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Time Course of 72-Kilodalton Heat Shock Protein Induction and Appearance of Trifluoroacetyl Adducts in Livers of Halothane-Exposed Rats

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SUMMARY

Previous studies have shown that exposure of phenobarbital-pretreated rats to halothane in 10% O_2 causes centrilobular necrosis, induces expression of the 72-kDa heat shock protein (HSP72), and produces several trifluoroacetylated adducts. In the present study the time course of development of the centrilobular lesion, as measured by histochemistry, was compared with the time course of appearance of both trifluoroacetylated adducts and HSP72, as measured by Western blotting. One group of 20 rats was pretreated with phenobarbital for 5 days, whereas a second group of two rats was left as untreated controls. Ten phenobarbital-pretreated rats were exposed for 2 hr to 1% halothane in 10% O_2 and 10 were exposed to 1% halothane in 20% O_2. At either 2, 4, 6, or 24 hr after exposure, livers were excised and frozen without fixation. Thin sections stained with hematoxylin and eosin demonstrated that centrilobular lesions occurred at 6 hr and became extensive at 24 hr in rats pretreated with phenobarbital and exposed to 1% halothane in 10% O_2. The time course of appearance of both trifluoroacetylated adducts and HSP72 was determined by Western blotting. Trifluoroacetylated adducts appeared in all rats exposed to halothane by 2 hr, lasted until 6 hr, and then diminished by 24 hr. In contrast, HSP72 was induced only in the rats pretreated with phenobarbital and exposed to 1% halothane in 10% O_2. HSP72 appeared in both the nuclear and supernatant fractions at 6 hr after exposure and was intense 24 hr after exposure.

The studies reported here are part of our ongoing effort to understand the role of heat shock proteins in the etiology of halothane hepatitis. The heat shock proteins are of interest in xenobiotic-induced liver injury because they comprise a family of proteins that are induced after physiologically stressful treatments, such as heat, oxidative stress, and xenobiotic exposure. These proteins are considered to be a part of cellular defense systems and, as such, are involved in folding and refolding proteins, in chaperoning proteins, and in cellular immunity (1-3). The HSP70 family is one of the major families of heat shock proteins; it includes both constitutive (HSP73) and inducible (HSP72) proteins.

The rat model of halothane hepatitis requires administration of the inhalation anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), in a hypoxic gas mixture of 10% oxygen, to rats that have been pretreated with phenobarbital (4-7). This treatment produces a centrilobular lesion that is characteristic of halothane hepatitis. The lesion involves three to 12 rows of cells that surround the central vein and show a range of abnormalities ranging from reversible vacuolization to necrosis. There is already considerable evidence that heat shock proteins are induced during this treatment. Recent immunocytochemical studies demonstrated that one of the major heat shock proteins, HSP72, was induced in livers of rats that were pretreated with phenobarbital and then exposed to halothane in a hypoxic gas mixture. The necrotic lesion, as determined by histological staining, and the distribution of binding of a commercial mouse anti-HSP72 IgG monoclonal antibody, as determined by immunocytochemistry, were superimposable (4). In addition, we have recently used both Western blotting and immunocytochemistry to show that 24 hr after this treatment HSP72 is induced and translocated to the nucleus (8).

In view of the role of heat shock proteins in both refolding damaged proteins (1, 2) and chaperoning irreparably damaged proteins to the lysosomes for destruction (9, 10), we hypothesized that heat shock proteins may be expressed soon after the stress of halothane exposure under hypoxic conditions and may ameliorate the possible damage. To test this hypothesis, the time course of induction of HSP72 was compared with the time course of the appearance of TFA adducts as well as the progression of the histological lesion. Rats were pretreated with

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ABBREVIATIONS: HSP70, 70-kDa heat shock protein(s); HSP72, 72-kDa heat shock protein; HSP73, 73-kDa heat shock protein; PBS, phosphate-buffered saline; RSA, rabbit serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetyl(ated).

phenobarbital and exposed to halothane in either hypoxic or normoxic gas mixtures. HSP72 and TFA adducts were determined at 2, 4, 6, and 24 hr after exposure. Nuclear and supernatant fractions were isolated and then HSP72 and TFA adducts were measured by PAGE, followed by Western blotting. The HSP72 was determined with a mouse anti-HSP70 IgG monoclonal antibody and the TFA adducts were determined with a rabbit polyclonal IgG antibody made monospecific against *N*-ε-TFA-L-lysine.

