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Heat Shock and Oxidative Stress-induced Exposure of Hydrophobic Protein Domains as Common Signal in the Induction of *hsp68**

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The hypothesis of a common signal for heat shock (HS) and oxidative stress (OS) was analyzed in C6 cells with regard to the induction of heat shock proteins (Hsps). The synthesis rate and level of the strictly inducible Hsp68 was significantly higher after HS (44 °C) compared with OS (2 mM H_2O_2). This difference corresponded to higher and lower activation of the heat shock factor (HSF) by HS and OS, respectively. OS, on the other hand, showed stronger cytotoxicity compared with HS as indicated by drastic lipid peroxidation and inhibition of protein synthesis as well as of mitochondrial and endocytotic activity. Lactic dehydrogenase also revealed stronger inhibition of enzyme activity by OS than by HS as shown in cells and *in vitro* experiments. Conformational analysis of lactic dehydrogenase by the fluorophore 1-anilinonaphtalene-8-sulfonic acid, however, showed stronger exposure of hydrophobic domains after HS than after OS which correlates positively with the Hsp68 response. Treatment of cells with deoxyspergualin, which exhibits high affinity to Hsps, the putative inhibitors of HSF, strongly increased only OS-induced *hsp68* expression. In conclusion, the results suggest that exposure of hydrophobic domains of cytosolic proteins represents the common first signal in the multistep activation pathway of HSF.

Stress proteins, initially termed heat shock proteins (Hsps)[1] after the first observation of Ritossa (1), are induced in response to a wide range of biological and physicochemical stressors, including heat shock (HS) and oxidative stress (1, 2). Prominent stress proteins are represented by members of the Hsp70 family, which consists of constitutive (Hsc70) and inducible isoforms (Hsp68). Hsp68 and Hsc70 act as chaperones and protect the cell by binding to malfolded proteins resulting from exposure to stress, thus preventing their aggregation and either helping these proteins to refold to their active state or targeting them to lysosomes for protein degradation (2, 3).

Activation of *hsp68* seems to be predominantly regulated by the heat shock transcription factor (HSF-1) which binds to heat shock elements (HSEs) in the *hsp68* promoter (4). HSF-1 is apparently regulated by trimerization, phosphorylation, redox modification, and compartmentation (4–6). Together with the expression of isoforms (7) these results suggest a multistep regulation of HSF (8, 9). In addition to HSE, the *hsp68* promoter contains binding sites for nuclear factor κB (NFκB) which is strongly activated by hydrogen peroxide (10). Other transcription factors such as signal transducers and activators of transcription, transcription factor IID, Sp-1, and CCAAT-box binding factor may also influence the induction of *hsp68* (11–13). The differential activation of these and other factors by HS and OS and their interplay may ultimately cause a differential induction of *hsp68*.

It is still unclear whether the activation of the *hsp68* gene by the great diversity of stressors depends on a common signal pathway. Initially, it was speculated that abnormal proteins may trigger HSF activation (14, 15). Later, it was demonstrated that Hsp70 forms a complex with HSF and that excess Hsp70 suppress HSF activation (16–18). It was hypothesized, therefore, that denatured proteins reduce the concentration of free Hsp70 by complex formation. This decrease of free Hsps relieves their inhibitory action on the HSF which can subsequently convert from the monomeric, non-DNA binding form into the trimeric active form (2). Binding of Hsp70 to the HSF-1 transactivation domain was shown to be responsible for the repression of *hsp70* transcription (19). Moreover, Hsp90 seems to be involved in the repression of HSF under physiological conditions (20).

On the other hand, studies exist which do not agree with the hypothesis of a feedback control mechanisms of HSF, because they failed to observe an effect of different amounts of Hsp70 on HSF activation (21–23). Noteworthy, purified HSF can be reversibly trimerized *in vitro* by heat shock, hydrogen peroxide, and low pH, indicating that the monomeric HSF can directly sense stressors in the absence of other factors (24–26). The monomer-trimer transition of HSF was shown to be autoregulated through intramolecular coiled-coil interactions of specific leucin zippers (25, 27, 28). Alternatively, different signal transduction pathways may be involved in *hsp68* induction by different stressors (29) which is consistent with different HSF-1 activation by various stressors (30). Different regulation of HSF activity may depend on different activation of protein phosphatases and protein kinases which control phosphorylation of HSF-1 at serine residues (31–33).

Reactive oxygen species which accumulate during oxidative and other types of stress are discussed as another general trigger for HSF activation, for example, by redox modifications (5, 34). Reactive oxygen species are elicited by external sources (heat shock, X-ray, and UV radiation, electrosmog, toxins etc.

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[1] The abbreviations used are: Hsp, heat shock protein; HS, heat shock; OS, oxidative stress; Hsc, constitutive heat shock protein; DSG, deoxyspergualin; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, neutral red; HSF-1, heat shock factor-1; TBARS, thiobarbituric reactive substances; LDH, lactic dehydrogenase; 1,8-ANS, 1-anilinonaphtalene-8-sulfonic acid; TBS, Tris-buffered saline.

(35, 36)), but are also generated intracellularly through electron leackage from mitochondria, activity of oxygen-utilizing enzymes and by macrophages generating superoxide anion radicals ($\cdot O_2^-$) (37, 38). These radicals either dismutate autonomously or are converted enzymatically by superoxide dismutase to hydrogen peroxide (H_2O_2). H_2O_2 is rather stable but decomposes to the highly reactive hydroxyl radical ($\cdot$OH) in the presence of trace metals such as iron by the Fenton reaction (39).

In our study, the induction of *hsp68* and HSF activation by heat shock was significantly stronger than by oxidative stress. We observed that HS (44 °C) also caused stronger exposure of hydrophobic domains of lactic dehydrogenase than OS (2 mM H_2O_2) which agrees with the assumption that denatured proteins represent a common signal for the Hsp68 response. Interestingly, this finding did not correlate with the suppression of LDH activity. Deoxyspergualin (DSG) which exhibit high affinity to constitutive Hsps strongly increased only OS-induced *hsp68* expression in contrast to the induction after 44 °C exposure. This implies that hydrophobic interactions between Hsps and denatured proteins are maximal only in response to HS. Our results thus suggest that hydrophobic domains of cytosolic proteins represent a common denominator in the multistep induction pathway of *hsp68* by heat shock and oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—Stock solution of hydrogen peroxide (Merck, Darmstadt, Germany) in H_2O was freshly made as a ×1000 concentrate prior to use. The concentration was routinely measured photometrically at 240 nm. Other chemcials were obtained from Sigma-Aldrich (Deisenhofen, Germany) and Biomol (Hamburg, Germany) unless otherwise noted.

