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Oxidative Stress Response in Iron-Induced Acute Nephrotoxicity: Enhanced Expression of Heat Shock Protein 90

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Iron overload with ferric nitrilotriacetate (Fe-NTA) induces acute renal proximal tubular necrosis, a consequence of oxidative tissue damage, that leads to a high incidence of renal adenocarcinoma in rodents. In the present study, we determined the proteins preferentially produced in response to the Fe-NTA-induced oxidative injury. A single intraperitoneal Fe-NTA treatment led to the enhanced production of a number of proteins with molecular masses of 85-95 kDa. These included heat shock protein 90 (HSP90) as determined by immunoprecipitation. The enhanced production of HSP90 was prominent in the renal tubular cells. Steady accumulation of HSP90 was observed in the subacute toxicity experiments with multiple injections of Fe-NTA, suggesting that the enhanced production of HSP90 is important in increasing resistance to subsequent injury caused by the Fe-NTA-induced oxidative stress.

There is a hypothesis that an elevated incidence of cancer is associated with chronic iron overload. In fact, the risk for primary hepatocellular carcinoma in idiopathic hemochromatosis is more than 200 times greater than that of the control population (1-3). One of the rationales for iron-induced carcinogenesis is its role as a catalyst for the generation of reactive oxygen species. It is well established that only loosely bound iron ('free' iron), not tranferrin-bound iron, can function as a catalyst. In human serum, non-transferrin-bound iron is observed in acute iron poisoning, severe idiopathic hemochromatosis, Bantu siderosis, and congenital atransferrinemia (4).

The appearance of 'free' iron in serum can be experimentally induced by intraperitoneal injection of ferric nitrilotriacetate (Fe-NTA) (5). A high incidence (92% in male Wistar rats; 60% in male ddY mice) of renal adenocarcinoma was observed in rodents after repeated intraperitoneal administration of Fe-NTA (6-8). It has been shown that oxidative stress plays a critical role in Fe-NTA-induced acute nephrotoxicity, which leads to the alteration of the fundamental function of the kidney, including reabsorption of filtered proteins from glomeruli and their proteolysis (9). Oxidative stress in vivo represents a degradative process, a consequence of the production and propagation of free radical reactions, leading to membrane lipid peroxidation that has been implicated in causing a wide range of biological effects including heart disease, aging, retinal degeneration, and cancer (10).

In the present study, based on the detection of a stress protein preferentially produced in the kidney of Fe-NTA-treated rats, we demonstrate the early stress response caused by the iron-induced oxidative stress.

MATERIALS AND METHODS

Animals and experimental groups. Male SPF slc: Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing

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Abbreviations: Fe-NTA, ferric nitrilotriacetate: HSPs, heat shock proteins; GRPs, glucose regulated proteins; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; TBS, tris-buffered saline.

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130 to 150 g (6 weeks of age) were used. They were kept in a stainless steel cage and given commercial rat chow (Funabashi F-2, Chiba) as well as deionized water (Millipore Japan, Osaka) *ad libitum.* The animals were divided into a time-course study group, a dose-dependency study group, and subacute toxicity groups. Each subgroup contained 3 animals. The animals were sacrificed by decapitation and both kidneys of each animal were immediately removed. In the case of subacute toxicity experiments, the animals were sacrificed 24 hours after the final injection.

Materials. Ferric nitrate enneahydrate, sodium carbonate, hydrogen peroxide, acetone, and ethanol were from Wako (Osaka); nitrilotriacetic acid disodium salt and 2,6-di-*tert*-butyl-*p*-cresol were from Nakarai Tesque Inc. (Kyoto, Japan). Fe-NTA solution was immediately prepared before use as reported previously (11) with a slight modification. Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin, and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Amersham. Protein A-Sepharose 4 Fast Flow was obtained from Pharmacia LKB.

Antibodies. Anti-HSP70 and anti-HSP90, anti-GRP78, and anti-GRP94 monoclonal antibodies (Stress Gen Biotechnologies Corp., British Columbia, Canada) were kindly provided by Dr. Y. Kitagawa (Nagoya University Bioscience Center).