Experimental Procedures

Materials. Mouse anti-HSP70 IgG1 monoclonal antibodies (clone BRM-22) that bind both HSP72 and HSP73 and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (whole molecule, absorbed with rat serum proteins) were from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy plus light chains) was from Bio-Rad (Richmond, CA).

Treatment of animals. Male Sprague-Dawley rats (200–250 g) were used. The experimental protocol was approved by the Henry Ford Health System Panel for Animal Studies. One set of two rats served as untreated controls. A group of 20 rats was given phenobarbital in the drinking water (0.2%, w/v) for 5 days and returned to untreated drinking water for 24 hr before the experiment. In a Plexiglas chamber, 10 phenobarbital-pretreated rats were exposed to 1% halothane in 20% O_2 and then 10 rats were exposed to 1% halothane in 10% O_2. The rats were then returned to their cages for 2 ($n = 2$), 4 ($n = 3$), 6 ($n = 3$), or 24 ($n = 2$) hr. These time points were selected because 1) in preliminary experiments HSP72 expression was high at 6 hr, 2) in previous studies the TFA adducts appeared at 2–6 hr (5, 11), and 3) in previous studies the centrilobular lesion was well developed at 24 hr (7). At each time point rats from both exposure groups were anesthetized with sodium pentothal (50 mg/kg, intraperitoneally) and then treated with heparin intravenously. The livers were perfused through the inferior vena cava with PBS (20 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4), excised, and then cut into cube-shaped samples. The samples were immediately placed in an isopropanol-dry ice slurry and then stored in a −70° freezer. The entire experiment was replicated with essentially identical results.

Histochemical staining. Thin sections (9 μm) were prepared from the frozen livers with a refrigerated microtome. They were placed on a microscope slide, stained with hematoxylin and eosin, and mounted with Glycergel. Photomicrographs of the centrilobular regions were made with a Nikon 20× objective (Tokyo, Japan) on a Nikon Diaphot microscope.

Isolation of supernatant and nuclear proteins. A frozen piece of each liver sample (100 mg) was homogenized with five strokes of a ground glass mortar and pestle (Kontes) in 2 ml of lysing buffer (50 mM Tris, 25 mM KCl, 1.5 mM $MgCl_2$, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). The homogenates were incubated on ice for 10 min and then EDTA was added to a final concentration of 10 mM. Nuclear pellets were prepared by centrifugation at 1300 × *g* for 5 min. The supernatants were collected and designated as supernatant fractions. The pellets were resuspended in 1 ml of lysing buffer and centrifuged again under the same conditions. These supernatants were discarded and the nuclear pellets were suspended in 1.2 ml of nuclear lysing buffer (1% Triton X-100, 0.1 M Tris, 10 mM EDTA, pH 7.4), by vortex mixing for 10 sec.

Production of hapten-specific IgG antibodies. *N*-TFA-RSA was prepared and injected into rabbits as described previously (12, 13). RSA (20 mg) was coupled to 6 ml of Affi-Gel 10 (Bio-Rad) to produce an affinity column that would remove nonselective but strongly binding antibodies. The serum (100 ml) from the sensitized rabbits was precipitated with 40% $(NH_4)_2SO_4$, dialyzed against 20 mM Tris, pH 7.4, and then passed through the RSA-Affi-Gel 10 column for 12 hr. Antibodies that bound to this affinity column were eluted with 3 M $MgCl_2$ and saved for use as a control for nonspecific binding (anti-RSA IgG). IgG antibodies specific for TFA-RSA were prepared by binding the IgG fraction that had flowed through the RSA-Affi-Gel 10 column to a TFA-RSA-Affi-Gel 10 affinity column and eluting the anti-TFA-RSA IgG antibodies with 3 M $MgCl_2$. A third affinity column was prepared by coupling 20 mg of *N*-ε-TFA-L-lysine (Senn Pharmaceuticals, Dielsdorf, Switzerland), dissolved in 10 ml of dimethylsulfoxide, to 6 ml of Affi-Gel 10 for 20 hr at 20°. The anti-TFA-RSA IgG antibodies eluted from the second affinity column were applied to this third TFA-lysine-AffiGel 10 affinity column. The third column was eluted with 3 M $MgCl_2$ to produce a fraction of antibodies specific for *N*-ε-TFA-L-lysine (anti-TFA-lysine IgG). A purified sample of IgG antibodies from sera of the same rabbits before immunization (preimmune IgG) was prepared (13) to use as a control for nonspecific binding.

