Heat Shock Protein Induction in Murine Liver After Acute Treatment With Cocaine

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The effect of cocaine on heat shock protein (hsp) induction in murine liver was examined using Western blotting and immunohistochemistry. A single dose of cocaine (50 mg/kg, intraperitoneal [i.p.]) was administered to naive, phenobarbital (PB)-induced or β-naphthoflavone (βNF)-induced mice, and the level of hsps in the liver analyzed 3, 6, and 24 hours after the cocaine dose. As measured by Western blotting, hsp70i levels were increased at all time points, and hsp25 levels at the 6- and 24-hour time points. Levels of hsp60, hsc70, and hsp90 remained unchanged. Pretreatment of mice with the cytochrome P-450 inhibitor SKF-525A eliminated both cocaine hepatotoxicity and the induced accumulation of hsp25 and hsp70i. Immunohistochemical localization of hsp25 and hsp70i in the liver showed that concentrations of both hsps were elevated only in cells with altered morphology. As has been observed previously, hepatic enzyme induction with PB or βNF shifted the location of the necrotic lesion within the lobule from zone 2, as observed in naive mice of this strain, toward zone 1 (PB) or zone 3 (βNF), respectively. Localization of induced accumulation of hsp25 and hsp70i was found to shift within the lobule in parallel with the necrotic lesion in these animals. Immunostaining of cocaine reactive metabolites bound to proteins was superimposable on the areas with hsp accumulation and cells with altered morphology. Our observations indicate a strong spatial correlation within the lobule between cocaine reactive metabolite formation, induced accumulation of hsp25 and hsp70i, and cytotoxicity (necrosis). (HEPATOLOGY 1997;25:1147-1153.)

Heat shock proteins (hsps) are ubiquitous in nature and can be grouped into a number of distinct families based on size and amino acid sequence relationships. Most widely recognized are the hsp90, hsp70, hsp60, and hsp25 families. Several families include members that are constitutively expressed as well as members whose synthesis is induced by heat stress and by a variety of other adverse stimuli.[1-4] In the liver of naive mice, for example, hsp90, hsc70, and hsp60 are expressed constitutively at appreciable levels. Levels of hsp70i are relatively low, and hsp25 is virtually undetectable.[5-7] In animals subjected to heat stress, levels of hsp70i and hsp25 are increased dramatically.[8-9]

A number of observations suggest that stress induction of hsp synthesis is mediated by the presence of non-native proteins. For example, overexpression of mammalian proteins unable to fold properly causes activation of hsp genes in bacteria,[10] and the injection of chemically-denatured proteins into vertebrate cells increases stress protein synthesis, whereas injection of the corresponding native protein does not.[11] Proteins of the hsp70 family are known to be capable of binding non-native proteins[12-13] and are thought to chaperone their refolding or elimination. During this binding, the heat shock transcription factor normally bound to hsp70 is released, whereupon it rapidly oligomerizes to become transcriptionally active.[14-16]

Many hepatotoxicants are bioactivated to reactive intermediates that bind cellular macromolecules including proteins.[17-18] It is conceivable that adducted proteins resulting from reactive metabolite binding are physiologically equivalent to proteins unfolded as a consequence of exposure to classical inducers of the heat stress response (e.g., heat, heavy metals, and so on), thereby activating hsp gene expression. Limited studies have suggested that hepatotoxicants forming reactive metabolites may increase the expression of hsps,[19] but there has been little direct examination of the relationship between hepatic reactive metabolite formation and hsp gene activation.

This report describes the results of a study of hsp induction in mice treated with hepatotoxic doses of cocaine. Cocaine, like a number of other hepatotoxicants, is metabolized to a reactive metabolite that binds to proteins.[20] The role of adducted proteins in the cytotoxicity of cocaine in the liver is unclear, but previous studies have shown that reactive metabolite binding occurs only in the region of the lobule where necrosis subsequently develops—zone 2 in naive ICR mice.[21] Unlike other classical hepatotoxicants, the location of reactive metabolite binding and toxicity within the hepatic lobule can be altered by pretreatment with hepatic enzyme inducing agents. In mice pretreated with phenobarbital (PB), protein binding and necrosis occur in zone 1, whereas in β-naphthoflavone (βNF)-pretreated mice they are localized primarily in zone 3.[21] In this study, we show a strong correlation between the intralobular location of cocaine reactive metabolite binding and the location of hsp accumulation, suggesting that cocaine reactive metabolite binding to protein plays a role in triggering hsp induction.

MATERIALS AND METHODS

Animals and Treatments. Adult ICR male mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing 25 to 30 g were used for these studies. Mice were housed on corn cob bedding in temperature- and humidity-controlled animal quarters with a 12-hour light/dark cycle and allowed free access to food and water before and during the

Abbreviations: hsp, heat shock protein; PB, phenobarbital; βNF, β-naphthoflavone; i.p., intraperitoneal; SKF-525A, α-phenyl-α-propylbenzeneacetic acid 2-[diethylamino]ethyl ester; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with Tween 20; BSA, bovine serum albumin; IgG, immunoglobulin G; ALT, alanine aminotransferase.

