Acute nephrotoxicity of a carcinogenic iron chelate Selective inhibition of a proteolytic conversion of α_{2U}-globulin to the kidney fatty acid-binding protein

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Abstract The mechanism of acute nephrotoxicity of an iron chelate in vivo has been investigated. Administration of a renal carcinogen ferric nitrilotriacetate (Fe-NTA) (15 mg Fe/kg body weight, intraperitoneally) led to selective loss of a renal protein with an apparent molecular mass of 17 kDa. Analysis of the 17 kDa protein by NH_2-terminal sequence demonstrated its identity over 16 NH_2-terminal residues as a kidney fatty acid-binding protein (k-FABP) that is a proteolytically modified form of α_{2U}-globulin, a major urinary protein of adult male rats. An immunochemical study using anti-α_{2U}-globulin polyclonal antibodies confirmed that a single injection of Fe-NTA led to a decrease in k-FABP levels. However, a 19-kDa protein identical to the α_{2U}-globulin progressively appeared in the kidney, suggesting that the proteolytic processing of α_{2U}-globulin in the renal proximal tubules was suppressed by the treatment with Fe-NTA. By monitoring k-FABP and its precursor α_{2U}-globulin, it was determined that repeated exposure to Fe-NTA caused suppression of both proteolytic and endocytotic activity of the kidney. The implications of these data in relation to the nephrotoxicity of Fe-NTA are discussed.

Key words: Ferric nitrilotriacetate; Renal carcinogen; Kidney fatty acid-binding protein; α_{2U}-Globulin

1. Introduction

There is a hypothesis that an elevated incidence of cancer is associated with chronic iron overload [1]. In fact, the risk for primary hepatocellular carcinoma in idiopathic hemochromatosis is more than 200 times greater than that of the control population [2,3]. Awai et al. [4] originally developed an experimental model of iron overload using ferric ion chelated with nitrilotriacetate (NTA). Intraperitoneal injections of Fe-NTA induced significant iron deposition in rat hepatic cells [5,6]. Repeated intraperitoneal injections of Fe-NTA were reported to induce acute and subacute renal proximal tubular necrosis and a subsequent high incidence of renal adenocarcinoma in male rats and mice [7–9]. Despite the pathological evidence for renal carcinogenesis mediated by Fe-NTA treatment, the role of iron or Fe-NTA has not been fully established, except for the induction of lipid peroxidation. Okada and his co-workers have shown that membrane lipid peroxidation, as seen biochemically by 2-thiobarbituric acid-reactive substances (TBARS) and histochemically by cold Schiff's method (detection of aldehydes), is one of the basic mechanisms of Fe-NTA-induced renal injury and is closely associated with renal carcinogenesis [10–14]. It can therefore be assumed that these renal oxidative injuries are associated with the alteration of the fundamental functions of the kidney, including reabsorption of filtered proteins from glomerular ultrafiltrate and their proteolysis.

In the present paper, based on the identification of a major kidney-specific protein, we demonstrate the alteration of proteolytic and endocytotic functions of the renal brush border membrane caused by Fe-NTA treatment.

2. Materials and methods

2.1. Animals and experimental groups

Male SPF slc: Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing 130 to 150 g (6 weeks of age) were used. They were kept in stainless steel cages and given commercial rat chow (Funabashi F-2, Chiba) as well as deionized water (Millipore Japan, Osaka) ad libitum. A total of thirty animals were divided into a time-course study group (Fe-NTA 15 mg Fe/kg body weight; 0, 1, 3, 16, and 24 h), a dose-dependency study group (1 h; Fe-NTA 0, 15, and 30 mg Fe/kg body weight), and subacute toxicity groups: *group A* (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); *group B* (3 weeks-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 days break, and 10 mg Fe/kg body weight for the next 4 days). Each subgroup contained 3 animals. The animals were sacrificed by decapitation and both kidneys of each animal were removed immediately. In the case of subacute toxicity experiments, the animals were sacrificed 24 h after the final injection.

2.2. Materials

Ferric nitrate enneahydrate, sodium carbonate, hydrogen peroxide, acetone, and ethanol were obtained from Wako (Osaka). Nitrilotriacetic acid disodium salt was obtained from Nacarai Tesque, Inc. (Kyoto). Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Cappel (Durham, NC) and Amersham, respectively. The protein concentration was measured using the BCA protein assay reagent obtained from Pierce.

2.3. Preparation and injection of Fe-NTA solution

The Fe-NTA solution was prepared immediately before use by the

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Abbreviations: Fe-NTA, ferric nitrilotriacetate; k-FABP, kidney fatty acid-binding protein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBARS, 2-thiobarbituric acid-reactive substances; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; TBS, Tris-buffered saline.

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method of Awai et al. [4] with only a slight modification. Ferric nitrate enneahydrate and nitrilotriacetic acid disodium salt were each dissolved in deionized water to form 300 mM and 600 mM solutions. They were mixed at a volume ratio of 1:2 (molar ratio 1:4) with magnetic stirring at room temperature. The pH was adjusted with sodium carbonate to 7.4. The Fe-NTA was injected intraperitoneally into the animals.