PAGE. The protein concentration of each lysate fraction was determined with bicinchoninic acid reagent (Pierce, Rockford, IL), and an aliquot containing 45 μg of protein was mixed with 8 μl of sampling buffer (10% of glycerol; 12.5% of 0.5 M Tris HCl, pH 6.8%; 20% of 10%, w/v, SDS; 5% of 2-mercaptoethanol; and 2.5% of 0.05%, w/v, bromophenol blue) (14). Each mixture was heated in a boiling water bath for 3 min. An aliquot (30 μg) for detection of HSP70 was loaded into a well of a 14- × 14-cm 10% tricine-SDS-PAGE gel prepared by a modification of the procedure of Schagger and von Jagow (15). In this modified procedure, only a 4% acrylamide mixture was used for the stacking gel and a 10% acrylamide mixture was used for the separating gel. A second aliquot (15 μg) for detection of TFA adducts was applied to a well of a mini-10% tricine-SDS-PAGE gel (Bio-Rad, Richmond, CA).

Western blotting. After PAGE the proteins were transferred to nitrocellulose by Western blotting (16) in a Bio-Rad mini-apparatus (Bio-Rad). After transfer the nitrocellulose membranes were blocked with either 2% BSA in PBS for 1 hr (HSP70) or 5% nonfat milk in PBS for 1 hr (TFA adducts). One set of membranes was incubated overnight with a 1/2000 dilution of mouse monoclonal anti-HSP70 IgG1 antibodies in PBS containing 1% BSA and 0.1% sodium azide. The membranes were washed in PBS containing 1% BSA and then incubated for 1 hr with a 1/10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibodies in PBS containing 1% BSA. These membranes were washed and incubated with alkaline phosphatase substrate (Sigma). The second set of identical nitrocellulose membranes was incubated overnight with 5 μg/ml anti-TFA-lysine IgG antibodies in PBS containing 1% BSA and 0.05% sodium azide. These membranes were washed in PBS containing 1% BSA and then incubated for 1 hr with a 1/2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies in PBS containing 1% BSA. This set of membranes were then washed and incubated with 18 mg of 3,3′-diaminobenzidine dissolved in 30 ml of 0.1 M Tris·HCl, pH 7.5, containing 30 μl of 30% H_2O_2. These membranes were scanned at a resolution of 203 pixels/inch. The resulting digital images were corrected for contrast (Fig. 1, A, C, and E) with the autocontrast feature of Photoshop (Adobe, Sunnyvale, CA). To discern faint bands of HSP72, the same digital images were converted to high-contrast images (Fig. 1, B and D).

Staining of nuclear and supernatant fractions with Commassie Blue. An additional experiment was performed to demonstrate that the proteins in the nuclear fraction were different from those in the supernatant fractions. Both the nuclear and supernatant fractions of a liver homogenate from a control rat were separated by PAGE as described above and then the gel was stained with Commassie Blue to detect the protein bands. The resulting stained gel was photographed while wet.