Cell Culture and Stress Treatment—The C6 rat glioma cell line, initially derived from a *N*-nitrosourea-induced astrocytoma (40) was grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum at 37 °C in a humidified, 10% CO_2 atmosphere. Cells were subcultured in culture flasks (Nunc, Wiesbaden, Germany) and passaged every 3 days. Viability of cells was routinely tested by the trypan blue exclusion assay. Before experiments, cells were seeded in 88-mm (1×10^6), 55-mm (4×10^5), or 35-mm (2×10^5) culture dishes and maintained for 3 days to establish a subconfluent monolayer. A heat shock of different temperatures was applied for 0.5 h by transferring cell culture dishes into a water bath. Oxidative stress was administered by incubating the cells with different concentrations of hydrogen peroxide for 1 h. After washing with phosphate-buffered saline, pH 7.4, cells were allowed to recover from the treatment for various times in normal medium at 37 °C.

In Vivo Labeling of Proteins with [^{35}S]Methionine and Autoradiography—After stress, medium was changed and 370 kBq (10 μCi)/ml of [^{35}S]methionine in methionine-free medium (ICN, Eschwege, Germany) added 2 h before harvest. After lysis (100 °C for 5 min) in sample buffer (5 mM Tris-base, pH 6.8, 5% 2-mercaptoethanol, 3% SDS, 10% glycerol) and subsequent centrifugation (18,000 × *g* for 10 min at 20 °C), the amount of trichloroacetic acid precipitable radioactivity was determined in a scintillation counter (Beckmann, München, Germany). Protein content was determined by the method of Neuhoff *et al.* (41). Protein synthesis is expressed as rate of incorporation in counts per minute (cpm)/μg of protein. Equal amounts of radioactivity (100,000 cpm/lane) were loaded on 7.5–15% polyacrylamide gels and separated by SDS-PAGE (42). The gels were fixed, vacuum-dried on a Whatman 3MM filter paper, then placed on Hyperfilm-β Max (Amersham Pharmacia Biotech) and kept at −20 °C during exposure.

Western Blot Analysis—Cell lysates were heated (100 °C, 5 min) in sample buffer and then sonified with a Branson-sonifier tip (Branson, Vésenaz-Genève, Switzerland) for 5 s to destroy DNA. After centrifugation (18,000 × *g* for 10 min at 20 °C), equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by electroblot (43). Blots were blocked with 0.2% Tween 20 in phosphate-buffered saline for 0.5 h at room temperature. Anti-Hsp68 conjugated to alkaline phosphatase (SPA 810-AP, monoclonal purified mouse IgG, clone C92F3A-5, 1: 1000, Biomol, Hamburg, Germany), which specifically recognizes Hsp68 as demonstrated by two-dimensional PAGE analysis[2] was incubated for 1 h followed by three washes in Tween 20 in phosphate-buffered saline. Immunocomplexes were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Electrophoretic Mobility Shift Assay—Directly after stress, cells were harvested with a rubber policeman, centrifuged in Eppendorf tubes (12,000 × *g*/2 min/4 °C), and resuspended in 100 μl of ice-cold 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 12% glycerol. Cells were then pulse-sonified with a Branson sonifier tip two times for 10 s on ice and subsequently centrifuged (18,000 × *g* for 15 min at 4 °C). Binding reactions were performed using a specific double-stranded synthetic HSE, carrying four GAAn repeats, two of them inverted (5′-ACTGTCT-GTCTGTCTGTCTGTCTATCTGCTAGAAGCTTCTAGAAGCGTTCTAG-3′). The HSE was labeled with digoxigenin-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) using DNA Taq polymerase (Biotherm, Genecraft, Münster, Germany) in a thermocycler (Progene, Thermo-Dux, Wertheim, Germany). Whole cell extracts (10 μg) were mixed with 68 ng of digoxigenin-labeled HSE oligonucleotide and 0.5 μg of poly(dI-dC) (Roche Molecular Biochemicals) in binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, and 12% glycerol), to a final volume of 30 μl. The binding reaction was performed for 20 min at room temperature. The samples were then electrophoresed on a nondenaturing 4% polyacrylamide gel, blotted on nylon membrane (Hybond N^+, Amersham Pharmacia Biotech), cross-linked with a transilluminator (Herolab, Wiesloch, Germany) at 254 nm for 30 s, and subsequently dried. After blocking (1% skim milk in 100 mM maleic acid and 150 mM NaCl), free HSE and HSF-HSE complexes were detected by the anti-digoxigenin antibody conjugated to alkaline phosphatase (polyclonal from sheep, 1:10000, Roche Molecular Biochemicals), and visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. For competition experiments, binding reaction mixtures contained a 50-fold excess of unlabeled HSE oligonucleotides.

Viability Assays—Cell viability was analyzed by the MTT and the neutral red (NR) viability assay. The MTT assay measures the conversion of the tetrazolium salt MTT to colored formazan by mitochondrial dehydrogenase activity (44), whereas the NR assay determines cell viability by endocytotic uptake of neutral red according to the method of Babich and Borenfreund (45). Briefly, at indicated times after heat shock or oxidative stress, either 150 μl of MTT solution (5 mg/ml phosphate-buffered saline) or 1.5 ml of NR solution (0.4% NR diluted 1:80 in Dulbecco's modified Eagle's medium + 10% NCS) was added to each 35-mm dish. The cultures were then incubated for 3 h at 37 °C and the supernatant discarded. For the MTT assay, solubilization of cells and formazan was achieved by adding 3 ml of lysis buffer (10% SDS in 50% dimethylformamide, pH 4.7). Color development was quantified photometrically at 570 nm. For the NR assay, neutral red was extracted by adding 3 ml of 1% acetic acid in 50% ethanol. After 10 min of gentle shaking, absorbance was determined photometrically at 540 nm. For both assays, viability is given in percentage of the control value.