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experiments. The following pretreatment regimens were used to induce or inhibit cytochrome P-450 activity in the liver: sodium phenobarbital, 80 mg/kg intraperitoneal (i.p.), daily for 4 days; βNF, 40 mg/kg i.p., once daily for 3 days; or SKF-525A (Proadifen HCl, Smith, Kline, and French Lab, Philadelphia, PA), 50 mg/kg i.p., 30 minutes before treatment with cocaine. After pretreatment, mice were administered a single i.p. dose of cocaine HCl (Sigma Chemical Co., St. Louis, MO) in saline. All pretreatment and cocaine doses were given with an injection volume of 10 mL/kg body weight. Mice were killed by carbon dioxide asphyxiation. Before initiation of the study, all procedures were assessed and approved by the Institutional Animal Care and Use Committee.

Polyacrylamide Gel Electrophoresis. Four hundred milligrams of liver was homogenized in 5 mL of sample buffer (0.05 mol/L Tris[hydroxymethyl] aminomethane (Tris), 2% sodium dodecyl sulfate, 10 mmol/L dithiothreitol, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, pH 6.8). Each sample was boiled for 5 minutes, passed through a 22-gauge needle three times to shear DNA, and stored at −80°C until use. Fifty micrograms of protein from each sample was aliquoted to separate tubes and bromphenol blue was added to a final concentration of 0.0025%. Each aliquot was boiled for 5 minutes, loaded onto separate lanes of a 12.5% sodium dodecylsulfate-polyacrylamide gel, and resolved by electrophoresis.[22]

Protein Blotting and Immunostaining. Proteins separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis were immediately blotted to Hybond-ECL Western membrane (Amersham Corp., Arlington Heights, IL) using a semi-dry blotting apparatus (Millipore, Bedford, MA) and one-half strength Towbin buffer (10% methanol, 96 mmol/L glycine, and 10 mmol/L Tris-base). Blotting was performed at 320 mA for 1.5 hours. Upon completion, the membrane was blocked in Tris-buffered saline (TBS) (20 mmol/L Tris, 500 mmol/L sodium chloride, pH 7.5) containing 3% gelatin. The membrane was washed two times for 5 minutes each in Tris-buffered saline with Tween 20 (TTBS) (TBS containing 0.05% polyoxyethylenesorbitan monolaurate) and probed with one of the following antibodies: anti-hsp25 (rabbit polyclonal), anti-hsp60 (mouse monoclonal), anti-hsc70 (mouse monoclonal), anti-hsp70i (mouse monoclonal), or anti-hsp90 (mouse monoclonal). Each of these antibodies were obtained from Stressgen (Victoria, B.C., Canada) and used at a 1:1,000 dilution in TTBS containing 1% gelatin. Incubation was for 18 hours at 24°C with continuous shaking. The membrane was washed two times for 5 minutes each with TTBS and then a sheep anti-mouse or donkey anti-rabbit (depending upon the primary antibody used) horseradish peroxidase-conjugated antibody (Amersham Corp., Arlington Heights, IL) at a 1:3,000 dilution in TTBS containing 1% gelatin was incubated with the membrane for 1 hour at 24°C with continuous shaking. Next, the membrane was washed three times for 5 minutes each in TTBS and once for 5 minutes in TBS. The chemiluminescent horseradish peroxidase substrate Luminol (Amersham) was added to the membrane and the membrane exposed to standard X-ray film to localize antibody binding.

Immunohistochemical Detection of Hsp25, Hsp70i, and Cocaine Adducts in Murine Liver. Five-millimeter-thick sections from several lobes of each liver were placed in tissue cassettes and fixed in neutral-buffered formalin for 3 hours. The livers were rinsed and stored in saline, processed routinely, and embedded in paraffin. Four sequential sections, 4- to 6-μm thick, were cut from the same block to facilitate, to the extent possible, cell-by-cell comparisons of cocaine reactive metabolite binding, histopathology, and induced accumulation of hsp25 and hsp70i. One section was stained with hematoxylin and eosin and examined for histopathology by light microscopy, whereas the remaining sections were immunohistochemically stained with an anti-hsp25 antibody, an anti-hsp70i monoclonal antibody, or an anti-cocaine antibody as follows. Preliminary experiments were performed to ensure that an adequate blocking reagent was used that would prevent nonspecific binding of immunoglobulins to necrotic areas. TBS containing 25% (vol/vol) bovine serum plus 3% (wt/vol) purified bovine serum albumin (BSA) (blocking solution) was found to prevent the nonspecific binding to necrotic areas of normal rabbit serum, mouse immunoglobulin G (IgG), or normal sheep serum used as negative controls for the anti-hsp25, anti-hsp70i, or anti-cocaine antibodies, respectively. Fab fragment goat anti-mouse IgG (H + L) (Jackson Immuno Research Laboratories, Inc., West Grove, PA) was added to the blocking solution (10 μg/mL final concentration) before blocking slides subsequently probed for hsp70i induction. The latter addition blocked any endogenous mouse IgG that was present in the sections and prevented false positive signals when probing with the biotinylated anti-mouse IgG secondary antibody. Secondary antibody-only–treated slides showed no binding.

