Preliminary Report

Purification and localization of a 25-kD porcine renal puromycin aminonucleoside-binding protein


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Abstract

Background. Reactive oxygen species (ROS) are considered to have a role in the progression of puromycin aminonucleoside (PAN) nephrosis. However, the exact mechanism by which PAN induces ROS in this model is little known. In the present study, we attempted to purify a candidate for the target protein from PAN nephrotoxicity.

Methods. Using PAN-affinity column chromatography, a series of PAN-binding proteins was isolated from porcine renal extracts. We produced a specific antibody against a 25-kD protein eluted from the PAN-affinity matrix, and then developed a method to purify this protein. A partial amino acid sequence of the 25-kD PAN-binding protein was determined, and its tissue distribution was examined by immunoblot and immunohistochemical studies.

Results. The purified 25-kD PAN-binding protein was identified as a renal homolog of a new member of NAD(P)H:quinone oxidoreductases (NQOs, EC 1.6.99.2) that suppress the semiquinone and superoxide anion formation in cells, designated NQO2. Immunoblot analysis revealed a higher expression of the 25-kD PAN-binding protein in the kidney, brain, and liver among porcine major organs. Immunohistochemical studies showed an intrarenal distribution of this protein in epithelial cells of the glomeruli and tubules, mesangial cells, and vascular smooth muscle cells.

Conclusions. We have purified the renal homolog of NQO2 as a PAN-binding protein, and shown its unique tissue expression. PAN may bind to the NQO2 homolog and inhibit its function in the renal target cells. This is presumed to result in an increase of ROS in the kidney with PAN nephrosis.

Key words: animal model; NAD(P)H:quinone oxidoreductase; nephrotic syndrome; puromycin aminonucleoside; reactive oxygen species

Introduction

Reactive oxygen species (ROS), including free radicals such as superoxide anion and hydroxyl radical, are mediators of ischaemic, toxic, and immune-mediated tissue injury. Numerous studies have shown that ROS are implicated in the progression of various kidney diseases in experimental animal models [1–4]. A single intravenous injection of puromycin aminonucleoside (PAN) induces massive proteinuria in animals, and early histological changes are similar to those of human minimal change nephrotic syndrome [5]. Thus, PAN nephrosis has been widely used as an animal model for human nephrotic syndrome. It has been demonstrated that ROS are also involved in the pathogenesis of PAN nephrosis [1,2,4]. Indeed, the administration of antioxidants, superoxide dismutase and allopurinol, can reduce proteinuria and prevent glomerular injury in PAN nephrosis [6]. However, the exact mechanism by which PAN induces ROS in this model is little known.

We have attempted to isolate renal target proteins from PAN nephrotoxicity, and recently purified a 17-kD PAN-binding protein from porcine kidney [7]. This protein was identified as the reported 17-kD protein kinase C inhibitor, but its function remained unclear. In the present study, we have purified another porcine renal PAN-binding protein with a molecular mass of 25 kD. This PAN-binding protein was identified as a renal homolog of a new member of NAD(P)H:quinone oxidoreductases (NQOs) [8], also known as DT-diaphorases (EC 1.6.99.2). Although this NQO member has not been purified or characterized as a protein so far, a role against oxygen toxicity is suggested [8,9]. We also examined the tissue distribution and intrarenal localization of the protein, by immunoblot analysis and immunohistochemical study using a specific antibody. Amongst porcine organs, a higher expression of the 25-kD PAN-binding protein was found in the kidney, mainly expressed in epithelial cells of the glomeruli and mesangial cells. We discuss the possibility that this new NQO member might be a target protein from PAN nephrotoxicity.
Subjects and methods

Purification of a 25-kD PAN-binding protein from porcine kidney (Method I)

PAN (100 mg; Sigma Chemica Co., St Louis, MO, USA) was coupled with 10 ml of activated CH-Sepharose 4B (Pharmacia LKB, Uppala, Sweden), according to the manufacturer’s instructions.