Thiobarbituric Acid Assay—Lipid peroxidation was quantified by the thiobarbituric acid assay according to the method of Bernheim *et al.* (46), which measures the production of malondialdehyde or other related substances, designated as "thiobarbituric reactive substances" (TBARS). This method is a first global measure of lipid peroxidation and was applied because of its sensitivity and simplicity (47). After stress, cells were harvested in 1 ml of ice-cold phosphate-buffered saline with a rubber policeman and transferred to 2-ml Eppendorf tubes. After vortexing, protein content was determined and 1 ml of 0.375% 2-thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl added. The tubes were placed in a water bath and kept at 95 °C for 45 min. After cooling, the mixture was centrifuged at 850 × *g* for 5 min. Absorbance was measured photometrically at 535 nm. Concentrations of TBARS were calculated using an extinction coefficient of 1.56×10^5/M/cm and expressed in nanomole of TBARS/mg of protein. Additionally, a calibration curve was established with malondialdehyde, produced by hydrolysis of 1.1.3.3 tetramethoxypropane in 0.1 N HCl as standard.

Enzyme Activity Assay—Enzyme activity of LDH either in cell extracts or pure solutions was quantified. For cellular LDH analysis, cells were harvested after stress treatment with a rubber policeman in 200 μl of ice-cold 0.01 M Tris-buffered saline (TBS), pH 7.4, and pulse-sonified two times for 10 s on ice with a Branson sonifier tip. Then, 5 μl of lysate were added to 0.17 mM NADH and 0.2 mM pyruvate in 1 ml of TBS. The decrease of NADH absorbance was then measured photomet-

[2] U. Neuhaus-Steinmetz, unpublished data.

rically every 30 s at 340 nm. Specific activity of LDH was calculated as units per mg of protein using $\epsilon = 6.22/\mu$M/cm. For *in vitro* experiments, 1.5 units of porcine muscle LDH (Sigma-Aldrich, Deisenhofen, Germany) in 1 ml of TBS, pH 7.4, supplemented with 10 μM $FeSO_4$ were treated with different concentrations of H_2O_2 for 1 h, incubated at 44 °C for 30 min or used as controls, respectively. After treatment, 15,000 units/ml of catalase (Roche Molecular Biochemicals) were added to each tube and incubated for 30 min. This was shown to inactivate the solution of 2 mM H_2O_2. Activity of LDH was determined by adding 5 μl of LDH solution to 0.17 mM NADH and 0.2 mM pyruvate and expressed in units/ml.

1-Anilinonaphtalene-8-sulfonic Acid (1,8-ANS) Fluorescence Assay—Conformational analysis of porcine muscle LDH was examined by extrinsic 1,8-ANS fluorescence analysis. 1,8-ANS is highly sensitive toward hydrophobic domains of proteins which are exposed during protein denaturation (48, 49). Absorption and fluorescence emission spectral analysis of 1,8-ANS (Sigma-Aldrich) revealed a maximum of 380 nm (absorbance) and 480 nm (emission). It was, therefore, excited at 380 nm. For experiments, 1 μM LDH in 100 μl of 0.1 M Tris buffer, pH 7.5, was incubated with different H_2O_2 concentrations or different temperatures. Then, a stock solution of 1,8-ANS (1.5 mM in H_2O) was added to the LDH solution to give a final concentration of 15 μM. After a 30-min incubation, fluorescence emission of 1,8-ANS was scanned from 400 to 650 nm at an excitation wavelength of 380 nm and expressed as arbitrary units (AU). All experiments were performed with a Hitachi-4500 spectrofluorimeter. The band pass was 5 nm for both excitation and emission wavelengths. Means of 25 repetitive scans (CAT mode of spectrofluorimeter) were recorded for each measurement.

RESULTS

Effect of Heat Shock and Oxidative Stress on Stress Protein Synthesis, Level of Hsp68, and HSF Activity—The effects of OS (2 mM H_2O_2, 1 h) and HS (44 °C, 0.5 h) on the synthesis of stress proteins were determined by autoradiography (Fig. 1*A*). HS induced the following heat shock proteins (Hsps) in descending order of induction strength: Hsp68, Hsc70, Hsp90, Hsp47, heme oxygenase-1 (HO-1), Hsp110, Hsp100, Hsp27, and αB-crystallin (αB-Cry), whereas OS induced Hsp47, HO-1, Hsp90, Hsp100, Hsc70, Hsp27, αB-Cry, Hsp110, and Hsp68 also in descending order of induction strength. Most stress proteins were induced stronger by HS (Hsp68, Hsc70, Hsp90, and Hsp110). αB-Cry, Hsp27, HO-1, and Hsp47 were almost equally induced by both stressors, whereas the synthesis rate of Hsp100 was significantly higher after OS. As evaluated by densitometry the strictly inducible Hsp68 exhibited the largest difference in response to the two stressors showing an ~20-fold higher synthesis rate after HS as compared with OS (data not shown). The induction of all stress proteins exhibited a faster response after HS compared with OS: αB-Cry, Hsp27, HO-1, Hsp47, Hsp68, and Hsc70 reached their maximum 6 h after HS, Hsp90, Hsp100, and Hsp110 12 h after HS, whereas OS induced maximal synthesis of αB-Cry, Hsp27, HO-1, and Hsp68 after 12 h recovery and of Hsp47, Hsc70, Hsp90, Hsp100, and Hsp110 after 24 h recovery.

