Nitric oxide inhibits the shedding of the glycosylphosphatidylinositol-anchored dipeptidase from porcine renal proximal tubules

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NO is related to the pathological condition acute renal failure, in which we previously observed that the level of soluble dipeptidase in urine was decreased. In this study the role of NO in the shedding of the glycosylphosphatidylinositol (GPI)-anchored form of renal dipeptidase (RDPase) was examined. The NO donors sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine rapidly inhibited the release of RDPase from porcine kidney proximal tubules. The substrate of NO synthase, L-Arg, also inhibited the release of RDPase, and this effect was reversed by the NO synthase inhibitor *N*^ω-nitro-L-arginine methyl ester. Western-blot analyses using antibodies raised against porcine RDPase and the inositol-1,2-cyclic monophosphate moiety formed on phospholipase C cleavage of the GPI anchor demonstrated that SNP mediated its inhibitory effect on the release of RDPase via a GPI-specific phospholipase C (GPI-PLC). Peroxynitrite

scavengers (deferoxamine and superoxide dismutase) or reducing agent (dithiothreitol) did not affect SNP's inhibition of the release of RDPase. Exposure to the G-protein activator AIF₄⁻ mimicked the L-Arg effect in the presence of a low concentration of L-Arg, and the effect was completely reversed by U73122, an intracellular phosphatidylinositol-specific PLC (PI-PLC) inhibitor. These results suggest a signal-transduction pathway involving NO, which is produced by NO synthase(s) following activation of a G-protein-coupled PI-PLC, resulting in inhibition of the GPI-PLC that cleaves and releases RDPase. Therefore, this indicates a role for NO as an inhibitory regulator of the shedding of the GPI-anchored RDPase in acute renal failure.

Key words: acute renal failure, GPI-specific phospholipase C, nitric oxide synthase, renal dipeptidase.

INTRODUCTION

In numerous mammalian cells and tissues, NO production from the terminal guanidino nitrogen of L-Arg is catalysed by NO synthases (NOSs), of which there are three isoforms [1–3]. NO is a highly reactive free radical involved in a number of physiological and pathological processes [4], such as vasodilation, neurotransmission and immune-mediated cytotoxicity. NO functions as a cytotoxic agent through its oxidative metabolites, nitrite and peroxynitrite, on invading micro-organisms in macrophages or on tumour cells, and induces death of various cell lines through apoptosis [5,6]. Intraperitoneally injected lipopolysaccharide, which is a cell-protective factor of bacteria, has also been shown to induce inducible NOS and further the elevation of NO in the kidneys and urinary bladder. The biologically active component of lipopolysaccharide, lipid A, also induces NO generation by renal proximal tubules and leads to NO-dependent cytotoxicity of tubular cells, causing endotoxaemia and acute renal failure accompanied by a sharp increase in cGMP within 24 h [7–10].

It has also been shown that NO acts as a signalling molecule by stimulating the soluble guanylate cyclase to yield the second messenger cGMP [11–13], which controls a variety of biological functions. The action of NO via cGMP is well established in the relaxation of vascular smooth muscle, which appears to be mediated by cGMP-dependent protein kinase Iα [14]. Sodium nitroprusside (SNP), an NO donor, or proinflammatory cytokines

(tumour necrosis factor-α, interleukin-6 and interleukin-8) dramatically elevate cGMP levels in cultured human airway smooth-muscle cells [15], in human fetal neuronal cells [16] and in urine accompanying pathological processes, such as acute urinary tract infections and renal transplant rejection [7,17,18]. These cytokines, as well as NO, also induce apoptotic and necrotic cell shedding from human proximal tubular epithelial cell monolayers, which is associated with acute tubular injury in sepsis [19]. In our *in vitro* system of porcine proximal tubules, carp bile (from *Ctenopharyngodon idellus*), which causes acute renal failure [20,21], produced NO and simultaneously decreased the release of the glycosylphosphatidylinositol (GPI)-anchored renal dipeptidase (RDPase; also known as membrane dipeptidase and dehydropeptidase I; EC 3.4.13.19) [22]. These observations raise the possibility that NO may play an important role in suppressing the shedding of RDPase, as well as in the inflammation and apoptosis of proximal tubules, through certain signal-transduction mechanisms.