Fresh porcine kidneys were obtained from a local slaughterhouse. All purification steps were performed at 4 °C. Minced porcine kidneys (500 g) were homogenized with 1500 ml of buffer A (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) containing 0.1% Triton X-100. After centrifugation at 20000 g for 20 min, the supernatant was dialysed against buffer A. After centrifugation at 20000 g for 20 min, the supernatant (1300 ml) was applied to a PAN–Sepharose column (1.5 × 5.2 cm) pre-equilibrated with buffer A. The column was washed with 100 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 1 M NaCl, followed by 100 ml of buffer A. Thereafter, PAN-binding proteins were eluted with 30 ml of buffer A containing 10 mM PAN. The 25-kD protein was further purified. Pooled fractions of the eluent were dialysed against buffer B (10 mM potassium phosphate buffer, pH 7.0), and applied to a hydroxyapatite (Bio-Rad, Richmond, CA, USA) column (2.5 × 15.0 cm) pre-equilibrated with buffer B. The column was washed with buffer B, the non-bound fractions were pooled. The pooled fractions were dialysed against distilled water and freeze-dried. The concentrated sample was then applied to SDS–PAGE gels. After electrophoresis, the gels were lightly stained. The 25-kD protein bands were carefully cut, and the protein was eluted from the cut gels by using a Sample Concentrator (Model 1750; Isco Inc., Lincoln, Nebraska, USA).

Antibody production

Polyclonal antibody against the 25-kD porcine renal PAN-binding protein was raised in rabbit by subcutaneous injection of the purified protein, emulsified in the Freund’s adjuvant. Booster shots were given three times at 2-week intervals. The rabbit was bled 10 days after the final injection.

SDS–PAGE and immunoblot

SDS–PAGE was performed by the method of Laemmli [10]. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Immunoblot was carried out according to the method of Towbin et al. [11]. For the first antibody, serum from a rabbit immunized with the purified 25-kD porcine renal PAN-binding protein was used at a dilution of 1:500. For the second antibody, peroxidase-conjugated anti-rabbit IgG (Bio-Rad) was used at a dilution of 1:500.

Purification of the 25-kD PAN-binding protein from porcine kidney without using PAN-affinity column chromatography (Method II)

All purification steps were performed at 4 °C. At each step, immunoblot analysis probed with antibody against the 25-kD PAN-binding protein was performed, in order to select the protein-rich fractions.

Minced fresh porcine kidneys (500 g) were homogenized with 1500 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl. After centrifugation at 20000 g for 15 min, the supernatant was fractionated by 40–50% saturated ammonium sulphate. The precipitate was recovered by centrifugation at 20000 g for 20 min, and dissolved in buffer C (10 mM Tris–HCl, pH 7.4). After dialysis against buffer C, the sample was centrifuged at 20000 g for 20 min. The supernatant (370 ml) was applied to a DEAE–cellulose (DE52; Whatman, Kent, UK) column (5.0 × 2.5 cm) pre-equilibrated with buffer C. After the column was washed with buffer C, the column was developed with a linear gradient of 0–0.5 M NaCl. The 25-kD protein was eluted in fractions at ~0.10–0.16 M NaCl. The eluent was dialysed against buffer D (10 mM potassium phosphate buffer, pH 7.0) and applied to a hydroxyapatite (Bio-Rad) column (1.8 × 25.0 cm) pre-equilibrated with buffer D. The column was washed with buffer D, and the 25-kD protein passed through the column with buffer D. Pooled unbound fractions were dialysed against buffer C, and applied to a Q-Sepharose (Pharmacia LKB) column (1.5 × 5.2 cm) pre-equilibrated with buffer C. After the column was washed with buffer C, the column was developed with a linear gradient of 0–0.5 M NaCl. The 25-kD protein was eluted in fractions at ~0.27–0.30 M NaCl. The eluent was applied to an AcA54 (LKB, Bromma, Sweden) gel filtration column (2.5 × 80 cm) pre-equilibrated with buffer E (10 mM Tris–HCl, pH 7.4, 0.1 M NaCl). Thereafter, the column was washed with 700 ml of buffer E. Pooled 25-kD protein-rich fractions of the eluent were dialysed against buffer C, and then applied to a second Q-Sepharose (Pharmacia LKB) column (1.5 × 5.2 cm) pre-equilibrated with buffer C. After the column was washed with buffer C, the column was developed with a linear gradient of 0–0.5 M NaCl. The 25-kD protein was eluted in fractions at ~0.23–0.34 M NaCl in its pure form.

The protein concentrations in the fractions eluted at each chromatographic step were measured by the method of Bradford [12].