Because Hsp68 showed the largest difference in response to HS and OS it was further examined by Western blot analysis comparing the dose dependence and kinetics of the induction by OS and HS. Untreated control cells did not express *hsp68* (Fig. 1, *A* and *B*). The level of Hsp68 was slightly increased after 42 °C, maximally elevated after a 44 °C treatment, and not detectable after 46 °C (Fig. 1*B, I*). The threshold for *hsp68* induction turned out to be 41 °C (data not shown). After OS, only high concentrations of hydrogen peroxide, starting from 1 mM up to 8 mM H_2O_2 increased the level of Hsp68 in a dose-dependent manner (Fig. 1*B, II*). 8 mM H_2O_2 induced *hsp68* expression maximally, the induction, however, reached only about 15% of the induction after HS (44 °C). Because 60 μg of protein were loaded on each SDS-PAGE lane in the case of OS (to obtain a good resolution of the bands) and 30 μg in case of HS, and because of the longer incubation time (1 h) with H_2O_2 as compared with 0.5 h exposure to high temperature, the difference in the amount of Hsp68 after HS and OS must be even greater. This was confirmed by separating equal amounts of proteins which showed approximately a 25-fold stronger expression of *hsp68* after 44 °C compared with 2 mM H_2O_2 (data not shown). The stronger induction of *hsp68* by HS was also confirmed by another slightly less sensitive antibody (SPA 820, Biomol) which was able to detect Hsp68 only after HS, but not after OS (data not shown).

In the next series of experiments we analyzed the induction kinetics after OS (2 mM H_2O_2, 1 h) and HS (44 °C, 0.5 h). After HS the increase of Hsp68 occurred earlier compared with the increase after OS: maximal amounts were reached about 15 h after HS (Fig. 1*B, III*). The same experiments after OS demonstrated a delayed response of Hsp68 with maximal expression only after about 36 h (Fig. 1*B, IV*). This agrees with the maximal Hsp68 synthesis rates after 6 h (HS) and 12 h (OS) recovery (Fig. 1*A*).

The stronger induction of *hsp68* by HS compared with OS is reflected also in a stronger HSF-HSE binding after HS of 44 °C compared with an OS of 8 mM H_2O_2 (Fig. 1*C*). The different HSF-HSE binding after HS and OS correlates with the different levels of synthesis and accumulation of Hsp68. Hence, HSF seems to be the main factor in mediating the induction of *hsp68* by both stressors. Because of the lower sensitivity of the electrophoretic mobility shift assay no binding activity of HSF was observed after treating the cells with 2 mM H_2O_2 for 1 h (data not shown). The HSF-HSE complex migrated as a doublet, possibly due to two distinct HSEs which were generated during the labeling reaction and which can be observed as two bands of free HSEs at the bottom of the gel. The sequence specifity of the retarded HSF band was confirmed by competition experiments using a 50-fold excess of the same but unlabeled HSE oligonucleotide.

Cytotoxic Effects of Oxidative Stress and Heat Shock—With the different *hsp68* inductions by OS and HS in mind, we wanted to find out whether these differences may correlate with other cellular effects caused by OS and HS. Therefore, we analyzed various cellular variables which are presently discussed as putative common signals in the induction pathway of *hsp68* in response to both stressors. First, we measured the effects of HS and OS on viability by the MTT and NR assays (Fig. 2*A*). Increasing concentrations of hydrogen peroxide (125 μM to 8 mM for 1 h) and increasing temperatures (42, 44, and 46 °C for 0.5 h) were tested. Compared with the NR assay (*right columns*) which indicates the degree of endocytotic NR-uptake (45), the MTT assay (*left columns*) which is assumed to measure the activity of mitochrondrial dehydrogenases (44) responded with higher sensitivity to both stressors. Oxidative stress caused by 1–8 mM H_2O_2 showed a stronger cytotoxicity compared with heat shock of 42 and 44 °C, as demonstrated by both assays. Noteworthy, 2 mM H_2O_2 showed stronger cytotoxic effects (37 and 70% viability for MTT or NR, respectively) compared with 44 °C (57 and 96% viability), whereas 2 mM H_2O_2 hardly induced *hsp68* as compared with the maximal induction by 44 °C (Fig. 1). The cytotoxic effects of HS (44 °C, 0.5 h) are equivalent to an OS of about 0.5 mM H_2O_2 (1 h) which, however, had no inducing effect on *hsp68*. Only a HS of 46 °C was as cytotoxic as 8 mM H_2O_2. These results indicate that general cytotoxic effects are not generating common inducing signals for *hsp68*.

To evaluate the influence of the two stressors on protein synthesis, cells were exposed to 2 mM H_2O_2 (1 h) and 44 °C (0.5 h). After 3, 6, 12, and 24 h recovery cells were incubated with [^{35}S]methionine for 2 h. Thereafter the labeling of proteins was analyzed (Fig. 2*B*). OS inhibited protein synthesis drastically stronger compared with HS. 3 h after oxidative stress, protein

