Characterization of the Hsp110/SSE gene family response to hyperosmolality and other stresses

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Abstract

A recently described subfamily of stress proteins is Hsp110/SSE, whose members are structurally related to the Hsp70/Bip gene superfamily (10, 19, 20). In mammals, this subfamily is comprised of heat stress protein Hsp110,1 osmotic stress protein Osp94,2 and Hsp70Ry. More distantly related members of this family include the SSE1 and SSE2 genes in yeast (28, 34), an orphan open reading frame from Caenorhabditis elegans (36, 43), the sea urchin sperm receptor (11), and mammalian glucose-regulated protein 170 (Grp170) (6). Based on structural homology to Hsp70, Hsc70, and Bip, members of the Hsp110/SSE gene subfamily likely encode a family of evolutionarily conserved molecular chaperones with putative ATP-binding and peptide-binding domains (22, 41).

METHODS

Cell culture and stress treatment. mlMCD3 cells were grown to confluence in plastic dishes in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 10% fetal bovine serum (JRH Biosciences) and 2% penicillin/streptomycin (Life Technologies). For hyperosmolality experiments, cells were treated with medium without serum for 18 h and then exposed to either isosmolar medium or hyperosmotic NaCl stress. In Chinese hamster ovary (CHO) cells, Hsp110 was greatly induced by heat shock (35). Heat shock also induced Osp94 expression in murine inner medullary collecting duct (mlMCD3) cells and TAMAC26 Sertoli cell line (17, 19). Analysis of Hsp70Ry, originally cloned from human B lymphocytes, showed that it is not regulated by heat shock (10, 13). The only study of non-heat stress showed Osp94 is induced by hyperosmotic NaCl in mlMCD3 cells (19). Thus the regulation and functions of the Hsp110/SSE gene family is largely unknown.

Characterization of the Hsp110/SSE gene family response to hyperosmolality and other stresses. Am. J. Physiol. 274 (Renal Physiol. 43): F1054–F1061, 1998.—Hsp110, Osp94, and Hsp70Ry are members of the recently described Hsp110/SSE subfamily of (heat and osmotic) stress proteins whose members are structurally related to the Hsp70/Bip gene superfamily. To date, little is known about the response of this gene family to stresses in vitro or in vivo. In this study, an analysis of mRNA expression showed that Hsp110 and Osp94, like Hsp70, are induced in renal murine inner medullary collecting duct (mlMCD3) epithelial cells by heat shock, hyperosmotic NaCl, and cadmium, whereas low pH had a suppressive effect on Osp94. H2O2 decreased expression of Osp94 while increasing levels of Hsp110 and Hsp70 message. Tunicamycin, hypertonic urea, and tumor necrosis factor-α had no effects. Hsp70Ry was responsive exclusively to cadmium chloride. Moreover, enhanced expression of Hsp110 and Osp94 was subsequently to induction of Hsp70 and was suppressed by inhibition of protein synthesis by cycloheximide. RT-PCR analysis showed Hsp110, Osp94, and Hsp70Ry are ubiquitously expressed in mouse tissues. In murine kidney, there was a corticomedullary gradient of expression of Hsp110, Osp94, Hsp70Ry, and Hsp70 but not Hsc70 or Bip. Furthermore, dehydron increased inner medullary expression of Hsp110 and Osp94. An analysis of stress tolerance in mlMCD3 cells showed that heat shock and hyperosmotic NaCl stress are cross-tolerant stresses, suggesting hyperosmolality is a physiological correlate of heat shock in mammalian kidney. Thus Hsp110 and Osp94 behave as heat shock proteins, although they are regulated differently than Hsp70.

heat stress protein 70; Bip binding protein; heat shock; stress tolerance; kidney; molecular chaperone

1 Coincident with Hsp110, another cDNA known as Hsp105 (Gen-Bank/EMBL Data Bank accession nos. D67016 and D67107) was cloned from mouse FM3A cells. Hsp105 encodes an 858 amino acid protein, whose predicted amino acid sequence is 96% identical to Chinese hamster Hsp110. Hsp110 and Hsp105 likely represent the same DNA.