The following procedure was used for immunostaining the sections. Paraffin-embedded sections were deparaffinized by passing through three changes of xylene for 5 minutes each. The sections were passed through 100% ethanol two times for 1 minute each, 95% ethanol for 1 minute, and double-distilled water two times for 2 minutes each. Endogenous peroxidase activity was quenched by submerging the slides in 3% hydrogen peroxide containing 0.1% sodium azide for 10 minutes. The slides were then washed in double-distilled water three times for 2 minutes each and then equilibrated in TBS for at least 2 minutes. All the following incubations were performed in a humidified chamber. Blocking solution was placed on each section and incubated at 37°C for 1 hour. The slides were washed two times in TBS for 2 minutes each. Anti-hsp25 antibody (rabbit polyclonal, Stressgen) was diluted 1:100 in blocking solution and placed on the appropriate slides, whereas anti-hsp70i (mouse monoclonal, Stressgen) was diluted 1:100 in blocking solution devoid of the Fab fragment goat anti-mouse IgG antibody and placed on the appropriate slides. Anti-cocaine antibody (sheep polyclonal, JEM Research Products, Inc., Arlington, VA) was diluted 1:100 in blocking solution and placed on the appropriate slides. The antibodies were incubated with the sections at 37°C for 1 hour and then at 24°C for 18 hours. The sections were washed three times in TBS for 2 minutes each. Biotinylated goat anti-mouse, goat anti-rabbit, or rabbit anti-sheep (Southern Biotechnology Associates, Inc., Birmingham, AL), depending upon the primary antibody used, was diluted 1:500 in TBS containing 3% BSA, placed on the slides and incubated at 37°C for 30 minutes. The sections were washed three times in TBS for 2 minutes each. Streptavidin-linked horseradish peroxidase (Southern Biotechnology Associates, Inc.) was diluted 1:200 in TBS containing 3% BSA, placed on the slides and incubated at 37°C for 30 minutes. The sections were washed three times in TBS for 2 minutes each. The horseradish peroxidase colorimetric substrate 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) supplemented with 0.03% $NiCl_2$ (wt/vol) was incubated with each section for 15 minutes at 24°C to provide a permanent location of antibody binding. The sections were then counterstained with hematoxylin and dehydrated by passing through graded alcohols and xylene in the reverse order as for deparaffinizing the sections. The sections were mounted using Permount (Fisher Scientific, Orlando, FL) and a glass cover slip. As a negative control, no binding of the anti-cocaine antibody, using the above procedure, was observed in livers from mice administered necrogenic doses of carbon tetrachloride, bromobenzene, or acetaminophen.

Protein Determination. Protein was measured with the Micro Protein Determination assay (Sigma Chemical Co.) using BSA as standard.

Serum ALT Activity. Blood for measurement of serum alanine aminotransferase (ALT) activity was collected by cardiac puncture immediately after carbon dioxide asphyxiation. Serum ALT activity was determined according to the method of Bergmeyer et al.[23] using a commercially available kit (Sigma Diagnostics, St. Louis, MO).

Statistical Analysis. Data were analyzed by a one-way ANOVA followed by a Student Neuman-Keuls post-hoc test. The level of significant difference was defined as the .05 level of probability.

RESULTS

Initial experiments used Western blot analysis to identify specific hsps induced in response to an hepatotoxic dose of cocaine. Representative Western blots showing the hepatic levels of hsp25, hsp60, hsc70, hsp70i, and hsp90 at 0 (control), 3, 6, and 24 hours after treatment of naive, male ICR mice with cocaine, 50 mg/kg, i.p., are shown in Fig. 1. The levels of hsp60, hsc70, and hsp90 in the liver were unaffected by cocaine treatment at any of these time points. Levels of hsp25, however, were elevated at 6 and 24 hours, and levels of hsp70i were higher than those in controls at each of the observation times. Concentrations of hsp25 and hsp70i were both greatest at 24 hours.

In a subsequent set of experiments, livers were removed for immunostaining and histopathologic evaluation 0, 3, 6, or 24 hours after a cocaine dose (50 mg/kg, i.p.). Based on the results of the Western blot experiments (described above),