In proximal tubules RDPase has been shown to be released both *in vitro* and *in vivo* from its GPI anchor through the action of a GPI-specific phospholipase C (GPI-PLC) [23,24]. The soluble form of RDPase has been suggested to be a marker of acute renal failure because its activity in the urine of such patients was decreased significantly compared with normal volunteers [25,26]. Why the release of RDPase is lowered in acute renal-failure patients, and the mechanism by which it occurs, remain unknown.

Abbreviations used: CRD, cross-reacting determinant; GPI, glycosylphosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; KRH, Krebs/Ringer/Hepes; L-NAME, *N*^ω-nitro-L-arginine methyl ester; NOS, NO synthase; PI-PLC, phosphatidylinositol-specific phospholipase C; RDPase, renal dipeptidase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

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However, we propose that the release of RDPase from its GPI anchor may be related to the level of NO in the urine, as NO is elevated in disease conditions of the kidney and urinary tract [17]. NO may thus play an important role (or roles) in regulating the action of the GPI-PLC responsible for the cleavage and release of RDPase.

In the present study, to examine the effect of NO on the activity of the GPI-PLC that releases RDPase and to investigate the related signalling pathway, porcine proximal tubules were incubated with NO-regulating agents, including SNP, *S*-nitroso-*N*-acetylpenicillamine (SNAP), *N*^ω-nitro-L-arginine methyl ester (L-NAME) and L-Arg. The nitrite concentration and the released RDPase were determined in the incubation supernatant after treatment with these agents, and the action of GPI-PLC was assessed by the identification of the inositol-1,2-cyclic monophosphate cross-reacting determinant (CRD) on the released RDPase.

MATERIALS AND METHODS

Materials and antibodies

Porcine kidneys were supplied by the Samho Slaughter House (Kwangju, Korea) and were transferred to the laboratory immediately after death. Cilastatin was a kind gift from Dr Helmut Kropp (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.). SNP, L-Arg, 1,3-dimethyl-2-thiourea, SNAP, L-NAME, deferoxamine mesylate, superoxide dismutase and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sulphanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, AlCl₃, NaF, U73122 {1-[6-((17β)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-dione}, 8-Br-cGMP, dibutyl-8-Br-cGMP and 8-Br-cAMP were also from Sigma-Aldrich. The bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL, U.S.A.). CNBr-activated Sepharose CH 4B and the enhanced chemiluminescence (ECL[®]) detection kit for Western-blot analysis were from Amersham Bioscience (Little Chalfont, Bucks, U.K.). SNAP and U73122 were dissolved in DMSO and the final concentration of DMSO was less than 1% (v/v). All procedures were carried out at 4 °C unless otherwise stated. The polyclonal antiserum raised against bacterial phosphatidylinositol-specific PLC (PI-PLC)-solubilized affinity-purified porcine RDPase was generated in rabbits [27]. The anti-CRD antibodies, which exclusively recognize the inositol-1,2-cyclic monophosphate CRD epitope that is generated by PLC cleavage of the GPI anchor, were isolated from the bulk of the anti-RDPase serum by affinity chromatography on a column of immobilized variant surface glycoprotein [28].

Preparation of porcine proximal tubules

Proximal tubules were prepared fresh for each experiment with the protocol described previously [23]. After perfusion of the kidneys with chilled PBS, the sliced cortex was homogenized with a polytron tissue homogenizer and a glass-teflon homogenizer (GlasCol, Terre Haute, IN, U.S.A.). The remnant from the crude homogenate that passed through a 253 μm nylon mesh, and was then retained on an 83 μm mesh, was washed by centrifugation (600 g, 5 min each) with chilled PBS and twice with Krebs/Ringer/Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 6 mM glucose and 25 mM Hepes, pH 8.0). The final pellet was resuspended in 5 vol. of KRH buffer and is referred to as the proximal tubules. The proximal tubules were used as the source of GPI-PLC as well as an RDPase-enriched compartment, i.e. the substrate of GPI-PLC. The proximal tubules were incubated in the various experiments followed by centrifugation (18000 g,

5 min), and the resulting supernatants referred to as the incubation supernatants.

