Abstract  Intoxication of rats with thioacetamide (TAA) is a model system to investigate mechanisms involved in liver cell death and tissue reconstitution. Our study was undertaken to determine by immunohistochemistry the expression pattern of the cytoprotective chaperone proteins HSC70 and HSP25 and proliferation markers cyclin D1 and PCNA in livers of Wistar rats intraperitoneally injected with TAA at a single dose of 50 mg/kg. For each protein studied we observed distinct dynamic changes in appearance and localization in liver lobules. During 24–36 h after TAA injection the HSC70 cytoplasmic immunoreaction gradually disappeared from hepatocytes localized around central veins and a shift of immunostaining to cell nuclei took place. Then, 36–48 h after TAA injection the HSC70 cytoplasmic immunoreaction reappeared with the highest intensity in hepatocytes surrounding the areas of inflammatory cells. HSP25, undetectable in control hepatocytes began to appear at approximately 36 h after TAA injection and HSP25-immunopositive cells formed a characteristic ring around areas of inflammation. Of the proteins studied, the most rapid reaction to TAA was observed for cyclin D1. As early as 15 min after TAA administration cyclin D1-positive hepatocytes appeared in intermediate and periportal areas of liver lobules and a subsequent shift of staining to centrilobular hepatocytes took place at 36 and 48 h. There was no correlation of cyclin D1 localization either with PCNA-positive cells or mitotic cells. Our observations suggest that in TAA-treated livers HSP25 and HSC70 proteins can play an anti-inflammatory role, and the early and distinct cyclin D1 expression is not related to proliferation of hepatocytes.

Keywords  Heat shock proteins · Cyclin D1 · Rat liver · Thioacetamide · Inflammatory reaction

Introduction  Thioacetamide (TAA) is a model hepatotoxin which prolonged administration of which may result in liver cirrhosis and cancer development in rat and mice (Becker 1983; Gervasi et al. 1989). When TAA is administered into rats at a single dose its metabolites form protein adducts (Dyroff and Neal 1981; Witzman et al. 1996) leading to apoptosis or necrosis of centrilobular hepatocytes (Ledda-Columbano et al. 1991) and infiltration of inflammatory cells. Concomitantly, autoprotective mechanisms induce proliferation of hepatocytes to overcome massive liver injury. It has been suggested that biochemical and morphological changes in hepatocytes induced by a single dose of TAA reflect the balance between death of cells and tissue reconstitution (Mangipudy et al. 1995).

It is a well known fact that treatment of cells with toxic compounds induces expression of heat shock genes (hsp genes) which code for molecular chaperones involved in cytoprotection (Morimoto 1998). However, there are only limited immunohistochemical studies on the expression pattern of HSPs in vivo in response to hepatotoxicants. It has been shown that in mouse liver the proteins HSP70 and HSP25 accumulated around areas of hepatocyte necrosis induced by cocaine, acetaminophen, or 2-acetylaminofluorene (Salminen et al. 1997a, b; Lindeman et al. 1998). In livers of TAA-treated rats the immunohistochemical analysis was applied to determine the expression pattern of the HSP70 genes (Andoh et al. 1994). It has not been unequivocally established, however, whether TAA activates expression of the stress-inducible HSP70 protein; it seems rather that translocation of the constitutively synthesized cognate HSC70 protein from cytoplasm into the nucleus takes place. Also the
pattern of the HSP25 protein distribution in livers of TAA-treated rats has not been investigated.

The two above-mentioned HSPs, the HSC70 and HSP25, are multifunctional proteins. The HSC70 is a cytoplasmic chaperone protein involved in the folding and assembly of proteins, intracellular transport and sorting of proteins to different subcellular compartments, signal transduction, and regulation of apoptosis (Bakau and Horwich 1998). There are several observations suggesting that the HSC70 protein can also be involved in the process of cell proliferation (reviewed in Helmbrecht et al. 2000). In turn the HSP25 protein was suggested to be a target protein in the signal transduction pathways involving mitogen-activated protein kinases (Helmbrecht et al. 2000). Molecular and cellular stressors including toxins can significantly increase the expression level of the hsp25 gene, and overexpression of the HSP25 protein was found to induce drug resistance and protect cells against apoptosis (reviewed in Jäättelä 1999). Recently, a novel role for this protein as an anti-inflammatory factor has been suggested (De et al. 2000; Hightower et al. 2000).

Some recent data indicate that an early event after treatment of rats and mice with carcinogenic or toxic compounds can be the elevation of cyclin D1 expression in the liver (Rininger et al. 1997; Ledda-Columbano et al. 2000). In a preliminary study we found that the level of cyclin D1 increases rapidly also in livers of rats treated with TAA (Matusecka et al. 2001a). Cyclin D1 belongs to G1 cyclins which control the transition through the G1 phase and the restriction point of the cell cycle. However, there were reports suggesting that the increased expression of cyclin D1 is not related to enhanced proliferation (Sgambato et al. 1995; Lee at al. 1997).