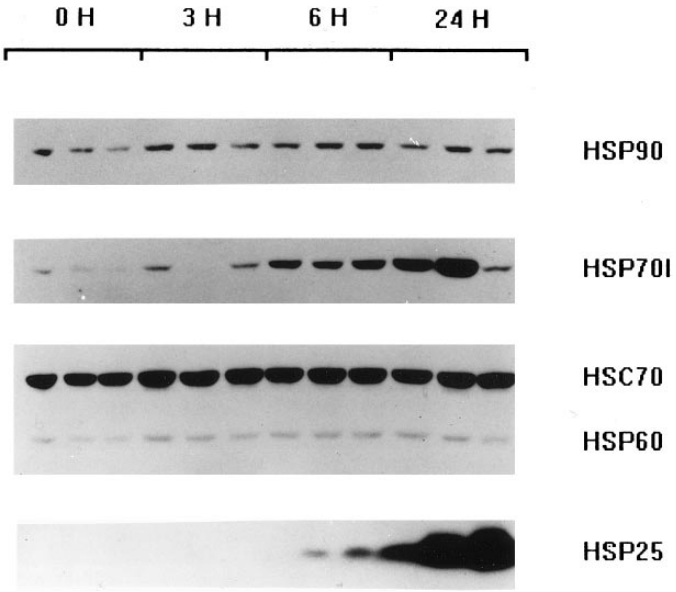

FIG. 1. Heat shock protein (hsp) levels in murine liver 0, 3, 6, or 24 hours after treatment with 50 mg/kg cocaine. Liver protein was resolved on a 12.5% sodium dodecyl sulfate-polyacrylamide gel, and the relative levels of various hsps estimated by Western blotting using antibodies specific for the indicated hsps. All samples were from the same experiment, with each lane showing hsp levels in a single mouse liver.

immunostaining for hsps was restricted to hsp25 and hsp70i. Following cocaine treatment, swelling of midzonal (zone 2) and centrilobular (zone 3) hepatocytes was observed at the earliest time point, 3 hours, and midzonal necrosis was evident by 6 hours after the cocaine dose (not shown). At 24 hours, the midzonal necrosis was extensive. Immunoreactive cocaine bound to protein was present beginning with the 3-hour specimens and was restricted to cells with altered morphology (i.e., cell swelling). Positive immunostaining for hsp25 and hsp70i was observed 3 hours after the cocaine dose. Hsp25 and hsp70i levels increased over time with the greatest accumulation observed 24 hours postadministration based on the intensity of immunostaining. As with the intralobular distribution of cocaine reactive metabolite binding, hsp25, and hsp70i expression was limited to cells with altered morphology. The extent of immunostaining appeared to be relatively uniform within the lesion. An example of the relationship between morphology, reactive metabolite binding, and hsp25 and hsp70i levels is shown in Fig. 2, which is from a liver specimen taken 24 hours after the cocaine dose. Hsp25 and hsp70i accumulation and cocaine binding (Fig. 2B, 2C, and 2D) were superimposable upon the hepatocytes with altered morphology in the midzonal regions (Fig. 2A). Liver sections from vehicle-treated control mice displayed normal histology and no detectable immunostaining for cocaine reactive metabolite, hsp25, or hsp70i (not shown).

To test if hsp accumulation following cocaine was a function of reactive metabolite binding and/or hepatotoxicity, rather than a direct effect of cocaine, the ability of the cytochrome P-450 inhibitor SKF-525A to inhibit cocaine binding and cocaine-induced hsp accumulation was measured through immunostaining. Consistent with previous observations,[21] SKF-525A pretreated animals had little or no hepatic necrosis from cocaine, and the increase in serum ALT activity associated with cocaine treatment, indicative of hepatic damage, was abolished (Table 1). Also lost in SKF-525A pretreated mice was any detectable immunostaining for cocaine reactive metabolite, hsp25, or hsp70i (not shown).

In an additional experiment, mice were pretreated with PB or βNF to determine if the association between cocaine reactive metabolite binding and hsp accumulation would be retained if the site of the cocaine-induced lesion was shifted. As with naive mice, preliminary experiments using Western blotting confirmed that among the hsps tested, only hsp25 and hsp70i were strongly induced by cocaine in either PB- or βNF-pretreated mice (not shown). Consistent with previous observations,[21] βNF pretreatment caused an apparent shift in the intralobular site of necrosis, as well as reactive metabolite binding, to the centrilobular region (zone 3) (Fig. 3). This was accompanied by an identical shift in the intralobular site of accumulation of hsp25 and hsp70i. As was the case with mice without pretreatment, hsp accumulation occurred only in cells with detectable reactive metabolite binding and altered morphology. Mice pretreated with PB, in contrast, displayed hepatic necrosis and reactive metabolite binding predominantly in the peripheral lobular region (zone 1) (Fig. 4). Again, the intralobular sites of accumulation of hsp25 and hsp70i corresponded precisely with the localization of the reactive metabolite binding and lesion.

DISCUSSION

Heat stress, the prototypical stimulus for increased hsp synthesis, characteristically induces the synthesis of an array of hsps. A survey of the response of the most common hsp families from 25- to 90-kDa to a series of hepatotoxicants in mice found that only hsp25 and hsp70i were increased significantly.[24] The results observed here with cocaine are consistent with this earlier report. The variability observed in the magnitude of hsp induction among mice at a given time point (Fig. 1) has been observed previously for other toxicants. Because the degree of toxicity varies also among individual animals within a given treatment group, this is perhaps not surprising.