Treatment of proximal tubules with various agents

In typical assays, the reaction mixtures (500 μl) containing proximal tubules and various agents, such as SNP, SNAP or L-Arg, were incubated for 30 min at 37 °C in a concentration- or time-dependent manner. To remove nitrite and peroxynitrite, 1,3-dimethyl-2-thiourea (10 or 100 μM), and deferoxamine mesylate (1 or 2 mM) or superoxide dismutase (0.1 or 0.5 units), respectively, were administered to the proximal tubules in the presence of 0.25 mM SNP. At 0.25 mM SNP, proximal tubules were treated with reduced glutathione (10 or 100 μM) and dithiothreitol (10 or 100 μM). To examine the signalling pathway of NO, proximal tubules were incubated with increasing concentrations of lipophilic cGMP analogues (8-Br-cGMP and dibutyl-8-Br-cGMP) or 8-Br-cAMP. Proximal tubules were pretreated with L-NAME (0, 1, 5 and 10 mM), and then reacted with L-Arg (10 mM). Proximal tubules were also pretreated with U73122 (0, 10 and 20 μM), then incubated further in the presence of AlF₄⁻ (40 μM AlCl₃ and 10 mM NaF). The proximal tubules were subjected to centrifugation as described above to obtain the incubation supernatants.

Purification of RDPase

Proximal tubules were incubated with *Bacillus cereus* PI-PLC (1 unit/50 mg of protein) for 2 h at 37 °C followed by centrifugation (18000 g, 5 min). The supernatant was subjected to ammonium sulphate precipitation (50–75% saturation). The final pellet was resuspended in Tris buffer (10 mM Tris/HCl, pH 7.6/0.5 M NaCl) followed by dialysis, and then subjected to cilastatin-Sepharose 4B affinity chromatography to purify RDPase (2156-fold purification) [27,29]. The protein concentrations were determined using a bicinchoninic acid assay kit with BSA as a standard. For Western-blot analysis, the incubation supernatants (140 μg) obtained following SNP treatment of the proximal tubules were loaded on to cilastatin-Sepharose 4B columns using 100 μl of resin in disposable 1 ml syringes, which were activated with 1 ml each of acetate buffer (0.1 M acetate, pH 4.0/0.5 M NaCl) and borate buffer (0.1 M borate, pH 8.0/0.5 M NaCl) three times in turn. After thorough washing with Tris buffer, the bound RDPase was eluted with 40 μl of 0.5 mg/ml cilastatin in Tris buffer. The eluates were mixed with SDS sample buffer and subjected to Western-blot analysis.

Assay of RDPase

The released RDPase in the incubation supernatants was assayed by the three-enzyme coupled spectrofluorimetric method [23] with slight modification as described by Ito et al. [30] using the following reaction mixture (2.5 ml): 1.5 μM L-Ala-L-Ala, 0.4 units of L-Ala dehydrogenase, 1.5 μM β-NAD⁺, 0.06 units of diaphorase and 0.1 μM resazurin sodium in 12.5 mM Na₂CO₃, pH 9.0. Resorufin, the fluorescent product of the coupled reactions, was measured spectrofluorometrically using its excitation and emission maxima of 568 and 589 nm, respectively (JASCO Co., Tokyo, Japan). The purified RDPase was assayed by the UV spectrophotometric method using glycyl-dehydrophenylalanine as the substrate [31] at 275 nm, based on its molar absorption coefficient ($\epsilon = 1.56 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and was expressed as μmol of glycyl-dehydrophenylalanine hydrolysed/min per ml.