2 Coincident with Osp94, another cDNA known as Apg-1 (GenBank accession no. D49482) was cloned from mouse testis cells, and this is identical to Osp94 (19).
factor-α (TNF-α, 50 ng/ml) (R & D Systems). Immediately after treatment and at appropriate time points, cells were washed twice with phosphate-buffered saline, and total RNA was isolated using the RNAzol B method (Tel-Test).

**cDNA cloning.** To clone murine-specific probes for Hsp70RY and Hsp110, total mRNA (5 μg) from mIMCD3 cells was reverse transcribed using random hexamer primers and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). For Hsp70RY, PCR was performed using sense (5’-GCAAGTGCCTCCACATGAGG-3’) and antisense (5’-CTTGGTGCCTTACACTGG-3’) primers corresponding to nucleotides 659 to 679 and nucleotides 1738 to 1717, respectively, of the human Hsp70RY cDNA (GenBank accession no. L12723) originally isolated from human Epstein-Barr virus-transformed B cells. For Hsp110, we used a nested primer strategy, in which the first amplification was performed with a degenerate sense primer (5’-CGGGGCCATCGAGAC-NATCGGC-3’) corresponding to nucleotides 86 to 107 and an antisense primer corresponding to nucleotides 1163 to 1143 (5’-TGCAATGCAATCCTTCTG-3’) of the CHO-derived Hsp110 cDNA sequence (accession no. Z47807). Subsequently, a second amplification was performed with a sense primer corresponding to nucleotides 164 to 184 (5’-GAACATGCTCGACCC-3’) and the same antisense primer used in the first reaction. In all cases, the 20 μL PCR mixture contained 1 μl of cDNA reaction mixture, 10 mM KCl, 10 mM Tris·HCl (pH 8.3), 2 mM MgCl₂, 200 μM dNTPs, 1.25 U Taq polymerase (Fisher), and 0.25 μM of each primer. The thermocycle protocol was as follows: 94°C for 3 min, 35 cycles of 55°C for 2 min, 72°C for 1 min, and 94°C for 2 min, followed by one cycle at 72°C for 5 min. Amplified cDNA fragments (~1 kb) were separated on a 1% agarose gel, isolated, ligated into the TA-cloning plasmid (Invitrogen), and cloned.

Northern analyses. Total RNA (10 or 20 μg) was fractionated in a 1% agarose and 0.7% formaldehyde denaturing gel and transferred overnight to a nylon membrane. For probes, murine-specific cDNA fragments of Hsp110, Hsp70RY, Osp94, and human cDNA fragments of Hsp70 (American Type Culture Collection (ATCC)) and BiP (ATCC) were labeled with [³²P]dCTP using a random hexamer labeling kit (Pharmacia). Blots were prewashed and then hybridized overnight at 42°C. Under these conditions, the Hsp70 probe detected both the constitutive and inducible Hsp70 transcripts. Autoradiography was performed with Reflection (NEN Research Products) film and an intensifying screen. Each blot was stripped and reprobed with different cDNAs, and the most representative blots are shown in Figs. 1–6.

RT-PCR analysis of tissue distribution. Murine tissues were homogenized, and total RNA was extracted by a standard CsCl ultracentrifugation method. For Hsp110 and Hsp70RY, RT-PCR was performed as described above using specific primers for each. For Osp94, PCR was performed under the same conditions described for Hsp110 and Hsp70RY, using a sense primer (5’-GCTTTCTCAACTGCTACATCG-3’) and an antisense primer (5’-CTCCTTGACTGCAGGAATCC-3’) corresponding to nucleotides 209 to 230 and 1239 to 1219, respectively, of the mouse Osp94 cDNA (accession no. U23921). PCR products were fractionated in 1.5% agarose and transferred overnight to a nylon membrane. Blots were probed with [³²P]-labeled murine-specific probes of Hsp110, Hsp70RY, and Osp94. Blots were prewashed and then hybridized overnight at 42°C, following the same protocol presented above. The blots were washed once at room temperature (2× SSC/0.1% SDS for 60 min), once at 65°C (0.2× SSC/0.1% SDS for 60 min), and autoradiographed.