2.4. Partial purification of 17 kDa protein and NH_2-terminal sequence determinations

The total cytosolic proteins were dialyzed against 20 mM Tris-HCl (pH 8.0), and the proteins were then applied to an ion exchange column (TSKgel DEAE-5PW) (TOSO). The samples were eluted with a linear gradient of 100% solvent A (20 mM Tris-HCl, pH 8.0) to 100% solvent B (0.5 M NaCl/20 mM Tris-HCl, pH 8.0) within 30 min at a flow rate of 1.0 ml/min. The proteins were monitored at 280 nm. The proteins from HPLC were then separated by a sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Automated Edman NH_2-terminal sequence determinations were performed with an Applied Biosystem Gas-phase Sequencer (Models 470/120/900).

2.5. Antibodies

Polyclonal antiserum against α_{2U}-globulin prepared according to the method of Ghosh et al. [15] was raised by immunizing New Zealand White rabbits with α_{2U}-globulin in a 50% emulsion with complete Freund's adjuvant at a final volume of 0.5 ml. After a booster injection over a period of one month, anti-α_{2U}-globulin antiserum was obtained.

2.6. Western blot

Rat kidneys were homogenized with 1.15% KCl (9 ml/g). The kidney tissue homogenate (1 ml) was centrifuged for 10 min at 400 × *g* and then the supernatant was centrifuged for 60 min at 105,000 × *g* to obtain the cytoplasmic fractions, which were then treated with Laemmli sample buffer [16] 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.

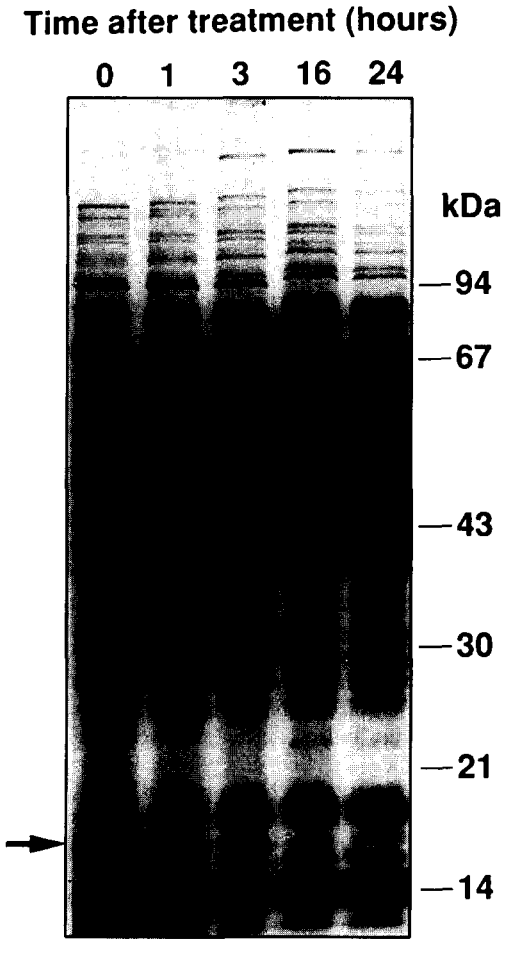

Fig. 1. Time-dependent changes in renal cytosolic proteins of rats treated with Fe-NTA (15 mg Fe per kg body weight). The arrow indicates a 17 kDa protein.

3. Results and discussion

As reported previously, a single intraperitoneal injection of Fe-NTA (15 mg Fe/kg body weight) leads to membrane damage assessed by the accumulation of lipid peroxidation-derived aldehydes [17]. Upon SDS-PAGE analysis, we found that this membrane damage was accompanied by significant alteration of the renal cytosolic proteins (Fig. 1). Particularly evident was the loss of a protein with apparent molecular mass of 17 kDa. In order to identify the 17 kDa protein, proteins from HPLC on a DEAE column were separated by SDS-PAGE and electroblotted onto a PVDF membrane for NH_2-terminal sequence analysis. Comparison of the 17 kDa protein sequence with the data base (GENETYX) indicated a 100% positive identification, over 16 residues, with the NH_2 terminus of the kidney fatty acid-binding protein (k-FABP) as follows:

NH_2-L-D-V-A-K-L-N-G-D-W-F-S-I-V-V-A-.

It is known that k-FABP, a proteolytically modified form of α_{2U}-globulin, is reabsorbed by endocytosis in the renal proximal tubules, is predominantly localized in the endosomes or lysosomes and is also associated with brush border membranes [18]. Whereas, α_{2U}-globulin is a secretory 18.7 kDa protein synthesized in the liver [19,20] and reabsorbed by a mechanism that includes the interaction of cationic groups with the molecule with anionic sites on the brush border membranes of the renal proximal tubules. It has been suggested that, during the time that α_{2U}-globulin is taken up from the urinary lumen into the endosomes, α_{2U}-globulin is proteolytically processed in the amino- and carboxyl-terminal regions and then converted to k-FABP by the protease associated with the brush border membrane. These facts lead to the assumption that the proteolytic conversion of α_{2U}-globulin to k-FABP followed by its endocytosis is an available index of structural and functional alterations of the brush border membrane of the renal proximal tubules caused by Fe-NTA.