Results

Western blotting of heat shock proteins. HSP72 was induced in both the supernatant (Fig. 1, A and B) and nuclear (Fig. 1, C and D) fractions from only those rats exposed to halothane under hypoxia after phenobarbital pretreatment. In each group, levels of the constitutive HSP73 remained constant in both the nuclear and supernatant fractions. In Fig. 1, B and

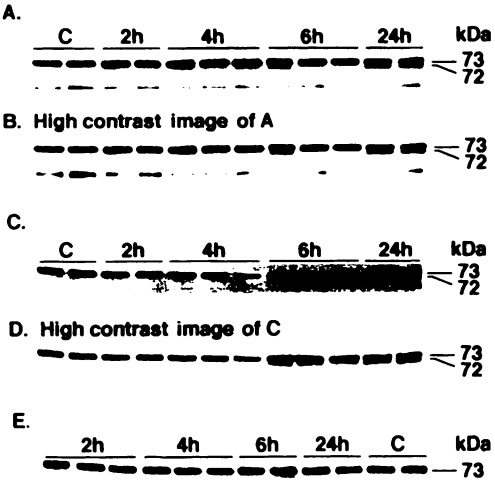

Fig. 1. Time course of HSP72 induction. Proteins were separated on 14- × 14-cm 10% tricine-SDS-PAGE gels and transferred to nitrocellulose membranes by Western blotting. The membranes were incubated with mouse anti-HSP70 IgG1 monoclonal antibodies and then visualized with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies. The staining of the bands was scanned into a digital format, and digital images of normal (A, C, and E) or equally enhanced (B and D) contrast were prepared using Adobe Photoshop. A, Supernatant fractions. *Lanes under C*, controls; *lanes under 2h*, rats pretreated with phenobarbital, exposed to 1% halothane in 10% O_2, and then returned to the cage for 2 hr before preparation of liver slices; *lanes under 4h*, as in the lanes under 2h but kept in the cage for 4 hr after treatment; *lanes under 6h*, as in the lanes under 2h but kept in the cage for 6 hr after treatment; *lanes under 24th*, as in the lanes under 2h but kept in the cage for 24 hr after treatment. B, Same digital image as in A, increased in contrast to reveal expression of HSP72 at 6 hr. C, Nuclear fractions, with the same lane labels as in A. D, Same digital image as in C, increased in contrast to reveal expression of HSP72 at 6 hr. E, Combined supernatant and nuclear fractions of normoxic rats. *Lanes under 2h*, rats pretreated with phenobarbital, exposed to 1% halothane in 20% O_2, and returned to the cage for 2 hr; *lanes under 4h*, as in the lanes under 2h but kept in the cage for 4 hr after treatment; *lanes under 6h*, as in the lanes under 2h but kept in the cage for 6 hr after treatment; *lanes under 24h*, as in the lanes under 2h but kept in the cage for 24 hr after treatment; *lanes under C*, control.

D, the contrast of the corresponding digital images in Fig. 1, A and C, was enhanced equally. Under these conditions, HSP72 was detectable in the nuclear fractions from all three rats sacrificed at 6 hr, whereas it appeared in the supernatant fraction of only one of these rats. Strong expression of HSP72 was observed in the supernatant fractions of both rats sacrificed 24 hr after exposure.

In the identical sets of rats pretreated with phenobarbital and exposed to 1% halothane in 20% O_2, HSP72 was not induced at any time point (Fig. 1E). Because HSP72 was not observed in either the nuclear or supernatant fractions, even under conditions of enhanced contrast as in Fig. 1, B and D, a Western blot of the combined fractions is shown in Fig. 1E.

Western blotting of TFA adducts. In sharp contrast to the delayed induction of HSP72, TFA adducts were observed 2 hr after exposure to 1% halothane in 10% O_2 and lasted for at least 6 hr (Fig. 2). The adducts were then cleared rapidly and virtually no TFA adducts were detectable 24 hr after exposure. These TFA adducts were present in both supernatant and nuclear fractions; two major proteins in the supernatant and one major protein in the nuclear fraction were trifluoroacetylated.