Hsp70i induction has been observed in rats treated with halothane.[25] Effects of this hepatotoxicant on other hsps is unknown, because this study focused specifically on hsp70i. Goering et al.[26] incubated liver slices from cadmium-treated rats with ^{35}S-methionine and observed increased *de novo* synthesis of proteins with relative molecular masses of 70-kDa, 90-kDa, and 110-kDa. Western blots probed with specific antibodies revealed the 70-kDa protein to be hsp70i (termed hsp72 in their report) and the 90-kDa protein to be grp94. We have found no increase in grp94 levels following cocaine treatment in mice (Salminen, Roberts, and Voellmy, unpublished observations, October 1994), an apparent difference in response that could be related to differences in either hepatotoxicant action between cocaine and cadmium, or to fundamental species differences in response to hepatotoxicants. It is unclear whether there was also a difference in hsp25 response. Goering and colleagues did not observe increased synthesis of proteins with a molecular mass at or near 25-kDa following cadmium treatment as was seen with cocaine. Induction of hsp25 synthesis could conceivably have been missed using ^{35}S-methionine labeling in their study, however, because rodent hsp25 lacks methionine.[27]

Metabolism of many hepatotoxic compounds leads to the formation of reactive metabolites, and binding of these metabolites with proteins has been postulated to play a role in their cytotoxic effects. In general, very little is known about the effects of adduction with these relatively small molecular weight metabolites on the conformation of target proteins. It is plausible, however, that adduction of target proteins could lead to their recognition as non-native, causing activation of hsp genes. Cocaine is among the hepatotoxicants whose oxidative metabolism leads to a reactive metabolite,[20] and it has been shown previously that immunohistochemistry can be used to identify the intraacinar sites of cocaine reactive