Amino acid sequence

The determination of the NH2-terminal amino acid sequence of the purified protein was performed with a model 477A protein sequencer equipped with an online 120A PTH analyser (Applied Biosystems, Foster City, CA, USA). Production and separation of peptides from the purified protein were carried out according to the method of Kawasaki et al. [13]. Separated polypeptides were also sequenced.

Analytical gel filtration

To determine the molecular mass of the purified protein, analytical gel filtration was performed as described previously [14].

Absorbance scanning of the purified 25-kD porcine renal PAN-binding protein

Absorbance of the purified protein solution was scanned with a spectrophotometer (Model 220A; Hitachi, Tokyo, Japan) at 300–600 nm.

Enzyme assays

To determine the NOQ activity of the purified protein, enzyme assays were performed according to the methods of...
Shaw et al. [15], using various quinones as substrates (2,6-dichlorophenolindophenol, menadione, 1,4-benzoquinone, 2,6-dimethylbenzoquinone, and naphthacenedione monohydrochloride).

Preparation of isotonic buffer-extractable fractions from porcine major organs

Each piece (1.0 g) of fresh porcine major organ (brain, heart, lung, liver, spleen, kidney, and small intestine) was homogenized with 3.0 ml Tris-buffered saline (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl). After centrifugation at 20000 g for 20 min at 4°C, the supernatants were stored at –80°C. Equal volumes of each sample were prepared for SDS–PAGE and immunoblot probed with antibody against the 25-kD PAN-binding protein.

Immunohistochemistry

Essential procedures for immunohistochemistry were previously described [16]. Briefly, a piece of fresh porcine kidney was embedded in compound (Miles Inc., Elkhart, IA, USA) and immediately frozen at −80°C. The embedded specimen was sectioned at 3 μm. Hydrated sections were incubated with serum from a rabbit immunized with the purified 25-kD porcine renal PAN-binding protein or with normal rabbit serum at a dilution of 1:100 at 4°C overnight. Immunohistochemical detections were performed using a Vectastain avidin-biotin-peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA).

Results

Purification of a 25-kD PAN-binding protein from porcine kidney (Method I)

By using PAN-affinity column chromatography, several PAN-binding proteins were isolated from a detergent extract of porcine kidney (Figure 1). Among them, a 25-kD protein was further purified by hydroxylapatite column chromatography, and by elution from SDS–PAGE gels. The purified protein produced a single band on an SDS–PAGE gel (Figure 2a, lane 2).

Antibody production

Polyclonal antibody against the purified 25-kD porcine renal PAN-binding protein was raised in a rabbit. Figure 2b of immunoblot shows its reactivity.

Purification of 25-kD PAN-binding protein from porcine kidney without using PAN-affinity column chromatography (Method II)

We next established a purification method for the 25-kD PAN-binding protein from an isotonic buffer extract of porcine kidney, by using DEAE-cellulose column chromatography, hydroxylapatite column chromatography, Q-Sepharose column chromatography, gel filtration, and second Q-Sepharose column chromatography. To determine fractions containing the 25-kD PAN-binding protein at each step, immuno-
Fig. 3. Purification of the 25-kD porcine renal PAN-binding protein, without using PAN-affinity column chromatography. (a) SDS–PAGE gel stained with Coomassie brilliant blue R-250. The numbers on the left indicate molecular mass standards as in Fig. 1. (b) Immunoblot probed with rabbit antibody against the 25-kD porcine renal PAN-binding protein and with peroxidase-conjugated anti-rabbit IgG. Lane 1. Pooled fraction after DEAE–cellulose column chromatography. Lane 2. Pooled fraction after hydroxylapatite column chromatography. Lane 3. Pooled fraction after Q-Sepharose column chromatography. Lane 4. Pooled fraction after gel filtration. Lane 5. Purified 25-kDa protein after second Q-Sepharose column chromatography.

Fig. 4. Protein solution of the purified 25-kD porcine renal PAN-binding protein and its absorbance scanning pattern. (a) Distilled water. (b) Protein solution of the purified 25-kD porcine renal PAN-binding protein. The purified protein solution is visible in light yellow. (c) Absorbance scanning pattern of the purified 25-kD porcine renal PAN-binding protein. There are two peaks close to 370 nm and 460 nm.

blots probed with specific antibody were performed. Figure 3 shows the results of each step in the purification of the 25-kD PAN-binding protein from porcine kidney extract. The yield of 1.6 mg of the purified protein was obtained by a series of procedures. The purified 25-kD protein solution had a light yellow colour (Figure 4b), and the absorbance scanning pattern of this solution revealed two peaks close to 370 nm and 460 nm (Figure 4c). These results indicated that the purified 25-kD PAN-binding protein was a flavoprotein.