Mouse treatment. ICR mice (4 wk old, 20 g) were allowed ad libitum access to water and food for 1 wk. Subsequently, three mice (dehydrated) had their access to water restricted for 24 h, and three control mice were allowed to continue with free water and food intake. All animals were killed, kidneys were removed, and renal cortex, outer medulla, and inner medulla were dissected and used to prepare total RNA for Northern analysis.

Stress-tolerance viability assays. mIMCD3 cells were seeded at 10³ cells/well in 100 μl of culture medium in 96-well plate flat-bottom (Corning) and incubated for 48 h in 5% CO₂ at 37°C. To evaluate hyperosmotic stress tolerance, cells were exposed for 15 min to 46°C and returned to 37°C, while control cells were maintained at 37°C. After 18 h, control and pretreated cells were exposed to hyperosmolar NaCl medium (+250 mM) for 8 h. To evaluate hyperosmolarity-induced thermotolerance, cells were treated with isosmotic medium (control) or hyperosmolar medium supplemented with either NaCl (+100 mM), urea (+200 mM), or glycerol (+200 mM) for 18 h and subsequently exposed to a heat shock (46°C for 75 min). After the hyperosmolar or heat shock treatment, viable cells were stained with 20 μl/well of 0.75% crystal violet in 30% acetic acid for 15 min, rinsed, and dried. Methanol (100%) was added to solubilize the stained cells, and the absorbance of each well was read at 630 nm with a Vmax Kinetic Microplate Reader ( Molecular Devices). Percent viability of treated cells was defined as the relative absorbance compared with control cells. To confirm the validity of this viability assay, cytotoxicity was also monitored by optical microscopic evaluation of the supernatant and adherent cells.

Statistical analyses. Statistical analyses were performed using True Epistat Software (Epistat Services, Richardson, TX). Data are expressed as means ± SE, and significance was assigned to P < 0.05, determined by using ANOVA followed by t-test analyses.

**RESULTS**

Stress-induced mRNA expression. A number of stress-related stimuli can trigger increased synthesis of stress proteins, but there is variability in the responses of individual stress genes to specific stresses. As members of the Hsp110 family have structural similarity to both Hsp70 and BiP, which are known to be regulated differently, we characterized the expression of the Osp94, Hsp110, Hsp70RY, Hsp70, and BiP in mIMCD3 cells under different stress conditions. As shown in Fig. 1, Osp94, Hsp110, and Hsp70 were induced by hyperosmotic NaCl and heat shock. Of note, Northern analyses showed two distinct Osp94 transcripts, representing alternative polyadenylation sites (19), whose expression levels varied in synchrony. The heavy metal cadmium increased expression of Hsp110, Osp94, Hsp70RY, Hsp70, and BiP, whereas low pH had a suppressive effect on Osp94. H₂O₂, an oxidant stress that can mimic...
reperfusion injury following hypoxia, decreased expression of Osp94, whereas it increased expression of Hsp70 and Hsp110. Tunicamycin, well known for its ability to induce endoplasmic reticulum-localized stress proteins, increased BIP expression, as anticipated, but failed to augment expression of Hsp70 or any of the members of the Hsp110 family. TNF-α increased none of these stress genes. Glyceraldehyde-3-phosphate dehydrogenase mRNA levels were also unchanged by the stresses.

As hyperosmolality represents the predominant physiological stress experienced by IMCD cells in vivo, we examined the kinetics of stress gene expression in mIMCD3 cells during continuous exposure to hyperosmotic NaCl. As shown in Fig. 2, Osp94, Hsp110, and Hsp70 were transiently induced, but none of the other stress genes was affected. Peak expression of Hsp70 mRNA was observed at ~4 h, and this preceded the increased expression of Osp94 and Hsp110, which appeared maximal at ~8 h.