Tissue Homogenate Preparation. Rat kidneys were homogenized with a Teflon homogenizer in ten volumes of 1.15% KCl. The homogenate was centrifuged at 100,000xg for 60 min, and the supernatants were used for Western blot or immunoprecipitation/Western blot analyses.

Western blot. For estimation of HSP levels in the kidney of rats treated with Fe-NTA, renal cytosolic proteins were treated with Laemmli sample buffer (12) for 3-5 min at 100°C. The samples were run on two 10% SDS-PAGE slab gels. One gel was used for staining with Coomassie Brilliant Blue; the other was transblotted to Immobilon PVDF membranes, incubated with 2% BSA in TBS/Tween for blocking, washed, and treated with the antibody. This procedure was followed by the addition of horseradish peroxidase conjugated to goat anti-rabbit IgG immunoglobulin and ECL reagents. The bands were visualized by exposure of the membranes to autoradiography film.

Immunoprecipitation. Renal cytosolic fractions (approximately 400 μg protein/0.1 ml) of Fe-NTA treated and untreated rats were incubated with 10 μl of anti-HSP90 monoclonal antibody (10 μg IgG) on ice for 3 hours. The mixture was then treated with 50 μl of Protein A-Separose 4 Fast Flow and incubated on ice for 1 hour. The mixture was then centrifuged (10,000g, 3 min), rinsed three times with 0.1 M Hepes buffer (pH 8.0), and then treated with Laemmli sample buffer for SDS-PAGE or SDS-PAGE/immunoblot.

Immunohistochemistry. The avidin-biotin complex method was used for Boulin's solution-fixed and paraffin-embedded sections as previously described (11) with a slight modification. The major change was the use of alkaline phosphatase instead of peroxidase. Normal rabbit serum (Dako Japan Co., Ltd, Kyoto; diluted to 1:75) for the inhibition of non-specific binding of secondary antibody, the anti-HSP90 monoclonal antibody (5 μg/ml), biotin-labeled rabbit anti-mouse IgG serum (Dako; diluted to 1:300), and avidin-biotin complex (Vector Laboratories; diluted 1:100) were sequentially used. The substrate for alkaline phosphatase (black) was obtained from Vector. Procedures using PBS or non-immune mouse IgG1 at the same concentration showed no or negligible positivity.

Gene expression. The probe of human HSP70 and HSP90, the construct made by Lee Weber (University of Nevada) (13), was kindly provided by R.I. Morimoto of Northwestern University. The probes were amplified and isolated according to standard methods (14). They were labeled with [α-^{32}P]dCTP by random oligonucleotide priming (Amersham). Total RNAs were extracted, size-fractioned by electrophoresis on a 1% agarose/6% formaldehyde gel and transferred to a Hybond-N$^+$ membrane (Amersham). The filters were prehybridized for 20 h at 42°C in a solution containing 50% formamide, 0.75 M NaCl, 0.075 M trisodium citrate, 5x Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 1% SDS, and 0.05 M sodium phosphate, pH 6.5. Hybridizations were performed overnight in the same medium at 42°C with the ^{32}P-labeled probe denatured at 100°C for 5 min. After two washes at room temperature, two final washes were performed at 50°C with 15 mM NaCl, 1.5 mM trisodium citrate and 0.1% SDS. In some experiments, a mouse β-actin probe (*HindIII-EcoRI* fragment of 4500 base pairs) was hybridized as indicated above after dehybridization of the filter with 15 mM NaCl, 1.5 mM trisodium citrate and 0.5% SDS. Autoradiography was performed at −70°C on Kodak X-Omat AR film.