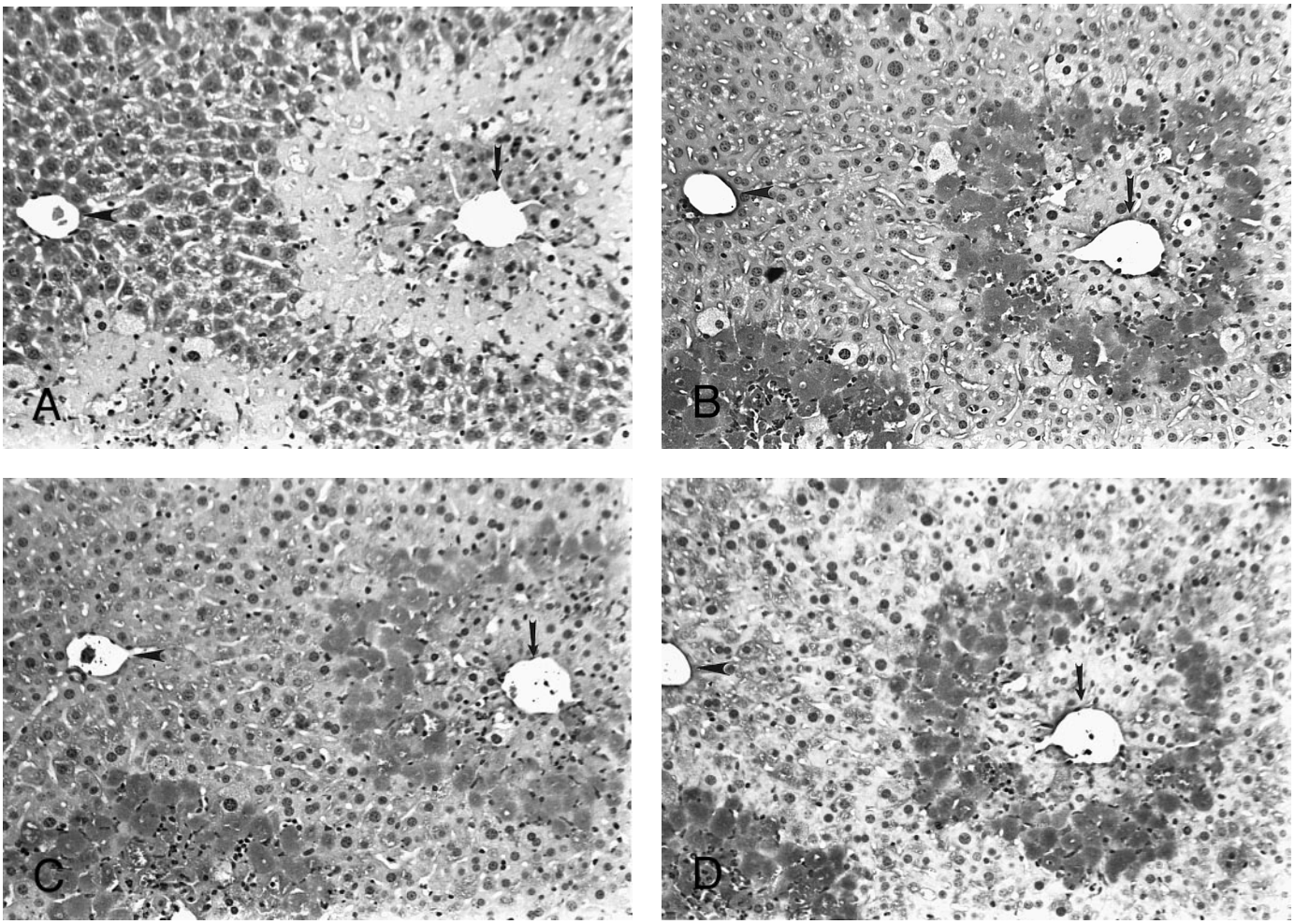

FIG. 2. Immunohistochemical detection of hsp25, hsp70i, and cocaine-adducted cellular macromolecules in murine liver 24 hours after treatment of naive mice with 50 mg/kg of cocaine. Sequential sections were stained as follows: (A) hematoxylin and eosin stain; (B) immunohistochemical stain using an anti-hsp25 antibody; (C) immunohistochemical stain using an anti-hsp70i antibody; and (D) immunohistochemical stain using an anti-cocaine antibody. All immunostained slides were counterstained with hematoxylin. *Arrow* indicates central vein and *arrow head* indicates portal triad. Midzonal (zone 2) necrosis is evident with some injury occuring closer to the central vein (zone 3). Field original magnification ×200.

metabolite binding.[21] To explore the relationship between reactive metabolite binding and increased hsp synthesis, this technique was used to compare the localization of cocaine-adducted proteins relative with accumulation of hsp25 and hsp70i.

The spatial correlation within the lobule between cocaine reactive metabolite binding, hsp25 and hsp70i accumulation, and cytotoxicity was found to be remarkably consistent. That is, immunostaining of sequential sections revealed that only cells with detectable cocaine metabolite binding had altered morphology and increased concentrations of hsp25 and hsp70i. This correlation existed whether the site of cocaine metabolite binding was in zone 2 (naive mice), zone 1, (PB-induced mice), or zone 3 (βNF pretreated mice). Cocaine reactive metabolite formation and hepatotoxicity result from cytochrome P-450 mediated oxidation of cocaine,[28] and both can be prevented by pretreatment of mice with the cytochrome P-450 inhibitor SKF-525A.[21] When reactive metabolite formation and binding was prevented by pretreatment with SKF-525A, no increases in hsp25 or hsp70i concentrations were observed. These observations are consistent with a mechanism in which cocaine-adducted protein provides a stimulus for activation of hsp25 and hsp70i genes. Of course, because reactive metabolite binding is closely correlated with cytotoxicity, the possibility cannot be ruled out that hsp induction occurs in response to some facet of toxicity rather than as a direct consequence of protein adduction. The difficulty in experimentally separating cocaine reactive metabolite binding and toxicity makes this possibility difficult to address.

TABLE 1. Effect of SKF-525A Pretreatment on Serum ALT Activities After Cocaine Administration

Treatment	Serum ALT Activity (IU/L)
Saline	54.4 ± 13.3
SKF-525A alone	58.0 ± 6.2
Cocaine alone	917.5 ± 410.3*
SKF-525A then cocaine	21.8 ± 9.8

NOTE. SKF-525A (50 mg/kg, i.p.) was administered 30 minutes before administration of cocaine (50 mg/kg, i.p.). Serum ALT activities were measured 24 hours after administration of cocaine. Data expressed as mean ± SEM (n = 5 animals per treatment group).

* Indicates significantly different from saline treated mice ($P < .05$).