To distinguish the nature of the osmotic stimulus responsible for enhanced stress gene expression, we evaluated the effects of increasing osmolality using different exogenous solutes. As shown in Fig. 3, mannitol, the least permeant solute, caused the most robust increase in Osp94, Hsp110, and Hsp70 transcript expression. NaCl and glucose had comparable stimulatory effects on these three transcripts. Interestingly, hyperosmolar glucose suppressed expression of BIP, which is well known to be induced by glucose deprivation (14, 38). Finally, little or no increase in mRNA expression of Osp94, Hsp110, and Hsp70 was observed with the permeant solutes glycerol and urea. Thus the stimulus, which elicits stress gene expression, is not simply hyperosmolality but rather it is hypertonicity associated with less-permeant solutes.

De novo protein synthesis can play an important role in the stress protein response. Consequently, we examined whether inhibition of protein synthesis affected NaCl-inducible mRNA expression, and, to exclude any intrinsic metabolic effect of NaCl, the same experiment...
was performed with hyperosmolar mannitol medium. As shown in Fig. 4, cycloheximide had little or no effect on basal expression of Osp94, Hsp110, and Hsp70. Inhibition of protein synthesis completely abrogated the NaCl-induced increase in Osp94 and Hsp110 mRNA expression but did not completely block induction of Hsp70. Similarly, mannitol-induced overexpression of Osp94, Hsp110, and Hsp70 were also suppressed by inhibition of protein synthesis (data not shown). Furthermore, cycloheximide alone appeared to promote a small increase in expression of Hsp70RY and BiP. Thus de novo protein synthesis is required to elicit an increase in expression of Hsp110 and Osp94 in response to hypertonicity.

Analysis of tissue expression. We next evaluated whether members of the Hsp110 family, like other known molecular chaperones, are ubiquitously expressed in vivo. Initial Northern analyses using total RNA indicated that the Hsp110 family members were generally undetectable in murine tissues under normal circumstances. Consequently, we performed RT-PCR analysis of a variety of mouse tissues using primers and probes specific for Osp94, Hsp110, and Hsp70RY. As evident in Fig. 5, Osp94, Hsp110, and Hsp70RY transcripts could be detected in all tissues examined, including brain, diaphragm, heart, liver, lung, stomach, and kidney, indicating these stress genes are ubiquitously expressed in vivo, even in the absence of any apparent stress. Not shown, these primers failed to amplify products from genomic DNA confirming that the RT-PCR products represented mRNAs.

Expression of heat shock proteins in the kidney. The renal medulla is well known to sequester high concentrations of NaCl and urea, and these levels significantly increase during states of dehydration. To evaluate whether in vivo osmotic stress could modulate expression of these stress genes, we examined mRNA levels in renal cortex, outer medulla, and inner medulla from control and dehydrated mice (Fig. 6). As previously noted (19), there was a corticomedullary gradient of Osp94 expression that parallels the known osmotic gradient. Moreover, expression was increased in inner medulla with dehydration. Similarly, Hsp110 and Hsp70 exhibited a corticomedullary gradient of increasing expression. Interestingly, Hsp70 mRNA level was not greatly altered in inner medulla with dehydration. Analysis of Hsp70RY revealed two transcripts, 3.2 and 4.4 kb, in outer and inner medulla, and both exhibited a slight increase of expression in response to dehydration. BiP was expressed constitutively in all kidney regions studied and was not modulated.