The multifunctional nature of the HSC70, HSP25, and cyclin D1 suggests a possible involvement of these proteins in similar or crossing signal transduction pathways in cells exposed to toxic compounds or to other molecular stresses. Interestingly, in our recent work on the HSPs expression in non-small cell lung carcinomas we found a statistically significant relationship between the cyclin D1 expression, high intensity of HSC70 immunostaining in tumor cell nuclei, and increased expression of HSP27, a human counterpart of the HSP25 protein (Matusecka et al. 2001b).

The observations mentioned above prompted us to investigate immunohistochemically the expression pattern of these proteins in rat livers in vivo after a single dose of 50 mg/kg of TAA. The aim of the present study was to find out if there are any correlations between the regional distribution of HSC70, HSP25, and cyclin D1 in liver lobules, especially in relation to inflammatory reaction and to proliferating cells referred to as those exhibiting proliferating cell nuclear antigen (PCNA) immunostaining. So far, such analysis has never been performed under similar experimental conditions.

Materials and methods

Animals and treatments

Male Wistar rats, 2.5–3 months old and weighing 250–300 g, were used. They were bred in the on-site Animal Facility of the Centre of Oncology in Gliwice. Rats were housed in a temperature-controlled environment (20–22°C) with a 12-h photoperiod and provided with laboratory fodder and water ad libitum. All animal procedures conformed with institutional regulations and the European regulations concerning the protection of animals. Rats received a single intraperitoneal injection of TAA (Sigma, St. Louis, Mo., USA) dissolved in 0.9% NaCl at a dose of 50 mg/kg body weight. The animals (two to four per time point) were killed 15 min and 1, 6, 12, 24, 36, 48, 60, 72, and 96 h after injection. Control rats received physiological saline. When indicated, rats were subjected to hyperthermia as described earlier (Krawczyk et al. 1989). For immunohistochemical analysis liver samples were fixed for 24 h at 4°C in 10% formalin in PBS and embedded in paraffin. For RNA and protein analysis livers were frozen in liquid nitrogen and pulverized.

Northern blot analysis

The analysis was performed essentially as described earlier (Lisowska et al. 1996). Total RNA was isolated from pulverized tissues by acidic phenol extraction (Chomczynski and Sacchi 1987). Normalized RNA samples were fractionated on 1.2% agarose–2.2 M formaldehyde gel (Maniatis et al. 1982) and blotted onto Hybond-N membrane (Amersham, Aylesbury, UK). Northern blot hybridization was performed under stringent conditions. The hybridization solution contained 20 mM sodium phosphate (pH 6.5), 50% formamide, 5×SSC, 5×Denhardt’s solution (where 1×Denhardt’s solution corresponds to 0.002% Ficol 400/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 250 μg/ml denatured salmon sperm DNA, and radioactive probe. Filters were hybridized with the probe detecting simultaneously transcripts of the two rat hsp70 genes, hsp70.1 (2.5 kb) and hsp70.2 (2.7 kb) (Lisowska et al. 1996). Probe (C-terminal part of the hsp70.1 gene coding unit amplified with primers C and F) was labeled with [α-32P]dCTP by random priming (Multiprime kit; Amersham) to a specific activity of about 108–109 cpm/μg DNA.

Western blot analysis

Livers were homogenized in a Dounce homogenizer in 4 vol lysis buffer (10 mM TRIS-HCl pH 7.5, 250 mM sucrose, 0.1 mM PMSF). The lysate was centrifuged for 10 min at 30,000 g to remove unbroken cells, nuclei, and other debris, and the supernatant was centrifuged for 90 min at 100,000 g at 4°C. Protein concentration was measured using the Bradford assay and bovine serum albumin was used as a standard. Proteins were separated in 10% SDS-polyacrylamide and electroblotted to a nitrocellulose filter (BA85; Schleicher and Schuell, Dassel, Germany). Non-specific binding sites were blocked with 5% non-fat milk in PBS. For detection of the HSP27 the specific antibody, clone BRM-22 (Sigma), was used. They were bred in the on-site Animal Facility of the Centre of Oncology in Gliwice. Rats were housed in a temperature-controlled environment (20–22°C) with a 12-h photoperiod and provided with laboratory fodder and water ad libitum. All animal procedures conformed with institutional regulations and the European regulations concerning the protection of animals. Rats received a single intraperitoneal injection of TAA (Sigma, St. Louis, Mo., USA) dissolved in 0.9% NaCl at a dose of 50 mg/kg body weight. The animals (two to four per time point) were killed 15 min and 1, 6, 12, 24, 36, 48, 60, 72, and 96 h after injection. Control rats received physiological saline. When indicated, rats were subjected to hyperthermia as described earlier (Krawczyk et al. 1989). For immunohistochemical analysis liver samples were fixed for 24 h at 4°C in 10% formalin in PBS and embedded in paraffin. For RNA and protein analysis livers were frozen in liquid nitrogen and pulverized.

Immunohistochemistry

Immunohistochemical staining was performed on 7-μm dewaxed sections using the following antibodies: anti-HSC70/HSP70i...