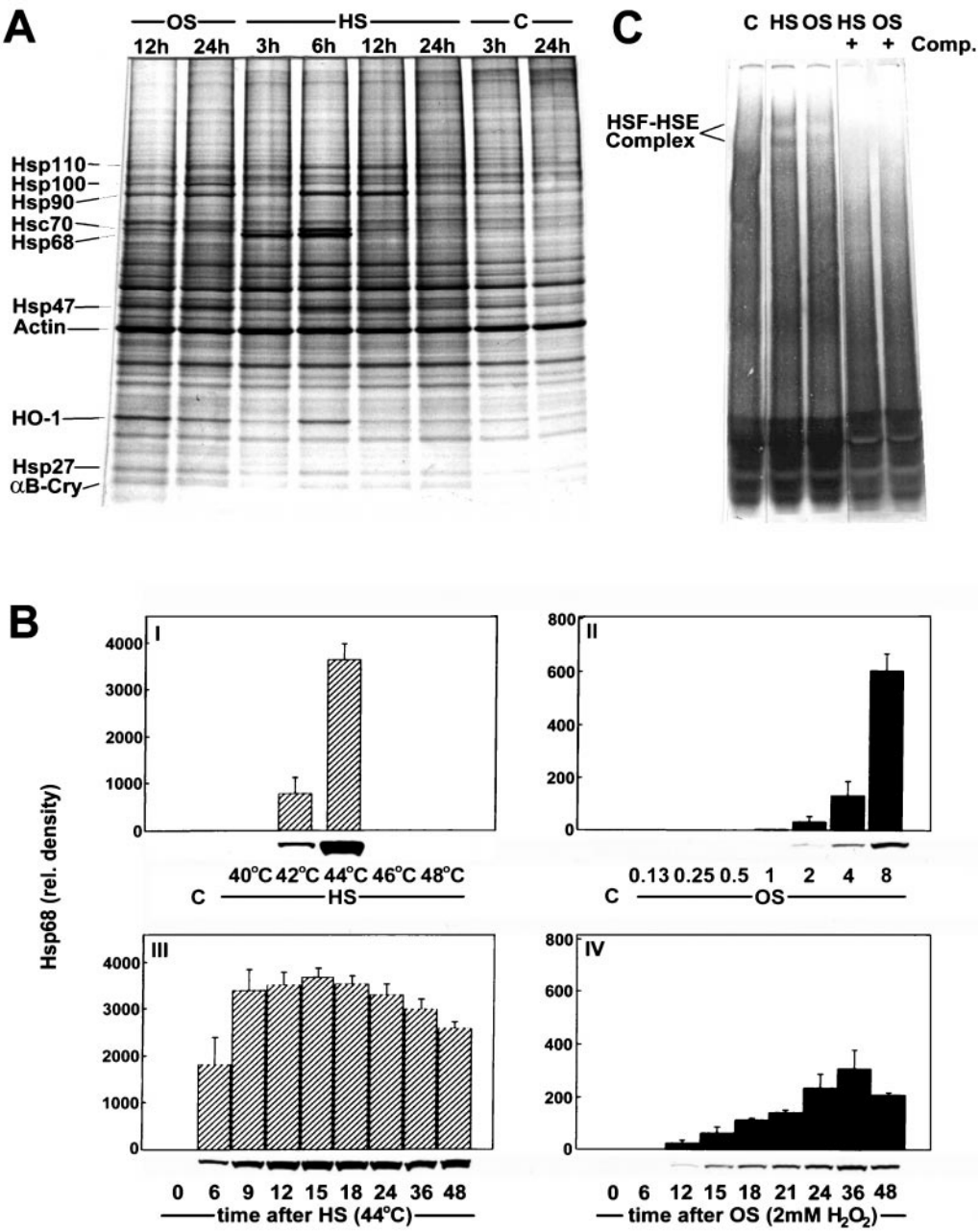

FIG. 1. **Induction of Hsp synthesis, level of Hsp68 and HSF activity by OS and HS.** *A,* synthesis rates of stress proteins after OS and HS. Cells were exposed to OS (2 mM H_2O_2, 1 h), HS (44 °C, 0.5 h) or remained untreated as controls. Cells were then labeled with [^{35}S]methionine during recovery for 2 h and harvested at the indicated recovery times. Equal amounts of labeled proteins (100,000 cpm) were separated by SDS-PAGE. In the case of oxidative stress, only cells which had recovered for 12 and 24 h are shown because of the strong inhibition of protein synthesis shortly after exposure. ^{35}S-Labeled proteins were then visualized by autoradiography. Positions of Hsps are indicated *B,* effects of HS and OS on Hsp68 accumulation. *I,* dose dependence of HS (different temperatures (°C) for 0.5 h, 12 h recovery); and *II,* of OS (different concentrations of H_2O_2 (mM) for 1 h, 18 h recovery). III, kinetics after HS (44 °C, 0.5 h); and *IV,* OS (2 mM H_2O_2, 1 h), followed by different recovery times. Equal amounts of protein (60 μg for OS or 30 μg for HS) were separated by SDS-PAGE for Western blot analysis. Means of at least three experiments ± S.E. Level of Hsp68 is expressed as relative densitometric units (*ordinates*). Representative blots are shown *below* the histograms. *C,* effect of HS (44 °C, 0.5 h) and OS (8 mM H_2O_2, 1 h) on HSF activity determined by electrophoretic mobility shift assay analysis. The HSF-HSE complex which migrates as a doublet is indicated.

synthesis was almost completely abolished and recovered to about 35% of the control level after 24 h. HS, in contrast, inhibited protein synthesis only briefly (3 h) during recovery (42% of the control). After 6 h, protein synthesis already reached control levels. The slight decrease of protein synthesis in the control cells during this time may be due to contact inhibition of confluent cells. These results demonstrate that inhibition of protein synthesis is also not a common signal for *hsp68* induction. The stronger inhibition by OS may, however, explain the delay of the induction after H_2O_2 exposure.

The level of lipid peroxidation was analyzed by the thiobarbituric acid assay. The thiobarbituric acid assay quantifies the products of lipid peroxidation such as malondialdehyde and related substances, designated as TBARS (47). The amount of lipid peroxidation induced by hydrogen peroxide increased in a concentration-dependent manner (Fig. 2*C*). A heat shock (44 °C) of 0.5 h did not enhance lipid peroxidation significantly. Longer exposure of the cells to HS (1 h) significantly increased lipid peroxidation ($p < 0.001$), however, much less compared with oxidative stress. The HS-induced level corresponds to a