In contrast to the lack of HSP72 induction under normoxia, more TFA adducts were formed under the normoxic conditions

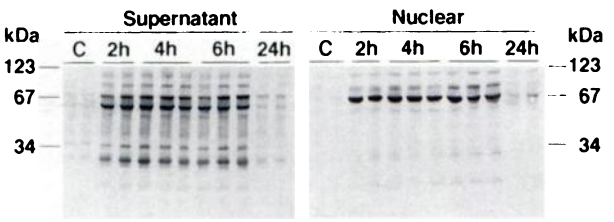

Fig. 2. Time course of formation of TFA adducts. Supernatant fractions and nuclear fractions were separated on a mini-10% tricine-SDS-PAGE gel and transferred to nitrocellulose membranes by Western blotting. The membranes were incubated with anti-TFA-lysine IgG antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies. The antibodies were visualized with 3,3′-diaminobenzidine and digital images were prepared as in Fig. 1. *Lanes under C*, controls; *lanes under 2h*, rats pretreated with phenobarbital, exposed to 1% halothane in 10% O_2, and then returned to the cage for 2 hr before preparation of liver slices; *lanes under 4h*, as in the lanes under 2h but kept in the cage for 4 hr after treatment; *lanes under 6h*, as in the lanes under 2h but kept in the cage for 6 hr after treatment; *lanes under 24h*, as in the lanes under 2h but kept in the cage for 24 hr after treatment.

(data not shown), as expected from the results of previous studies (6, 17). Under normoxia, TFA adducts were formed at the same time as under hypoxia but were cleared more slowly. There was a small amount of some persistent TFA adducts still present in equal amounts in both supernatant and nuclear fractions 24 hr after exposure.

Histochemical staining. Thin slices from the frozen livers of all of the rats were stained with hematoxylin and eosin; representative photomicrographs are shown in Fig. 3. The slices from a control rat (Fig. 3, *upper panel*) were essentially identical to slices from rats pretreated with phenobarbital and then sacrificed after 24 hr, as well as slices from rats pretreated with phenobarbital, exposed to 1% halothane in 20% oxygen, and sacrificed after 24 hr. In contrast, a slice from a rat pretreated with phenobarbital, exposed to 1% halothane in 10% oxygen, and sacrificed after 24 hr (Fig. 3, *lower panel*) showed extensive centrilobular damage. Only very minor alterations were observed in rats given the latter treatment and sacrificed at 6 hr. These findings are consistent with many other published studies that have shown that the centrilobular lesion appears about 24 hr after exposure and either resolves or results in death of the animal in 2–3 days (4, 5).

Commassie Blue staining of PAGE gels. The nuclear and supernatant fractions used in the Western blot analysis were prepared by a single centrifugation step. This division into two fractions was based on our previous studies in which nuclear translocation of HSP72 was observed by immunocytochemistry (8). In those studies the binding of gold-conjugated antibodies corresponded to the location of the nucleoli. The rapid isolation procedure was chosen to avoid the known problems of proteolysis and autoproteolysis (18, 19) of heat shock proteins. However, the simple preparation of a nuclear pellet could result in some contamination of the pellet with supernatant proteins and vice versa. To demonstrate that this possible cross-contamination did not lead to the results observed in Fig. 1, A and C, a PAGE gel of the nuclear and supernatant fractions was prepared and stained with Commassie Blue (Fig. 4). It is clear that the two fractions contain distinct sets of proteins; in each case there are bands that appear in one of the lanes but not in the other. This result is corroborated by the results of the enhanced-contrast images (Fig. 1, B and D), in which at 6 hr there was expression of HSP72 in the supernatant fraction