RESULTS AND DISCUSSION

As the earliest event, we have previously shown that the intraperitoneal treatment of Fe-NTA leads to the rapid and temporary accumulation of membrane lipid peroxidation products in rat kidney (11,15). It was anticipated that this event could be a signal for cellular response, leading to the recovery of the kidney. As a matter of fact, a number of proteins newly appearing in the kidney were detected upon SDS-PAGE analysis of the renal cytosolic proteins (data not shown). Particularly, the appearance of proteins with molecular masses arround 90 kDa was evident. We expected that these included stress-inducible proteins such as HSP90. Hence, we attempted to detect HSP90 by means of immunochemical procedures. Immunoblot analysis of HSP90 in renal proteins from Fe-NTA-treated and untreated rats demonstrated multiple immunoreactive protein species with molecular masses of 85–95 kDa (Fig. 1A). Apparently, the monoclonal anti-HSP90 antibody used in this study also cross-reacted with the proteins except for HSP90. Therefore, the SDS-PAGE/

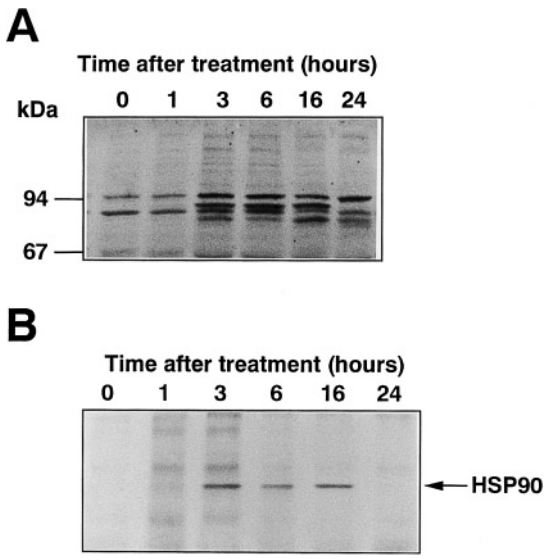

FIG. 1. Immunochemical detection of HSP90 in the kidney of rats after acute treatment with Fe-NTA. A, SDS-PAGE/ immunoblot analysis of HSP90. B, Immunoprecipitation of HSP90. Renal cytosolic proteins were treated with anti-HSP90 monoclonal antibody and precipitated with Protein A-Sepharose 4 Fast Flow. The antigen-antibody complexes were then analyzed by Western blot using anti-HSP90 monoclonal antibody.

immunoblot analysis was considered unfeasible for identification of HSP90. Taking advantage of the fact that the antibody strongly favors native HSP90 over denatured or complexed HSP90, we then performed immunoprecipitation prior to Western blot, which allowed the specific detection of HSP90. As shown in Fig. 1B, the protein level of HSP90 in the kidney was clearly increased at 3 hours and progressed through 16 hours of Fe-NTA treatment. The amount of HSP90 returned to the undetectable level at 24 hours. In contrast, HSP70 constitutively produced in the renal cytoplasm of untreated control rats disappeared within 3 hours and did not recover within 24 hours after Fe-NTA treatment (data not shown). Other stress proteins including glucose regulated proteins (GRP78 and GRP94) were scarcely detected immunochemically.

As shown in Fig. 2, the induction of HSP90 was prominent in the renal tubular cells. The kidneys of untreated control rats showed faint staining in the cytoplasm of the proximal tubular cells (Fig. 2A). The staining was increased in the proximal and distal tubules 1 h after the administration of Fe-NTA (Fig. 2B). The enhanced positivity persisted in the remaining proximal and distal tubular cells up to 24 h after the Fe-NTA treatment (Fig. 2C). The fact that the proximal tubules are major target organs of Fe-NTA suggest that the Fe-NTA-induced oxidative stress resulted in the enhanced production of HSP90 in the renal tubules. It is of interest to note that the immunostaining was also observed in the nuclei 3 to 24 h after the Fe-NTA treatment, although HSP90 is the most abundant cytosolic protein in eukaryotic cells. This phenomenon may be interpreted by the fact that HSP90 functions in the transportation of several receptor complexes to the nucleus (16).