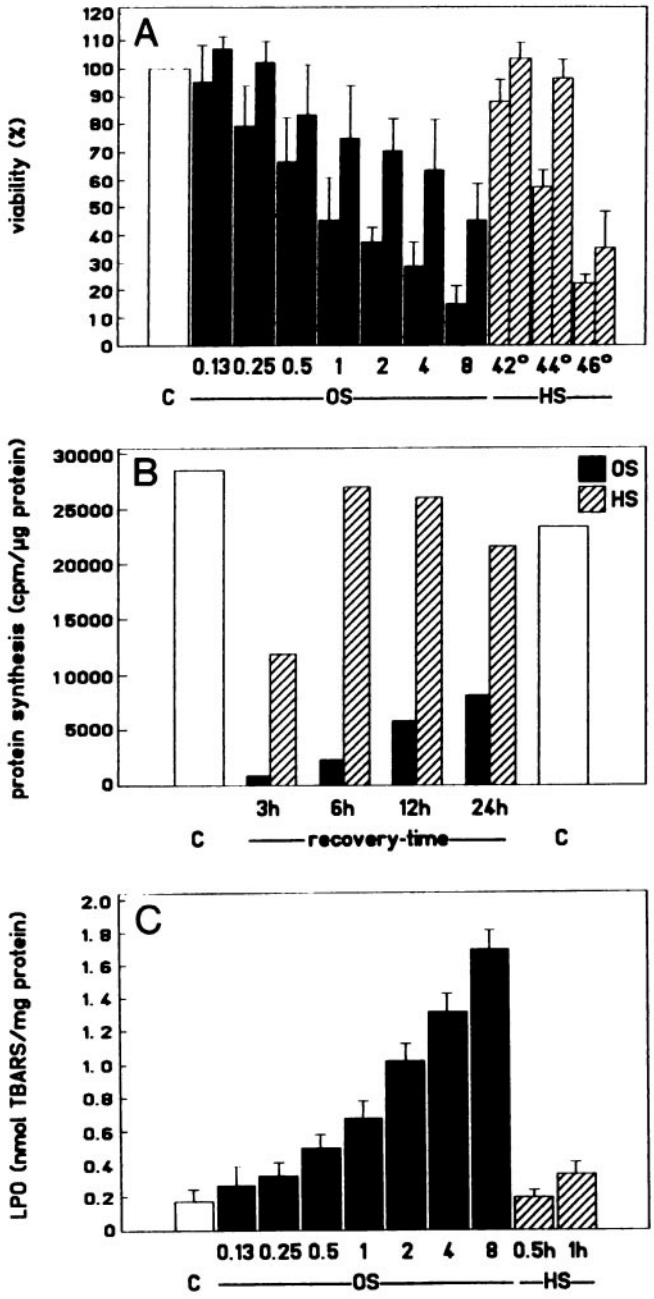

FIG. 2. **Cytotoxic effects of OS and HS.** *A,* cells were exposed to different OS (mM H_2O_2) for 1 h or different HS (°C) for 0.5 h. After 24 h recovery the viability was determined by the MTT assay (*left columns*) and the NR assay (*right columns*) and expressed in % of the control value (*ordinates*). Means of at least three independent experiments ± S.D. *B,* cells were treated with OS (2 mM H_2O_2, 1 h) or HS (44 °C, 0.5 h), labeled with [^{35}S]methionine (2 h) and harvested during recovery at the indicated times (*abscissa*). Protein synthesis rate is expressed as incorporated [^{35}S]methionine (cpm) per µg of protein (*ordinate*). Controls were analyzed at the beginning and end of the experiment (*white columns*). *C,* cells were exposed to different OS for 1 h or to HS (44 °C) for either 0.5 or 1 h. Lipid peroxidation was measured by the thiobarbituric acid assay directly after stress and expressed as thiobarbituric reactive substances (TBARS) per mg of protein. Means of five and seven experiments ± S.D. for OS and HS, respectively.

level reached after incubation with about 0.25 mM H_2O_2, which implies that HS generates reactive oxygen species only poorly. These results show that OS causes lipid peroxidation at low concentrations of H_2O_2 where no induction of *hsp68* is observed. HS, in contrast, causes little lipid peroxidation, but maximal induction. Hence, induction of *hsp68* is not or only little mediated by products of lipid peroxidation. The strong

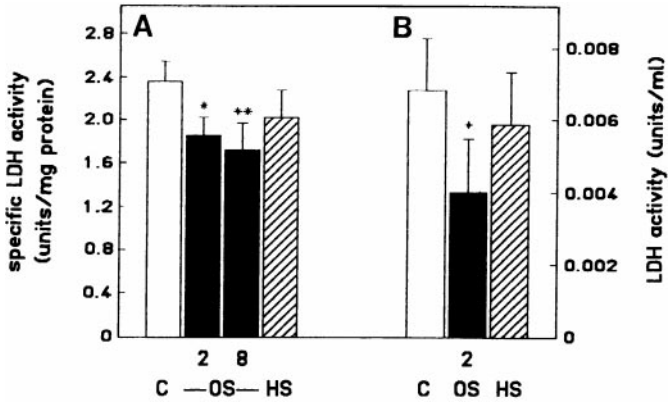

FIG. 3. **Effect of OS and HS on LDH activity in cells and *in vitro*.** *A,* cells were treated by OS (2 and 8 mM H_2O_2, 1 h), HS (44 °C, 0.5 h), or remained untreated as controls. LDH activity was determined directly after stress and expressed as specific activity in units per mg of protein. *B,* for *in vitro* experiments, solution of purchased LDH in TBS, pH 7.4, supplemented with 10 µM $FeSO_4$ was exposed to OS (2 mM H_2O_2) and HS (44 °C) for 1 or 0.5 h, respectively, then treated with 15,000 units/ml of catalase for 0.5 h. Thereafter the enzyme activity was measured as described under "Experimental Procedures." Means of four experiments ± S.D. (* or ** indicate significant differences with respect to the control with $p < 0.05$ and 0.01, respectively).

OS-induced lipid peroxidation may be particularly responsible for its strong cytotoxicity and deleterious effects on membranes which also result in mitochondrial damage as observed in electron micrographs.[3]

Effects of OS and HS on Protein Denaturation—It is generally believed that denatured proteins are involved in the induction of stress proteins (2). To get an estimate of the stress-induced denaturation of proteins by OS and HS we chose LDH for a detailed analysis. The degree of protein denaturation was determined indirectly by measuring enzyme activity by the common photometric assay as well as conformational changes by using the fluorophore 1,8-ANS which is highly sensitive toward hydrophobic surfaces (48).

First, we analyzed the changes of LDH activity of C6 cells in response to both stressors. Oxidative stress inhibited LDH activity stronger than heat shock (Fig. 3*A*). The inhibition of LDH activity by 2 mM H_2O_2 and 8 mM H_2O_2 was significant ($p < 0.05$ and $p < 0.01$), whereas the LDH activity was only slightly but not significantly suppressed by HS (44 °C). To exclude potential cellular regulatory mechanisms induced by OS or HS, experiments with purchased LDH were performed *in vitro* (Fig. 3*B*). Again, HS suppressed LDH activity slightly but not significantly, whereas oxidative stress (2 mM H_2O_2) decreased the activity of LDH remarkably ($p < 0.05$). The concentration of hydrogen peroxide, at which the level of inhibition is similiar to HS-induced inhibition of enzyme activity ranged between 0.25 to 0.5 mM H_2O_2 (data not shown). The latter concentrations, however, did not induce *hsp68*. The suppression of enzyme activity by OS seems to be due to the generation of hydroxyl radicals induced by the Fenton mechanisms, because the hydrogen peroxide-induced inhibition of LDH activity was clearly less effective without supplementation of 10 µM $FeSO_4$ (data not shown). These experiments show that there is also no correlation between *hsp68* induction by both stressors and their general inhibitory effect on enzyme activity which may be due to oxidative damage of the active site.