On analytical gel filtration, the molecular weight of the purified protein was estimated at 18 000 (data not shown). The molecular mass of the purified protein on SDS–PAGE gels under both reduced and non-reduced conditions was equal (25-kD; data not shown). From these results, the purified protein was considered to exist as a monomer and a non-globular protein.

Partial amino acid sequence of the 25-kD porcine renal PAN-binding protein

The NH₂ terminus of the purified 25-kD porcine renal PAN-binding protein was blocked. The purified proteins obtained by both purification methods (I and II) were digested with lysylendopeptidase, and cleavage products were separated by reverse-phase high-performance liquid chromatography (Figure 5). The pattern of elution profile was the same in the two samples. The separated digested peptides were then sequenced. The amino acid sequences of eight lysylendopeptidase-digested fragments of the purified protein were determined. Figure 6 summarizes the determined 111 amino acid sequences of the 25-kD porcine renal PAN-binding protein. These sequences were identical (82%) to those deduced from nucleotide sequence of a cDNA encoding the 25-kD human liver protein, reported as a second form of NQO [8]. Thus, our purified protein was identified as a porcine renal homolog of NQO₂.

Enzymatic assays of the 25-kD porcine renal PAN-binding protein on NQO activity

NQO activity of the purified 25-kD porcine renal PAN-binding protein, obtained by Method II, was examined. The activity was assayed using six different quinone substrates, but the protein did not show any NQO activity (data not shown).

Distribution of the 25-kD PAN-binding protein in porcine major organs

Immunoblot analysis probed with antibody against the 25-kD PAN-binding protein indicated a higher expression of this protein in the brain, liver, and kidney than in other main organs (Figure 7).
Fig. 5. Reverse-phase high-performance liquid chromatography elution profile of lysylendopeptidase-digested peptides of the purified 25-kD porcine renal PAN-binding protein. The purified protein was digested with lysylendopeptidase according to the method of Kawasaki et al. [13]. Cleavage products were separated by reverse-phase high-performance liquid chromatography on a C18 column with a 60-min gradient (0–64% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. Designated fragments (L1–L8) were then sequenced.

Discussion

In this study, we purified a 25-kD PAN-binding protein, at first as a denatured protein, from porcine kidney, and produced a specific antibody against the purified protein. By using the antibody as a probe, we next developed a method to purify this protein in a native form. The purified protein had characteristics of a flavoprotein. On the basis of its molecular mass on SDS–PAGE and partial amino acid sequence, this protein was identified as a porcine renal homolog of NQO2 [8].

NQOs (DT-diaphorases) are flavoproteins that catalyse the two-electron reduction of quinones to hydroquinones using either NADH or NADPH as the electron donor. By one-electron reduction of quinones, semiquinones are generated which react spontaneously with molecular oxygen to give the parent quinone and superoxide anion via a redox cycling mechanism. Therefore, NQOs have a function in the cellular detoxification of quinones, by decreasing the formation of the reactive semiquinone intermediate [17].

It has been suggested that several different forms of NQOs are present in mammalian tissues. A cDNA encoding human liver NQO2 was first isolated by Jaiswal et al. [8] as a second member of the NQO family. This cDNA codes for a low molecular weight protein (Mw=25,956), and its deduced amino acid sequences were 48% identical with those of the human dioxine-inducible NQO [8]. The genomic organization of...
Fig. 7. Distribution of the 25-kD PAN-binding protein in porcine major organs. (a) SDS–PAGE gel stained with Coomassie brilliant blue R-250. The numbers on the left indicate molecular mass standards as in Fig. 1. (b) Immunoblot probed with rabbit antibody against the 25-kD porcine renal PAN-binding protein and with peroxidase-conjugated anti-rabbit IgG. Lane 1. Purified 25-kD porcine renal PAN-binding protein. Lanes 2–9. Extracts from normal porcine major organs: 2, cerebrum; 3, cerebellum; 4, heart; 5, lung; 6, liver; 7, spleen; 8, kidney; 9, small intestine.