Next, we examined whether the change in the level of HSP90 was due to transcriptional changes or a posttranslational event. Northern blot analysis of total mRNA from the kidney of Fe-NTA-treated rats was performed using an oligonucleotide probe complementary to HSP90 mRNA sequences (Fig. 3). The level of HSP90 mRNA was found to increase approximately 2-fold at 6 h of Fe-NTA treatment. Therefore, the increase in HSP90 messages coincided with a substantial rise in the HSP90 protein levels.

The facts that the renal injury was less severe than anticipated when the kidney was rechallenged with toxins and that the exposure to one toxin increased renal resistance to a second unrelated toxin

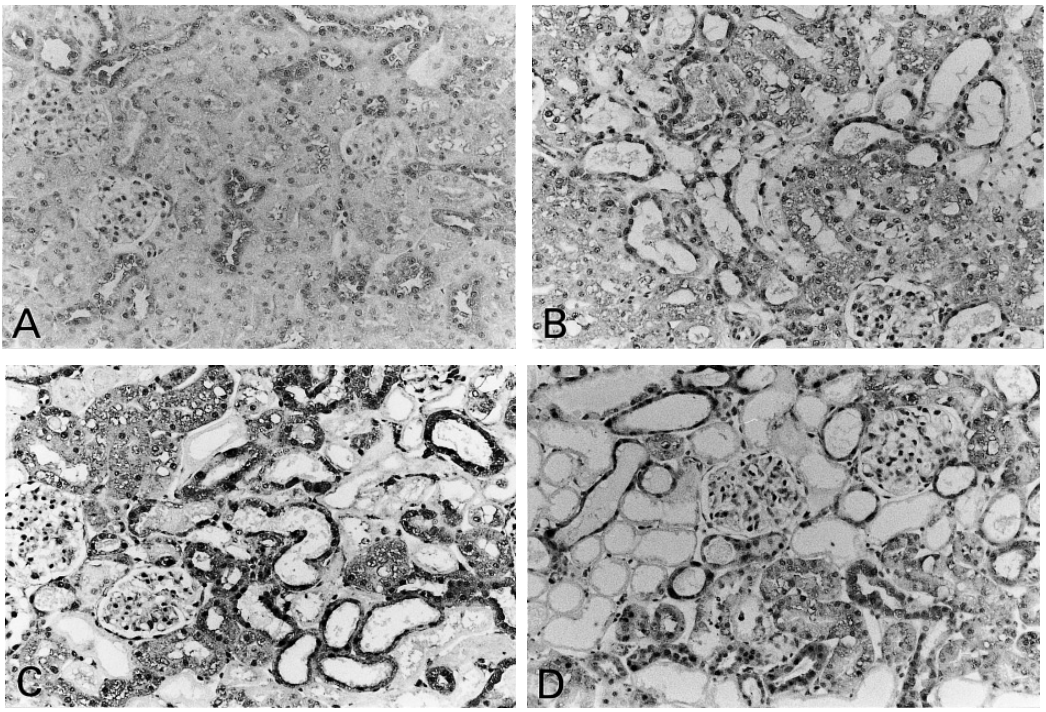

FIG. 2. Immunohistochemical detection of HSP90 in renal cortex of rats after acute treatment with Fe-NTA (Serial paraffin-embedded section, ×50). A, Untreated control; B, 1 h; C, 3 h; D, 16 h.

(17) led us to examine the production of HSP90 in the subacute toxicity experiments with multiple treatments of Fe-NTA. As shown in Fig. 4, the multiple injections of Fe-NTA resulted in a steady accumulation of HSP90. The result supports the idea that the repeated exposure to the Fe-NTA-induced oxidative stress mediates cell repairs or increased resistance to subsequent injury by virtue of the enhanced production of HSP90 in the renal tubules. Curiously, this phenomenon coincides with the previous observation that multiple injections of Fe-NTA develop tolerance to the exposure to Fe-NTA (18–20). HSP90,representing an important adaptive response to stress, may thus mediate acquired resistance to the injury in this carcinogenesis model. Furthermore, acquiring resistance by inducing HSP90 may be linked to carcinogenesis. HSP90 is in fact known to be present in association with several different Src-related oncogenic nonreceptor tyrosine kinases including $pp60^{v\text{-}src}$ (21).