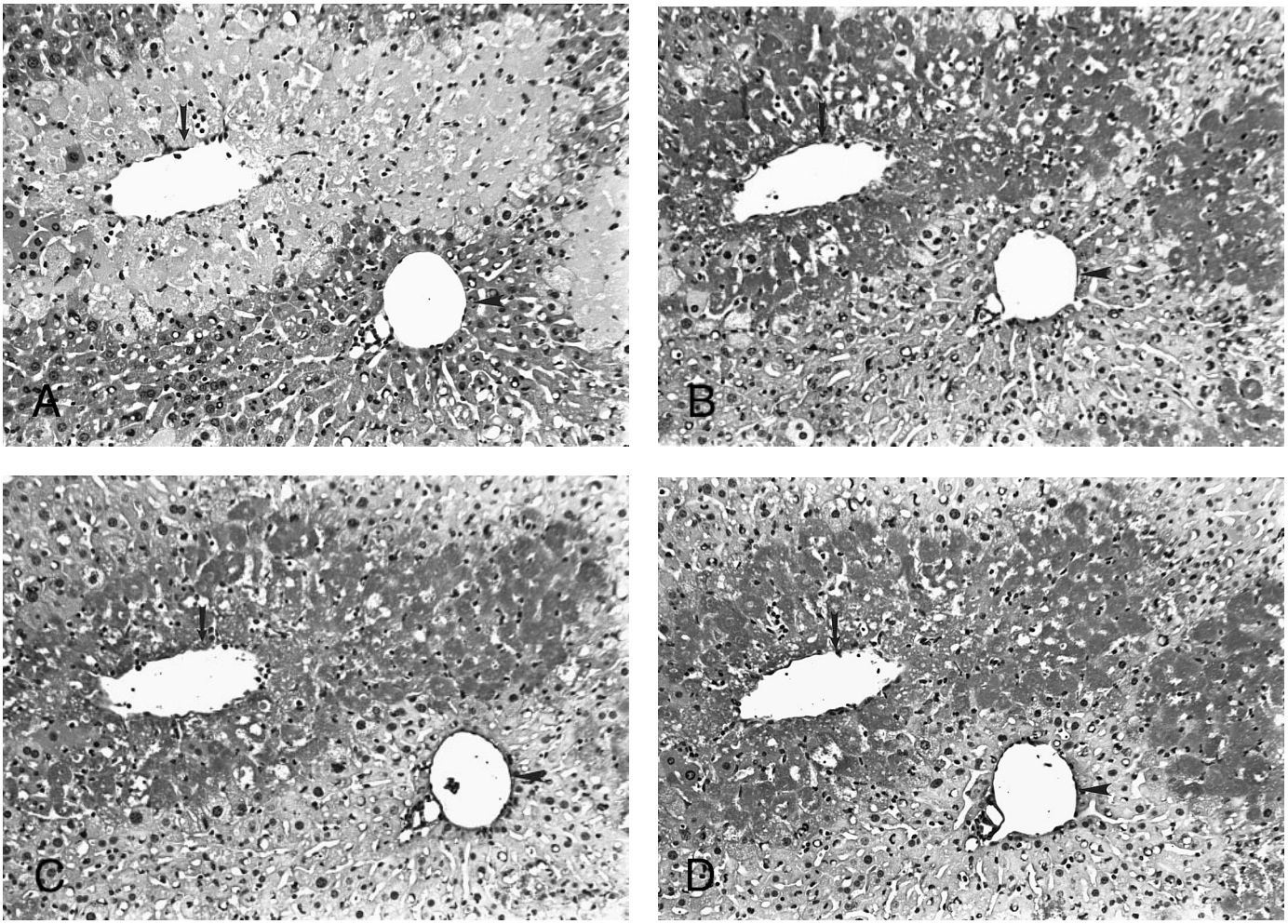

FIG. 3. Immunohistochemical detection of hsp25 or hsp70i induction and cocaine adduction of cellular macromolecules in murine liver 24 hours after treatment of βNF-pretreated mice with 50 mg/kg of cocaine. Sequential sections were stained as follows: (A) hematoxylin and eosin stain; (B) immunohistochemical stain using an anti-hsp25 antibody; (C) immunohistochemical stain using an anti-hsp70i antibody; and (D) immunohistochemical stain using an anti-cocaine antibody. All immunostained slides were counterstained with hematoxylin. *Arrow* indicates central vein and *arrow head* indicates portal triad. Extensive centrilobular (zone 3) necrosis is evident. Field original magnification $\times$200.

Another interesting observation arising from this study was the relative uniformity of the immunostaining within the affected areas, both for cocaine reactive metabolite binding and the hsps. To the extent that the intensity of staining is reflective of intracellular concentrations, relatively equivalent levels of hsps were observed in cells within the lesions, up to the margins of the affected areas. Cells immediately adjacent to cells in the affected area had no apparent staining. This suggests that hsp induction in response to cocaine may be an "all or none" phenomenon. The absence of a graded hsp response may be a function of a threshold for cocaine reactive metabolite binding to proteins. This would be consistent with the hypothesis that cocaine reactive metabolite binding to protein triggers hsp induction, and would explain why cells lacking cocaine reactive metabolite binding (i.e., cells surrounding and away from the necrotic lesions) did not show hsp induction. The existence of a threshold for reactive metabolite binding is well established for other hepatotoxicants such as acetaminophen and bromobenzene,[29-31] and is related to the capacity of intracellular detoxification mechanisms such as glutathione conjugation. The presence of a similar threshold for cocaine has not been explicitly shown but the apparent absence of a graded response among cells for cocaine reactive metabolite binding in this study implies that such a threshold exists.

Increased levels of hsp25 and hsp70i were detectable in cocaine-treated mice at the earliest time point examined, 3 hours postadministration, and the hsp concentrations continued to increase during the 24-hour period following the cocaine dose (Fig. 1 and data not shown). The apparent continued synthesis of the hsps between 6 and 24 hours postadministration was somewhat surprising, given the severity of toxicity experienced by the cells within the lesion. By the 6-hour time point, a number of the cells within the affected zone were necrotic, and by 24 hours severe coagulation necrosis was evident. Continued increases in hsp levels during this time frame suggest that hepatocytes are capable of sustaining hsp synthesis until very near cell death, at least in response to cocaine intoxication.