Fig. 8. Immunohistochemical localization of the 25-kD PAN-binding protein in porcine kidney. Porcine renal sections (original magnification, ×1000) focusing on the glomerulus. Details of the conditions are given in the text. (a) Section incubated with rabbit antiserum against the 25-kD PAN-binding protein. (b) Control section incubated with normal rabbit serum. Immunoreactivities are observed in glomerular epithelial cells (thick arrows), mesangial cells (thin arrows), and epithelial cells of the Bowman’s capsule (arrow heads).

of the NQO2 gene is characterized by the presence of an antioxidant response element and xenobiotic response elements in the promoter region [9]. This suggests that the expression of this gene is inducible in response to oxidative stress and toxic agents in cells [18,19]. However, the potential substrates for the cDNA-derived NQO2 protein have not been identified [9]. In the present study, we were unable to find catalyzing activities of the purified porcine renal NQO2 homolog on a selection of quinones, which are known as common substrates for NQOs. These results suggest that NQO2 carries an activity for quite specific substrates.

According to the results of Northern blot analysis by Jaiswal [9], the human NQO2 gene was expressed in several human tissues, but not in the placenta. Our immunoblot analysis probed with antibody against the NQO2 homolog indicated a higher expression of the protein in the brain, liver, and kidney, among porcine major organs. Thus, NQO2 appears to have a tissue-specific function in these organs. Our immunohistochemical studies showed a specific localization of the NQO2 homolog mainly in neurons in the brain, and in epithelial cells of bile ducts in the liver (manuscript in preparation). In the kidney, its unique expression was found in glomerular epithelial cells, mesangial cells, epithelial cells of the Bowman’s capsules, epithelial cells of distal tubules and collecting ducts, and vascular smooth muscle cells.

It is now generally accepted that ROS have roles in the pathogenesis of PAN nephrosis [4]. Since early histological changes mainly occur in glomerular epithe-
Fig. 9. Immunohistochemical localization of the 25-kD PAN-binding protein in porcine kidney. Porcine renal sections (original magnification, ×400) focusing on distal tubules (a) and (b), collecting ducts (c) and (d), and vessels (e) and (f). Details of the conditions are given in the text. (a, c, and e) Sections incubated with rabbit antiserum against the 25-kD PAN-binding protein. (b, d, and f) Control sections incubated with normal rabbit serum. Immunoreactivities are observed in epithelial cells of distal tubules and collecting ducts, and vascular smooth muscle cells.

lial cells in PAN nephrosis, these cells have been considered to be the targets for PAN nephrotoxicity [20]. Recently, Kawaguchi et al. [21] demonstrated that cultured glomerular epithelial cells enhance the generation of ROS in response to the exposure to PAN. Contrary to this, the continuous administration of PAN into animals produces additional histological changes: glomerulosclerosis, proliferation of mesangial cells, adhesions of glomerular basement membranes to the Bowman’s capsules, and crescent formations [16,22]. Thus, a variety of glomerular components seem to be affected by PAN in this animal model. Indeed, Zent et al. [23] recently demonstrated that PAN inhibits mesangial cell function by stimulating production of ROS. Several studies using scavengers of ROS suggested a specific role for superoxide anion and hydrogen peroxide [24], xanthine oxidase-generated superoxide anion [6], and hydroxyl radical [25] in this animal model. Accordingly, the generation of these ROS in the target cells from PAN nephrotoxicity seems to result from a complex series of reactions.

Although the potential substrates for NQO₂ have not been identified as yet, its structural characteristics suggest that NQO₂ acts as an intrinsic antioxidant enzyme. As shown in this study, the renal homolog of NQO₂ has a PAN-binding property, and is expressed in target cells of injury in PAN nephrosis. We therefore suggest that the renal NQO₂ homolog is a candidate for the target protein from PAN nephrotoxicity. It could be considered that an enzymic dysfunction of the NQO₂ homolog caused by PAN participates in the generation of ROS including the superoxide anion in the target cells. This possibility is supported by the fact that the administration of adriamycin (a cytotoxic anti-tumour quinone) induces nephrosis in animals with morphological changes similar to those observed in PAN nephrosis [26]. In this animal model, adriamycin appears to be converted to a semiquinone metabolite by one-electron reduction, which is able to cause superoxide anion generation via a redox cycling mechanism [4].

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References


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