2 mg/mL), rabbit IgG (1:300, v/v), or antibody specific to recombinant murine Hsp25 (Ab₂₅) (1:300, v/v). Results are means \pm 1 SE; $n = 3$.

To confirm if the mitosHsps protect complex I from oxidative stress, we monitored the NADH dehydrogenase activity of complex I in the presence and absence of H₂O₂ by measuring the reduction of ferricyanide spectrophotometrically (Fig. 5). Prior to incubation with H₂O₂, NADH dehydrogenase activity was lower in pre-heat-stressed mitochondria, compared to unstressed controls ($P < 0.02$), and was negatively affected by H₂O₂ in both prestressed and unstressed SMVs ($P < 0.0001$). However, activity rates were about 100% higher in SMVs from pre-heat-stressed cells incubated in the presence of H₂O₂ than SMVs from unstressed cells incubated in the presence of H₂O₂ ($P < 0.0001$), indicating that an acclimation had occurred in pre-heat-stressed mitochondria. This acclimation again seemed to be due entirely to the production of the mitosHsps, because addition of Ab₂₅ decreased NADH dehydrogenase activity in SMVs from pre-heat-stressed cells ($P < 0.05$) to rates approximately equal to the activity of SMVs from unstressed cells incubated in the presence of H₂O₂. As before, addition of IgG (Fig. 5) or BSA (data not shown) had no effect.

DISCUSSION

In summary, we provide evidence for the association of multiple sHsps with the mitochondria in murine PC12 cells during heat stress that have antigenic homology to the "crystallin" family of sHsps. Using a polyclonal antibody specific to recombinant murine Hsp25, we provide *in vitro* evidence that functional inactivation of these mitochondrial sHsps (i) decreased electron transport through complex I from NADH to

ubiquinone (complete complex I activity) during both heat stress and oxidative stress and (ii) decreased NADH dehydrogenase activity alone (partial complex I activity) during both heat stress and oxidative stress. To our knowledge, results from this study are the first evidence that sHsps in mammalian cell systems may play a role in the thermotolerance and oxidative tolerance of mitochondrial electron transport and oxidative phosphorylation.

Several factors may account for the lack of detection of sHsps in mammalian mitochondria prior to the present study and the focus of most sHsp research on single Hsps in human (Hsp27) and murine cells (Hsp25). Because the previously characterized sHsps in human and murine cells contain few or no sulfur-containing amino acid residues, detection of many sHsps may be missed in metabolic labeling studies employing [³⁵S]methionine or [³⁵S]cysteine (13). Furthermore, the type of tissue or cell line examined, the effect of particular growth factors or hormones, the method of heat stressing (e.g., degree of temperature and duration of stress), and the type of stress imposed can affect the induction of Hsps (13, 14, 33). For example, it is well documented that murine Hsp25 cannot be induced in certain cell lines under conditions that induce production of other Hsps (12), illustrating that sHsp expression is highly regulated and that induction

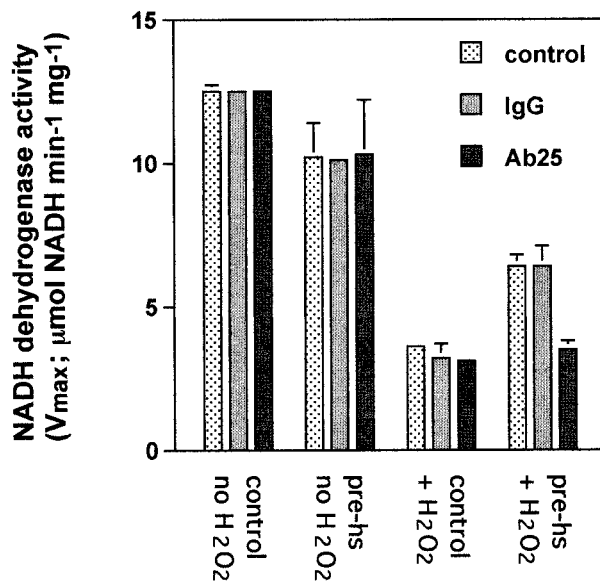


FIG. 5. The effect of antibodies to recombinant murine Hsp25 on NADH dehydrogenase activity. NADH dehydrogenase activity was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm. Submitochondrial vesicles purified from either unstressed (control) or heat-stressed PC12 cells were incubated in the absence or presence of 150 μ M H₂O₂. Either no protein was added, or BSA (0.2 mg/mL), rabbit IgG (1:300, v/v), or antibody specific to recombinant murine Hsp25 (Ab₂₅) (1:300, v/v). Results are means \pm 1 SE; $n = 3$.

of sHsps may only be observed under specific conditions and in specific tissue/cell types. Interestingly, several studies examining the induction of heat-shock proteins in various mammalian tissue/cell types using both [³H]leucine and [³⁵S]methionine contain evidence for multiple heat-stressed-induced low-molecular-weight proteins (13–16). Compared to many previous studies that utilized heat-stress treatments of less than 1 h and observed production of only one sHsp (e.g., 32–33), the prolonged heat stress treatment used in this study was designed to account for recent observations that novel or multiple low-molecular-weight Hsps are induced with longer heat stress regimes, at temperatures above 41°C, or during post-heat-stress recovery (13–16, 33). In the present study, detection of the multiple larger sHsps of the mitochondria (i.e., the sHspDs in Fig. 1) only in purified mitochondrial samples, and not in whole-cell samples as with HspA25, suggests that the sHspDs accumulate to such low amounts that detection of them is extremely difficult and/or that binding of Ab₂₅ to the sHspDs is reduced compared to HspA25. Previous studies have indicated that Hsp25 (equal to HspA25 here) localizes to either the cytosol or nucleus (10–12). Results from the present study indicating that Hsp25 localizes to the mitochondria suggest either that there are multiple Hsp25 isoforms or localization of Hsp25 varies with type of cell, species, stress, etc.