It has been proposed that Fe-NTA can be easily activated by reducing agents including cysteine and γ-glutamylcysteine. These reducing agents are amply supplied by the decomposition of glutathione within the lumen by the action of γ-glutamyltranspeptidase and dipeptidase situated at the proximal tubular brush border membrane, thereby effectively promoted the membrane lipid peroxidation [12,21,22]. To determine the effect of Fe-NTA on the proteolytic and endocytotic functions of the kidney monitoring with k-FABP and α_{2U}-globulin as markers, rabbit polyclonal IgG against α_{2U}-globulin was raised and used for the immunochemical analysis of these proteins. In the control rats, an immunoblot analysis of the renal cytosolic proteins revealed that only a single protein band representing k-FABP

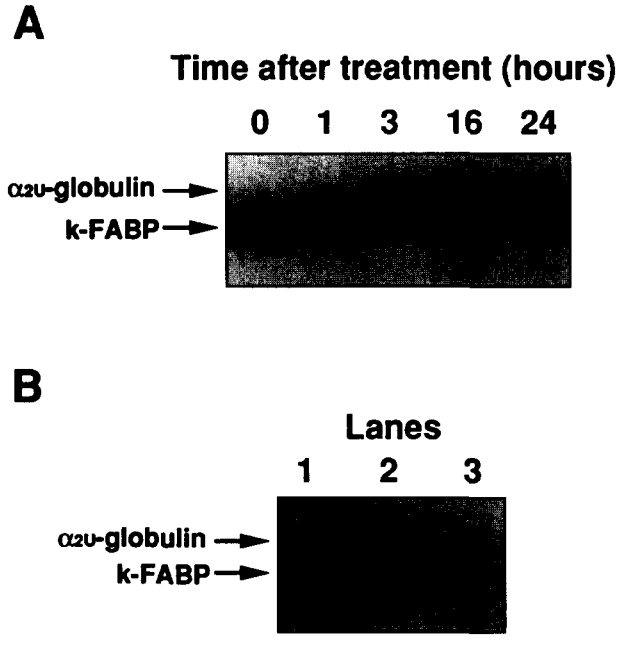

Fig. 2. Western blot analysis of k-FABP and α_{2U}-globulin in the renal cytosolic fraction of rats treated with Fe-NTA. (A) A time course study with a single injection of Fe-NTA (15 mg of Fe per kg body weight). (B) A subacute toxicity experiments with multiple injections of Fe-NTA. Lane 1, control; lane 2, group A (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 3, group B (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 days break, and 10 mg Fe/kg body weight for the next 4 days). Cytosolic proteins were isolated from the rat kidney and analyzed by Western blot using anti-α_{2U}-globulin antibody.

showed an apparent immunoreactivity with the anti-α_{2U}-globulin antibodies (Fig. 2A). A single injection of Fe-NTA (15 mg Fe/kg body weight) resulted in the time-dependent appearance of α_{2U}-globulin, accompanied by a decrease in k-FABP. These observations suggest that the endocytotic activity of proximal tubules in the reabsorption of a filtered protein (α_{2U}-globulin) from the glomerular ultrafiltrate was not altered; however, the proteolytic activity was progressively suppressed by treatment with Fe-NTA. It was observed that the suppression of proteolytic conversion of α_{2U}-globulin to k-FABP was clearly dependent upon the period of time after Fe-NTA treatment, because little or no suppression was observed for a period up to an hour; however, the suppression of the conversion started 3 h after Fe-NTA treatment (Fig. 2A). This and the fact that the renal membrane damage reached the maximum within an hour after Fe-NTA treatment (15 mg Fe/kg body weight) [17] suggest that the proteases in the renal brush border membranes were inactivated or depleted by an Fe-NTA-induced membrane lipid peroxidation. These renal proximal tubular dysfunctions caused by Fe-NTA treatment are consistent with the fact [23] that the injection of Fe-NTA causes a number of time-dependent morphological alterations in the structure of the renal proximal tubules including the loss of the brush border from the apical membrane of the epithelium at an early stage of injury. Furthermore, it should be noted that, although a single dose of Fe-NTA altered only the proteolytic activity of proximal tubular cells (Fig. 2A), multiple injections of Fe-NTA led to the suppression of both proteolytic and endocytotic activities of the kidney (Fig. 2B, lanes 2 and 3). The α_{2U}-globulin/k-FABP may play an important role in the transport and metabolism of fatty acids in the rat kidney. Thus, the inhibition of reabsorption of the filtered proteins may implicate severe damage of the proximal tubular epithelium, eventually leading to renal carcinogenesis.

Disturbances of the proximal tubular function are often observed in a lot of clinical situations. They may be caused by nephrotoxic agents such as certain antibiotics and chemotherapeutic drugs and by hypotension and/or ischemia. These conditions are often associated with membrane lipid peroxidation. Therefore, monitoring the proteolytic conversion of α_{2U}-globulin to k-FABP followed by its endocytosis may be useful to identify the chemicals that cause membrane damage in the rat kidney.

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