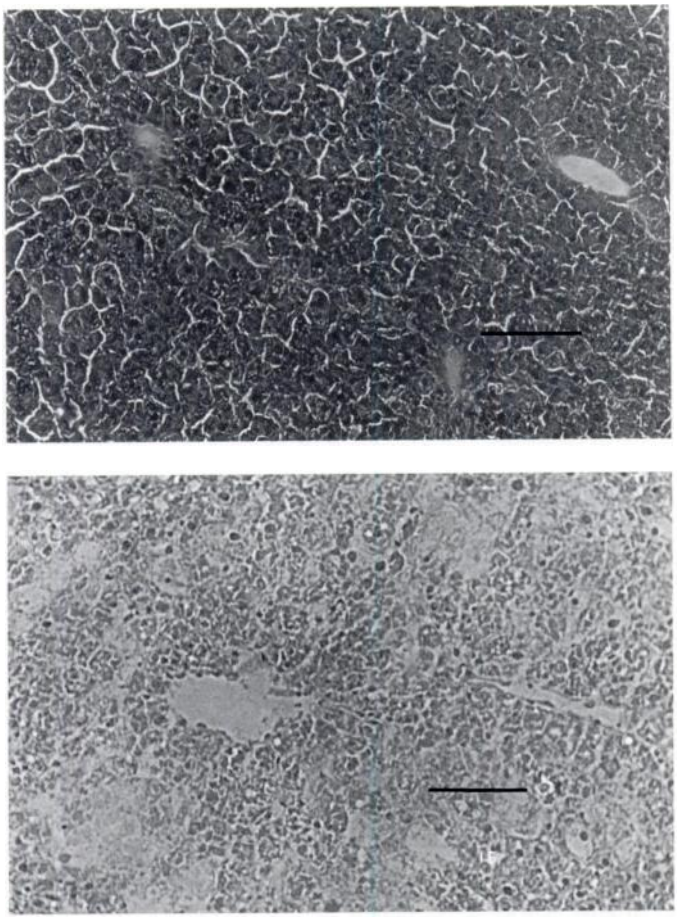

Fig. 3. Photomicrographs of 9-μm slices of liver stained with hematoxylin and eosin. The slices are from a control rat (*upper*) and from a rat pretreated with phenobarbital, exposed to 1% halothane in 10% oxygen, and sacrificed after 24 hr (*lower*). Only the latter slice shows extensive centrilobular damage. *Scale bar*, 100 μm.

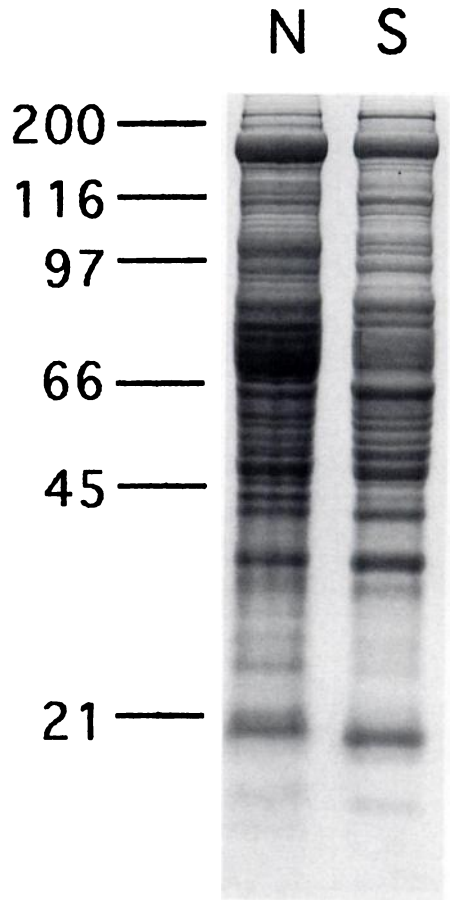

Fig. 4. Equal amounts of protein from the nuclear (*lane N*) and supernatant (*lane S*) fractions were separated on a PAGE gel and the protein bands were visualized with Commassie Blue staining. *Numbers to the left*, molecular mass markers (in kDa) were separated in an adjacent lane.

from only one of the rats but there was expression in the nuclear fraction from all three rats.

Discussion

In this study the time course of the development of centrilobular lesions was compared with that of the formation of TFA adducts and the expression of HSP72. The centrilobular lesions were observed in livers from only those rats that had been pretreated with phenobarbital and exposed to halothane in 10% oxygen (Fig. 3, *lower panel*). This result is consistent with many previous studies in which a centrilobular lesion was first observed at 6 hr, damage was extensive at 24 hr, and early stages of resolution were evident by 48 hr (4, 5, 7, 20). TFA adducts appeared immediately and then diminished, such that they were nearly gone at 24 hr (Fig. 2). This time course is consistent with a body of evidence from radiolabeling (21) and autoradiography experiments (11), as well as with results of Western blotting with antibodies specific for TFA adducts (22). The new data provided by the present study are that HSP72 induction begins at 6 hr and is very strong at 24 hr in only those rats that had been pretreated with phenobarbital and exposed to halothane in 10% oxygen (Fig. 1). This finding is complementary to a previous study in which we showed that neither phenobarbital pretreatment alone nor phenobarbital pretreatment plus hypoxia induced HSP72 24 hr after exposure (8). The finding that only the treatment that resulted in centrilobular lesions caused expression of HSP72 is also consistent with a previous immunocytochemistry study in which we showed that the pattern of anti-HSP72 antibody labeling is superimposed on the cells involved in the centrilobular lesions (4).