We therefore measured the exposure of hydrophobic domains of LDH by means of 1,8-ANS-fluorescence analysis. Oxidative stress caused increasing emission intensity compared with native LDH (control) in a dose-dependent manner starting from

[3] A. Gosslau, unpublished results.

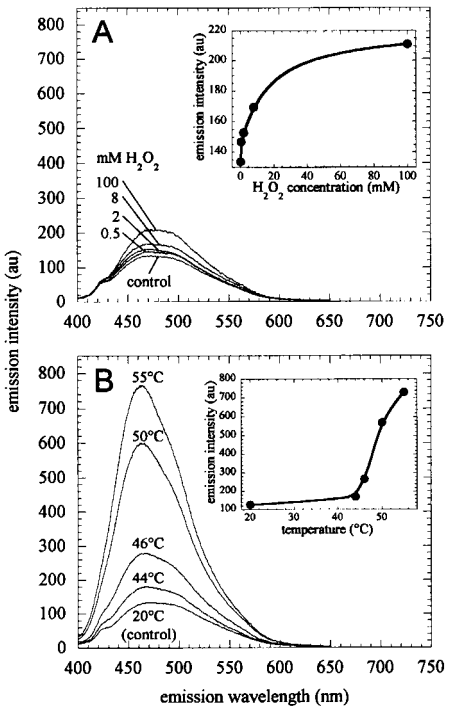

FIG. 4. ***In vitro* effect of OS and HS on exposure of hydrophobic domains of LDH.** A solution of purchased LDH in TBS, pH 7.4, was treated by: *A,* different OS (0.5–100 mM H_2O_2, 1 h); or *B,* different HS (44–55 °C, 0.5 h) or remained untreated as controls (20 °C). The degree of exposure of hydrophobic domains was determined by 1,8-ANS fluorescence analysis and expressed as emission intensity in arbitrary units (*ordinates*). *Insets* indicate dependence of OS (mM H_2O_2) and HS (°C) on maximal ANS-emission intensity excited at 380 nm.

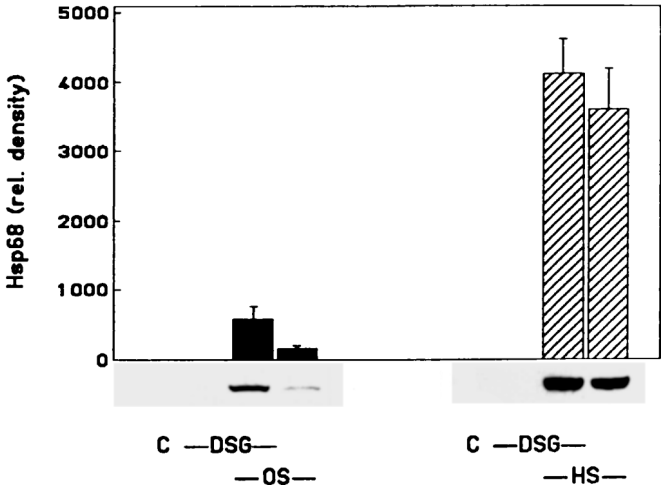

FIG. 5. **Effect of DSG on the level of Hsp68 after OS and HS.** Cells were pretreated with DSG (5 μg/ml) for 5 h followed by 18 h recovery (control), treatment by OS (2 mM H_2O_2 for 1 h, 18 h recovery), or by HS (44 °C for 0.5 h, 12 h recovery). Equal amounts of protein (60 μg (H_2O_2) or 30 μg (HS)) were separated by SDS-PAGE for Western blot analysis. Means of five experiments ± S.E. Level of Hsp68 is expressed as relative densitometric units (*ordinates*). Representative blots are shown *below* the histogram.

0.5 to 100 mM H_2O_2 (Fig. 4*A*). Heat shock from 44 to 55 °C showed increasing emission intensity which is stronger compared with OS (Fig. 4*B*). Since free ANS does not contribute significantly to the total fluorescence, the emission intensity is a reflection of bound ANS. Thus, OS and HS caused increased accessibility of the interior hydrophobic core of LDH. In contrast to the degree of LDH activity (Fig. 3), the effects of OS on the exposure of hydrophobic domains were less compared with HS. Noteworthy, 2 mM H_2O_2 caused lesser binding of 1,8-ANS compared with 44 °C as indicated by the lower emission intensity. Higher temperatures starting from 65 °C caused precipitation of LDH which resulted in lesser binding of ANS (data not shown). The *insets* in Fig. 5, *A* and *B,* demonstrate the dose dependence of OS and HS on maximal ANS binding as indicated by emission intensity. The temperature which induced strongest *hsp68* expression (around 44 °C) caused an emission intensity of about 180 arbitrary units. This corresponds to the effects of an OS of about 20 mM H_2O_2.

Effects of DSG on hsp68 Induction—The assumption that hydrophobic domains of proteins play an important role in *hsp68* induction may be related to their interactions with chaperones which subsequently relieve the negative feedback effects of Hsp70 on HSF activity as postulated by Morimoto and others (2). To test this hypothesis, we used the peptide DSG which shows strong affinity to stress proteins, specifically to Hsp70 and Hsp90 (50, 51). Cells were pretreated with DSG (5 μg/ml) for 5 h, exposed to HS (44 °C, 0.5 h) or OS (2 mM H_2O_2, 1 h) followed by 12 and 18 h recovery, respectively. Treatment of the cells with DSG either alone or before HS and OS did not affect cell viability as determined by the MTT assay (data not shown). DSG treatment before HS only slightly enhanced the level of Hsp68 (Fig. 5*A*). In contrast to HS, the effect of DSG before OS was drastically stronger as indicated by an almost 4-fold increase of Hsp68 after OS (Fig. 5*B*). One may speculate that OS (2 mM H_2O_2) causes a lower exposure of hydrophobic domains of proteins as demonstrated for LDH (Fig. 4) and thus a lesser binding of Hsps to these proteins, which causes a lower expression of *hsp68*. In this case DSG may bind to Hsps and attenuate their inhibition of the HSF. An HS of 44 °C, on the other hand, causes stronger hydrophobic interactions between denatured proteins and Hsps leaving little additional effect for DSG on the induction of *hsp68*.