![Fig. 4. Northern analysis of stress protein mRNA expression in the presence of protein synthesis inhibition by cycloheximide. Total RNA was isolated from mIMCD3 cells exposed to isosmolar (−) or hyperosmolar NaCl (+) for 5 h in the presence (+Chx) or absence (−Chx) of 10 µg/ml of cycloheximide. Cycloheximide blocked NaCl-induced Osp94 and Hsp110 mRNA expression totally and partially inhibited Hsp70. Hsp70RY mRNA increased in isosmolar and hyperosmolar condition, when cycloheximide was added to the medium. GAPDH mRNA level was unchanged. Same blot was utilized for the various probes and is representative of 3 different experiments.](http://ajprenal.physiology.org/)
Stress tolerance. In the foregoing experiments, gene expression during adaptation to hyperosmotic NaCl was similar to that seen with heat shock, suggesting that renal medullary hyperosmolality may be a physiological correlate of heat shock. To investigate this further, we performed cross-tolerance stress experiments to determine whether mIMCD3 cells could be protected from osmotic stress by prior exposure to heat shock and vice versa. As shown in Fig. 7, cells treated for 18 h with hypertonic NaCl (+100 mM) exhibited an enhanced ability to survive 75 min of 46°C heat stress (71% viable) compared with heat shocked control cells (57% viable). This tolerance to high temperature was not different from that induced by pretreatment with heat for a short period of time (data not shown). Not shown, hyperosmotic urea and glycerol (+200 mM) failed to elicit resistance to heat shock treatment in keeping with their inability to elicit enhanced expression of Hsp. Conversely, exposure to heat stress conferred increased survival to subsequent exposure to hyperosmotic NaCl (+250 mM) with viability increasing from 21 to 61% (Fig. 7). Thus mIMCD3 cells exhibit cross-tolerance between heat stress and hyperosmotic NaCl stress.

**DISCUSSION**

In the present study, analysis of the response of mIMCD3 cells to a variety of stresses demonstrated that Hsp110 and Osp94 are regulated in a manner similar to that seen for Hsp70 but not BiP. In particular, heat shock, hyperosmotic shock, and cadmium significantly increased expression, whereas tunicamycin failed to elicit a response. Thus it appears Hsp110 and Osp94 should be classified as heat shock proteins in terms of their regulation. The failure of tunicamycin, a known inducer of endoplasmic reticulum chaperones, to affect expression is consistent with the observation that Hsp110 and Osp94 lack a consensus endoplasmic reticulum retention signal. In comparison, Hsp70RY appeared to be a poorly inducible member of the Hsp110 family, responding exclusively to cadmium, in much the same way that constitutive Hsp70 shows only minor changes in expression with a variety of stresses (13).

Hyperosmolality is a physiologically important stress in mammalian kidney, and a role for molecular chaperones in the adaptation to hyperosmolality has been suggested from studies of mammalian cells in culture. Renal epithelial cells exhibited increased expression of Hsp70 and mitochondrial Hsp75 with hypertonic stress (8, 31, 33). Hyperosmolality increased expression of Hsp27 in MCF breast cancer cells (18), αβ-crystallin in astrocyte cells (16), and Hsp70, BiP, and Grp95 in mouse fibroblasts (38). In the present study, we found that exogenous addition of relatively impermeant solutes elevated mRNA levels of Hsp110, Osp94, and Hsp70, whereas urea or glycerol had little or no effect. This suggests that hypertonicity, as opposed to hyperosmolality per se, is the stimulus for increased expression, and cell shrinkage may be involved. A similar phenomenon was observed for hyperosmolality-induced expression of genes responsible for accumulation of organic osmolytes. For example, expression of aldose reductase was maximally stimulated by hyperosmolar raffinose and NaCl but not affected by hyperosmolar urea or glycerol (39).

In mouse, Hsp110, Osp94, and Hsp70RY expression were detected in all tissues examined, including brain, diaphragm, heart, liver, lung, stomach, and kidney. Prior studies demonstrated expression of Hsp110 in brain, liver, ovary, spleen, heart, lung, and muscles (20) and Osp94 expression in testes and kidney (17, 19). Hsp70RY expression was previously detected in B lymphocytes and a variety of human hematopoietic cell lines (9). This profile of ubiquitous expression for the three members of Hsp110 family is similar to that described for other known molecular chaperones, a finding that has underscored their functional significance in basic cellular processes, such as protein synthesis, protein degradation, and signal transduction (15).