It has been established that, in response to adverse changes in their environment, cells from all

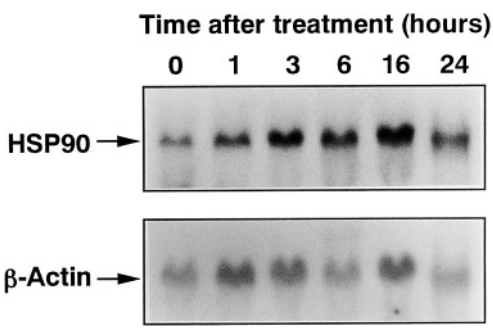

FIG. 3. Northern blot analysis of expression of HSP90 gene in the kidney after acute treatment with Fe-NTA.

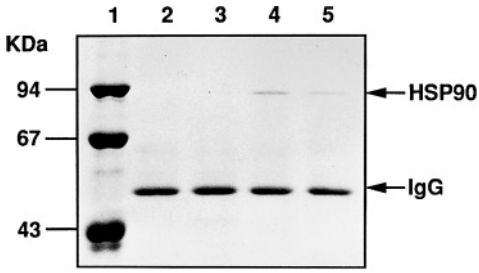

FIG. 4. Steady accumulation of HSP90 in the kidney of rats after subacute treatment with Fe-NTA. Lane 1, marker; lane 2, untreated control; lane 3, a single injection of Fe-NTA (24 hours; 15 mg Fe/kg body weight); lane 4, subacute toxicity study group (group A) with multiple injections of Fe-NTA (1 week-treatment: 5 mg Fe/kg body weight daily for 3 days and 10 mg Fe/kg body weight daily for the next 3 days); lane 5, subacute toxicity study group (group B) with multiple injection of Fe-NTA (3-week treatment: 5 mg Fe/kg body weight daily for 3 days, 10 mg Fe/kg body weight for the next 3 days, one-day break, 10 mg Fe/kg body weight for the next 5 days, 2 day-break, and 10 mg Fe/kg body weight for the next 4 days). Total renal cytoplasmic proteins were treated with anti-HSP90 monoclonal antibody and precipitated with Protein A-Sepharose 4 Fast Flow. The antigen-antibody complexes were then analyzed by SDS-PAGE. The HSP90 protein band was visualized by Coomassie staining.

organisms increase the expression of proteins referred to as stress protein, including HSPs in response to elevated temperature (22), GRPs after nutrient or oxygen deprivation (23), and others, including heme oxygenase after ultraviolet radiation (24). One of the major functions of stress proteins is to prevent damage to other cellular proteins and allow denatured proteins to reacquire their native conformation (25). Oxidative stress is known to generate abnormal proteins oxidatively modified by a variety of active species including free radicals and membrane lipid peroxidation-derived aldehydes (26). Indeed, the alteration of renal proteins with cytotoxic aldehydes during Fe-NTA-induced oxidative stress has been reported (11,15). Accumulation of these abnormal proteins may therefore represent a stress able to induce HSP90.

It is of interest to note that the HSP90 appeared in parallel with the disappearance of HSP70. In relation to this observation, it has been shown that the available levels of HSP70 are reduced as a result of global protein denaturation after heat shock treatment and that the cells respond by increased expression of new HSPs as the levels of free or available HSP70 decline (14). In addition, the HSP70 is known to interact with heat shock factor which controls the transcription of all of the heat shock genes (14,27). These suggest that the HSP70 modulates the expression of other heat shock proteins including HSP90, which can be responsible for increasing resistance to oxidative damages. Therefore, it is not unlikely that the reduction in the level of constitutive HSP70 leads to the activation of a stress response in the renal tubules of Fe-NTA-treated rats. However, the reason for the specific induction of HSP90 among HSPs remains unclear.

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