Long-term (as in this study) or chronic heat stress is known to result in the development of oxidative stress, as does prolonged exposure to a wide variety of environmental and biomedical toxins (e.g., doxorubicin, arsenate, cadmium, 1-methyl-4-phenylpyridinium (MPP), or rotenone) (1, 25). Furthermore, several important genetically linked or age-related diseases are known to involve increased susceptibility to oxidative stress or increased levels of active oxygen species (e.g., Parkinson's disease, Alzheimer's disease, Down's syndrome, and some forms of cardiovascular disease) (26, 27, 36, 37). Complex I is thought to be the major site of stress-related inhibition of oxidative phosphorylation, including oxidative stress (26). For example, reduction of complex I activity by 25% in PC12 cells significantly affected overall respiration, indicating that reduction in complex I activity not only severely affects redox balance within the mitochondria, but also severely affects oxidative phosphorylation (26). Because of complex I's importance to mitochondrial function and its relative susceptibility to heat stress, oxidative stress, and inhibitory toxins, complex I is thought to play a vital role in the etiology of many neurodegenerative and cardiovascular diseases (for reviews, see 26, 27, 36, 37). For example, studies demonstrating strong correlations between complex I deficiencies and idiopathic Parkinsonianism coupled with studies examining the induction of symptomatic Parkinsonianism by the com-

plex I-specific toxin, MPP, strongly suggest that disrupted complex I activity may be etiologically involved in neurodegeneration (38). Several published studies, as well as unpublished data from our own lab, demonstrate that induction of Hsps protects mitochondrial and complex I activity and promotes cell survival against rotenone, MPP, doxorubicin, and active oxygen species (i.e., H₂O₂) (28, 39, 40). Protection of complex I from oxidative stress by the mitosHsps *in vitro* suggests the possible importance of these sHsps either in diseases that involve complex I-specific oxidative damage to electron transport and oxidative phosphorylation or in diseases that involve cells that are highly resilient to oxidative damage (e.g., cancer) (25, 26, 40).

Although several well-characterized NADH:quinone oxidoreductases and alternative electron transport systems exist in plants and fungi, popular perception holds that only one NADH:quinone oxidoreductase complex exists in mammalian systems. However, several recent studies have documented the presence of both an endogenous as well as an exogenous complex I in the mitochondrial inner membrane and an NADH:quinone oxidoreductase in the outer mitochondrial membrane (41, 42). It is unknown if the mitosHsps associate with only the endogenous complex I or if they associate with either of the alternative NADH:quinone oxidoreductases. The anthracycline, doxorubicin (trade name, Adriamycin), undergoes redox recycling by NADH:ubiquinone oxidoreductase, creating doxorubicin semiquinone radicals and reactive oxygen species, which in turn produces oxidative stress-induced complex I inactivation (43). Recently, Nohl and co-workers demonstrated that this doxorubicin-mediated oxidative-stress toxicity is generated specifically by the exogenous NADH:ubiquinone oxidoreductase (44). Several recombinant genetic studies provide evidence that cancer and fibroblast cells become resistant to doxorubicin-induced oxidative stress when transfected with Hsp27 (for review, see 40). These studies, and the data presented here, suggest that the mitosHsps could be protecting exogenous complex I. Protection of exogenous complex I might also account for the tissue-specific accumulation of murine Hsp25, since evidence exists that exogenous complex I expression is also tissue specific (44).

The mechanism of protection or the specific site of interaction between the sHsps and Complex I is unknown, but the results from the NADH:CoQ and NADH dehydrogenase assays suggest that the sHsp might be interacting at the junctures of the hydrophilic/hydrophobic arms or the ubiquinone binding site(s) on the hydrophobic arm of complex I, since the greatest amount of protection occurred after the FMN and iron-sulfur N-1 site as indicated by greater protection of electron transport from NADH to ubiquinone vs electron transport from NADH to FeCN. This site of pro-

tection differs from the suggested site of protection on complex I for the plant mitochondrial sHsp (8). It is well documented that acid-ethanol-heat treatments induce a decomposition of complex I to smaller fragments, giving rise to conformational changes, transiently altered catalytic properties, decreased proton-transfer ability, and changes in substrate specificity (31). One hypothesis is that during heat and oxidative stress, the mitochondrial sHsps may stabilize the structural interactions between the hydrophilic and hydrophobic arms of complex I or the ubiquinone binding site(s), thereby maintaining complex I's function of reducing ubiquinone and proton translocation. An alternative hypothesis is that the sHsp is protecting complex I by scavenging active oxygen species in a site-specific fashion, as hypothesized for the chloroplast sHsp (45). Clearly, further work is needed in order to elucidate the mechanism of interaction between the mitochondrial sHsp and complex I.

ACKNOWLEDGMENTS

This work was funded by NSF Grant IBN-9728041 (S.A.H.) and an Oak Ridge Associated Universities Junior Faculty Enhancement Award (L.R.J.). We thank Sandra Downs, Stephanie Dellis, Joanne Baylis, and the anonymous reviewers for critical review of the manuscript. We especially thank Sandra Downs for insightful discussions and technical advice.

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