In vivo, the renal inner medulla is a site of continual stress, as it typically accumulates very high concentrations of NaCl and urea to enable the kidney to generate a normal, concentrated urine. With dehydration, renal medullary osmolality can exceed 1,200 mosM in humans and 3,000 mosM in rodents. Previous investigators observed a renal corticomedullary gradient of Hsp70, Hsp25, and Osp94 expression that paralleled the known osmotic gradient (19, 29). In the present...
study, Hsp110, Osp94, Hsp70RY, and Hsp70 expression exhibited a corticomedullary gradient of expression with expression generally greater in inner medulla than cortex. In contrast, BIP levels did not vary significantly among different regions of the kidney. Mice dehydrated for 24 h to enhance renal medullary osmolality expressed increased levels of Hsp110 and Osp94 in inner medulla, whereas Hsp70 and Hsp70RY levels appeared to be unchanged. Thus it appears that there is an important role for heat shock proteins in renal medulla in vivo and that hyperosmolality represents a powerful physiological stimulus for induction of the heat shock response in mammals.

Stress-related transcriptional regulation of heat shock proteins has been shown to be mediated by heat shock factors (HSFs) (reviewed in Ref. 24). HSF1 is present in unstressed cells in a monomeric non-DNA binding form and is rapidly converted to the trimeric form in response to adverse stress. Trimerization enables HSF1 to bind to a consensus nucleotide sequence (heat shock element) in the promoter region of heat shock protein genes (1, 32). The signal for activation of HSFs is unknown, but previous studies showed that unfolding of mature cellular proteins or misfolding of nascent polypeptides can both trigger the heat shock response (2, 3, 26, 27). Furthermore, stresses that cause misfolding of newly synthesized polypeptides fail to elicit a stress response when cycloheximide is used to block protein synthesis. For example, cycloheximide can totally block Hsp70 mRNA expression in response to nitric oxide (44) and establish a different concentration threshold of arsenite to induce the heat shock response (37).

In our experiments, addition of cycloheximide abolished the hyperosmolality-induced increase in Hsp110 and Osp94 expression. There are at least two potential mechanisms to explain this observation. On one hand, if Hsp110 and Osp94 proteins function in the de novo protein synthesis process, as is known for Hsp70 (12), their expression would be increased under conditions when nascent peptide synthesis is adversely affected, as with hyperosmotic stress and heat shock (7, 15). Hence, cycloheximide, by preventing synthesis of nascent proteins, would prevent stress-induced aberrant protein synthesis and thus fail to stimulate the corresponding stress response system (3, 4, 26). Alternatively, increased expression of Hsp110 and Osp94 may require the prior synthesis of a signaling molecule, such as a transcription factor other then HSFs such that inhibition of protein synthesis would prevent the synthesis of this activating protein. Further study is needed to define both the functions of the Hsp110 family members, as well as the signaling pathways involved in regulating their expression.

The expression profile of stress proteins indicated that there is considerable similarity between the response of a cell to hypertonicity and heat shock. Furthermore, as hypertonicity, like thermal stress, is known to adversely affect cellular protein functions and alter biosynthetic processes (5, 8), hypertonicity may represent the physiological correlate of heat shock in mammalian cells. Prior investigations in many systems documented that exposure to one stress (e.g., heat shock) can confer tolerance to another, related stress (e.g., ischemia) (42). Consequently, we examined whether mMCD3 cells exhibited such cross-tolerance with exposure to hyperosmolality and heat stress. As hypothesized, it was possible to demonstrate, for the first time in mammalian cells, that those preconditioned with mild heat shock exhibited increased tolerance to hyperosmotic NaCl. Conversely, exposure to NaCl stress conferred increased survival to thermal stress. Thus there must be considerable overlap in the adaptive mechanisms responsible for cellular adaptation to these two stresses. Of note, cross-tolerance appeared to be most effective when heat shock was used as the preconditioning stress. A similar cross-tolerance between heat and hyperosmolality was demonstrated in bacteria (40) and recently in yeast (21), suggesting an evolutionarily conserved phenomenon.

In conclusion, members of Hsp110/SSE family are ubiquitously expressed in mammalian tissues. One member of the family, Hsp70RY, was induced exclusively by cadmium chloride, and two members of the family, Hsp110 and Osp94, were induced by heat stress, heavy metal stress, or hypertonicity, and synthesis of new protein was essential for their expression. Their inducibility and corticomedullary gradient of expression in the kidney indicates an important role in the preservation of cell viability and function during exposure to the physiologically harsh environment where they may serve as a primary protective measure.

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REFERENCES