In conclusion, the results of this study indicate that cocaine hepatotoxicity results in a strong, but selective induction of hsp accumulation in the mouse. Induction of hsp accumulation, as well as cytotoxicity, is confined to cells with detectable cocaine metabolite binding, suggesting that this binding may be responsible for both phenomena. The role of this induced hsp accumulation in the hepatotoxicity of cocaine and

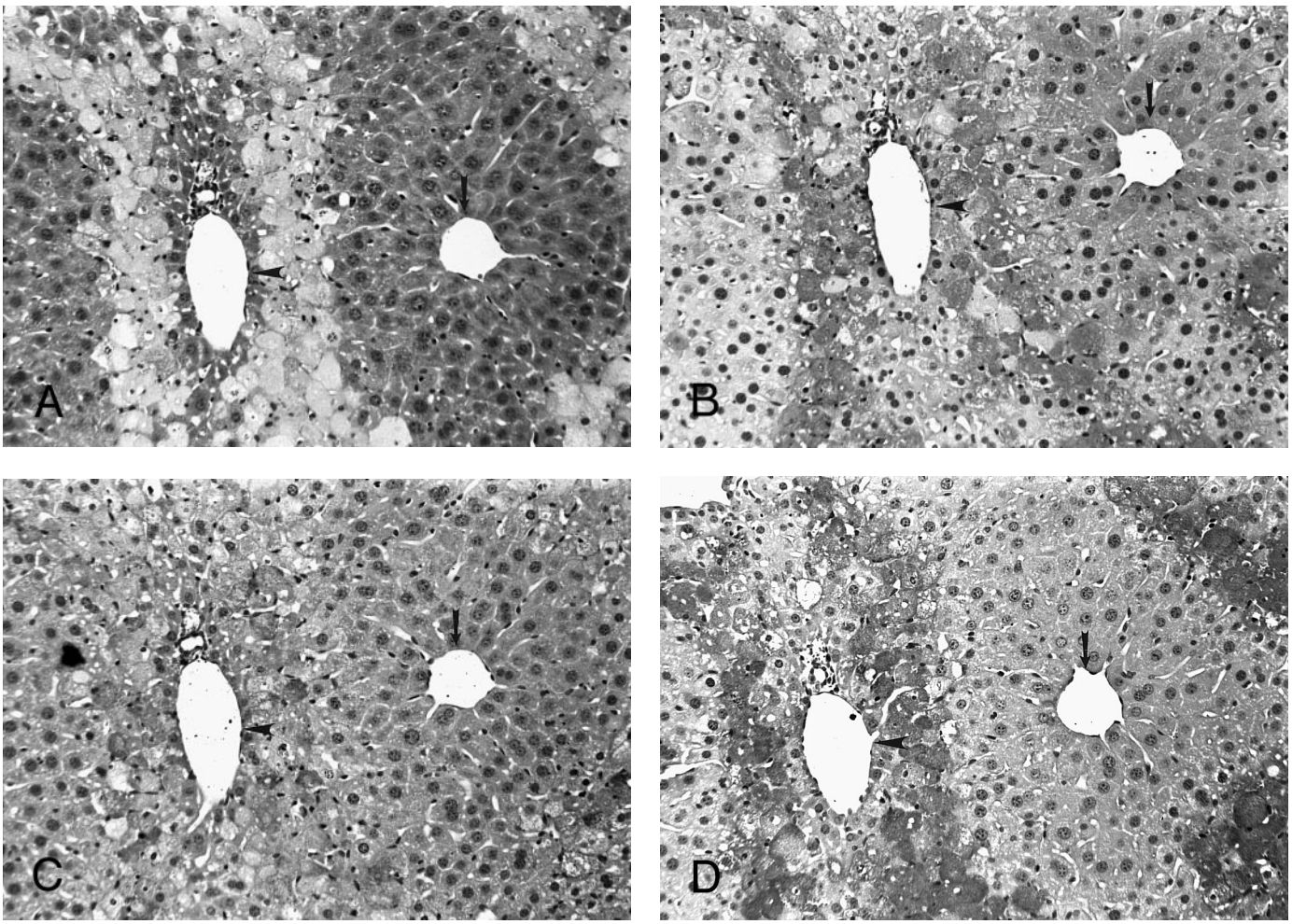

FIG. 4. Immunohistochemical detection of hsp25 or hsp70i induction and cocaine adduction of cellular macromolecules in murine liver 24 hours after treatment of PB-pretreated mice with 50 mg/kg of cocaine. Sequential sections were stained as follows: (A) hematoxylin and eosin stain; (B) immunohistochemical stain using an anti-hsp25 antibody; (C) immunohistochemical stain using an anti-hsp70i antibody; and (D) immunohistochemical stain using an anti-cocaine antibody. All immunostained slides were counterstained with hematoxylin. *Arrow* indicates central vein and *arrow head* indicates portal triad. Extensive periportal (zone 1) necrosis is evident. Field original magnification ×200.

other compounds remains to be determined. On one hand, hsp induction may constitute a cellular defense mechanism to enhance the correct refolding of damaged proteins and/or their targeting for degradation.[19,32-33] On the other hand, the continued synthesis of these proteins may contribute to toxicity by further perturbing metabolism of cells attempting to respond to a toxicant insult.[26] Delineation of the role of hsps in toxicant action will be an important step in understanding the fundamental mechanisms of hepatotoxicity.

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