DISCUSSION

Responses to HS and OS in C6 rat glioma cells were compared to test the hypothesis of a common signal involved in the induction pathway of stress proteins. Expression of *hsp68* was significantly stronger and faster induced by HS compared with OS as reported previously (34, 52, 53). In some previous studies no induction after OS was reported (9, 54, 55). In the present study, the lower induction of *hsp68* by OS correlated with a weaker HSF activity as described previously (5, 53, 55) which indicates that HSF-1 is the main regulator in the induction of *hsp68* by both stressors. This agrees with the observation that *hsp70* induction is independent of NFκB (56).

Besides autoregulatory (25, 27, 28), phosphorylation or redox-dependent (5, 32–34) mechanisms, HSF-HSE interaction seems to be controlled by a repressor, the so-called "constitutive HSE binding factor." The dissociation of constitutive HSE binding factor from the HSE is responsible for the transcriptional activity of HSF-1, *i.e.* transcription of *hsp68* (9, 57). This inhibitory action of the constitutive HSE binding factor may explain the enhanced HSF-1 binding activities by various stressors determined *in vitro* without an apparent transcription of stress genes *in vivo* (55, 57, 58). Recently, another protein was characterized (HSBP-1) which negatively affects HSF-1 activity (59). These repressors thus represent candidates whose inhibitory influence may prevent activation of heat shock gene expression after oxidative stress.

We found no correlation between cytotoxic effects of OS and HS and the degree of *hsp68* induction. OS-induced *hsp68* expression was observed only at high doses of hydrogen peroxide and at a degree of cytotoxicity which suppressed *hsp68* expression when caused by HS. Mitochondrial dehydrogenase activity was strongly inhibited by OS as indicated by the MTT assay

(44) which is consistent with a significant damage of mitochondria as demonstrated by electron microscopy.[3] The strong inhibition of protein synthesis by OS may be due to the impairment of the elongation factor-2 (EF-2) (60) as well as to a depletion of ATP (61). Inspite of the fact that translation of stress proteins seems to be less sensitive to stress compared with the synthesis of other proteins (2), the strong toxic effects of OS on the protein synthesis machinery may explain at least the delayed kinetics of *hsp68* expression. In addition, an earlier increase of Hsp68 may be prevented by unknown factors (for example, constitutive HSE binding factor, HSBP-1, or EF-2) which may inhibit the inducing signal or strengthen the influence of these inhibitory factors and thus limit or even close the window for *hsp68* induction. The OS-induced cytotoxicity correlated strongly with lipid peroxidation which causes perturbation of ion homeostasis (*e.g.* increase of intracellular Ca^{2+}) and subsequently activates Ca^{2+}-dependent phospholipases, proteases, endonucleases, NO synthetase, and xanthin oxidase (39, 62).

Denatured proteins were hypothesized as signal for HSF-1 activation by means of their binding and sequestering of Hsp70 which is assumed to act as inhibitor of HSF-1 (2). A first analysis of lactic dehydrogenase activity which may serve as indicator of cytosolic protein denaturation revealed a stronger suppression by OS compared with HS which may be due to oxidative damage of the active site. In more specific experiments, however, we observed stronger exposure of hydrophobic domains in response to HS (44 °C) compared with OS (up to 8 mM H_2O_2) as demonstrated by ANS fluorescence analysis. Binding of ANS identifies molten globule intermediate states which are converted from the native state under denaturating conditions (48). The fairly compact molten globules display significant native-like secondary structures, but increased exposure of hydrophobic surfaces and a lack of rigid tertiary structure compared with the native protein (49, 63). Consistent with our results it was postulated recently that the molten globule intermediate, which is prone to aggregate may represent the critical parameter for the heat shock response (64). While HS may cause a direct conversion to a molten globule state (65), OS may induce protein destabilization due to fragmentation and formation of non-native disulfide bonds which secondarily result in a transition to molten globule intermediates (63, 66–68).

LDH hydrophobicity caused by HS (44 °C) corresponded to an exposure of hydrophobic domains caused by OS of about 20 mM hydrogen peroxide which does not induce *hsp68* due to its letal effects on cells. We hypothesize that hydrogen peroxide on the one hand interrupts the negative feedback control of Hsps on HSF activation due to the increase of hydrophobic domains of cellular proteins. On the other hand, hydrogen peroxide may strongly suppress phosphorylation- or redox-dependent HSF activation due to its strong molecular damaging capacity. In response to low hydrogen peroxide concentrations which hardly causes exposure of hydrophobic domains, induction of *hsp68* may be increased by products of the lipoxygenase pathway (29). As hydrophobicity of LDH increases dose dependently up to high temperatures, the question arises why *hsp68* induction by HS is sharply abrogated already by relative nontoxic temperatures (*e.g.* between 44 and 46 °C), a question that we cannot answer at present.

Deoxyspergualin which exhibits strong affinity specifically to Hsp70 and Hsp90 (50, 51) drastically increased only OS-induced *hsp68* expression in contrast to HS. An HS of 44 °C may saturate the binding of constitutive Hsps such as Hsp70 and Hsp90 to cytosolic proteins due to strong exposure of hydrophobic domains (69, 70). In response to OS, on the other hand, a lesser exposure of hydrophobic domains may be responsible for a lesser binding of these domains to Hsps which may be compensated by DSG.

Our results support the assumption of a common signal as trigger for the induction pathway of *hsp68* by HS and OS. Specific hydrophobic interactions between molten globule-like cytosolic proteins and Hsp70 and Hsp90 may interrupt their negative feedback on HSF (19, 20). HSF should then convert to a trimer which represents probably a primary step in the activation of the *hsp68* gene. Specific phosphorylation- or redox-dependent modifications of the HSF and other transcription factors by HS or OS may be responsible for their different inducing effects on *hsp68*.

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