At 6 hr after exposure HSP72 was detected in the nuclear fraction of all three rats that had been pretreated with phenobarbital and exposed to halothane in 10% oxygen but in the supernatant fraction of only one of the same rats (Fig. 1). HSP72 was expected in the nuclear fraction because of our previous immunocytochemistry study that showed a strong localization of anti-HSP72 antibodies in the nucleoli at 24 hr after exposure (8). However, the time course of this induction has not been described and it is of interest that the expressed proteins are first seen in the nuclear fraction. It is seen that no HSP72 is detectable in either nuclear or supernatant fractions at 4 hr, even in the enhanced-contrast images in Fig. 1, B and D.

It is of interest that the time courses of appearance and disappearance of TFA adducts in supernatant and nuclear fractions were nearly the same, even though different proteins were labeled in the two fractions. Because the reactive TFA chloride is formed by cytochrome P450 2E1 in the endoplasmic reticulum (23), it would be expected that the first TFA adducts would be formed there. On the other hand, the cytosol contains

the lysosomes as well as the highest levels of HSP72 and ubiquitin (9, 10). On this basis it would be expected that degradation of TFA adducts would be more rapid in the cytosol. Although differences in turnover rates may be revealed by future studies with higher time resolution, it appears that either the formation of halothane adducts of nuclear proteins (24) or their exchange from the cytoplasm into the nucleus is rather rapid. Although a single centrifugation step was used to separate the fractions (to avoid degradation), it was sufficient to allow examination of the time course of adduct formation in the two fractions. The protein band at approximately 65 kDa in Fig. 2 was detected only in the cytoplasm and serves as a good marker for the disappearance of the TFA adducts by 24 hr. The conclusion that the separation of fractions was adequate to follow the time course is substantiated by the clear differences in protein bands on a PAGE gel stained with Commassie Blue (Fig. 4) and by the differences detected between the nuclear and cytoplasmic fractions in Fig. 1.

It would appear that the amount of TFA adducts formed was unrelated to the expression of HSP72, because HSP72 was not expressed under normoxic conditions but even more TFA adducts were formed under normoxia than under hypoxia. There are several possible explanations for the requirement of both halothane metabolism and hypoxia for expression of HSP72. It may be that the two stresses are additive and, in liver, neither one is sufficient to cause expression of HSP72 alone. For example, although hypoxia alone did not cause expression of HSP72 in rat liver (8), transient focal cerebral ischemia caused its expression in rat brain (25). It would be interesting to repeat these studies in guinea pigs, in which hypoxia is not required to produce halothane hepatitis (26). Another possible explanation for the requirement for halothane metabolism and hypoxia in the rat model is that the 1-chloro-2,2,2-trifluoroethyl radicals formed during reductive metabolism of halothane (27, 28) are somehow responsible for the expression of HSP72. Several studies have suggested that expression of another important protein, p53, is induced by either radiation or free radical damage (29, 30). Either of these explanations would be consistent with the previous immunocytochemical result that showed preferential centrilobular binding of anti-HSP72 antibodies (4); the centrilobular region becomes the most hypoxic (31) and the most 1-chloro-2,2,2-trifluoroethyl radicals are produced under hypoxic conditions (27, 28).

This study did not support the hypothesis that heat shock proteins were expressed soon after exposure to halothane in a hypoxic gas mixture. As a result, this study provides no evidence that they could have ameliorated the damage from the exposure by refolding damaged proteins and chaperoning irreparable proteins to the lysosomes. On the other hand, the study revealed a temporal association between the appearance of centrilobular lesions and expression of HSP72. Both events required hypoxia and their time courses were very different from those of the TFA adducts. These results may lead to future studies that will address the role of heat shock proteins in liver damage.

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