Determination of nitrite

Aliquots of the incubation supernatant (100 μ l) were transferred to 96-well plates and mixed with 100 μ l of Griess reagent [0.8 % sulphanilamide/0.75 % *N*-(naphthylethylene)diamine in 85 % H_3PO_4] [8]. After a 15 min incubation at room temperature, the nitrite concentrations were determined by measuring the absorbance at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) and were expressed as nmol/mg of protein. Sodium nitrate was used as the nitrite standard up to 100 μ M, where the slope was linear.

Western-blot analyses

Western-blot analyses were carried out according to Park et al. [23]. The protein bands resolved on an SDS/polyacrylamide (12 %) gel were transferred to a nitrocellulose filter. The filter was incubated for 1 h with either the polyclonal antiserum raised against RDPase in blocking buffer (3 % non-fat dry milk and 0.5 % Tween 20 in PBS) or the anti-CRD serum in a milk-free blocking buffer. The bound primary antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG and the immune complex was identified with an ECL[®] kit. To destroy the CRD, the incubation supernatants were incubated with 1 M HCl for 30 min at 23 °C, neutralized with NaOH, and acetone-precipitated to remove excess salt prior to SDS/PAGE [23].

Data presentation

For data analysis, the Student's *t* test was performed by the SigmaPlot program and a *P* value of less than 0.05 was considered significant. Comparisons were made with the control as described in each legend.

RESULTS

SNP and SNAP inhibit the release of RDPase from proximal tubules

Incubation of the kidney proximal tubules at 37 °C for 30 min resulted in the release of RDPase into the incubation supernatant as described previously [23], and this control release is expressed as 100 %. Release of RDPase from the proximal tubules decreased with increasing concentrations of the NO donor SNP and was 39.6 ± 5.1 % at 1 mM SNP ($P < 0.01$ versus control; Figure 1A). SNAP also reduced the release of RDPase from the proximal tubules (Figure 1A) but to a lesser extent than SNP. In a time-course experiment the inhibition of RDPase release (Figure 1B) was noticeably effective at 0.5 mM SNP in relation to the control at 10 min, and thereafter the release of RDPase decreased to less than 50 % of the control. Administration of 100 μ M 1,3-dimethyl-2-thiourea, an NO-scavenging agent, to proximal tubules in the presence of 0.5 mM SNP completely restored the release of RDPase (Figure 1D). The suppression of RDPase release by SNAP was also recovered with 1 and 5 mM 1,3-dimethyl-2-thiourea to 89.6 ± 7.0 and 101.9 ± 4.8 %, respectively (compared with 70.4 ± 4.4 % for 1 mM SNAP; $P < 0.05$, $n = 3$).

Because of the rapid inhibitory action of SNP on the release of RDPase, we investigated whether NO may directly modulate the activity of RDPase. Although the inhibition of RDPase release was very effective, neither SNP nor SNAP significantly affected the activity of the purified RDPase (Figure 1C). Together these data demonstrate that NO does not act by inhibiting the activity of RDPase itself but rather inhibits the release of RDPase from the proximal tubules.

Considering that SNP is more potent than SNAP, we checked whether cyanide, which is released from SNP prior to NO generation, may have caused any unspecific side effects. However, cyanide did not affect RDPase release up to a concentration of 1 mM (results not shown).

L-Arg reduces the release of RDPase via NO production

To assess the action of NO on the release of RDPase in more detail, proximal tubules were incubated with various concentrations of L-Arg, a substrate of NOS, followed by determination of nitrite concentration and RDPase released into the incubation supernatant (Figure 2A). The concentration of nitrite in the incubation supernatant increased with increasing concentrations of L-Arg up to 3.3-fold at 20 mM, whereas the release of RDPase decreased in inverse proportion to the concentration of L-Arg (release was 25.1 ± 3.1 % at 20 mM), thus demonstrating the inhibitory action of L-Arg on the release of RDPase via production of NO. The reciprocal correlation between RDPase release and nitrite concentration is demonstrated by the crossing-over of the two lines as shown in Figure 2(A). Half-maximal inhibition of RDPase release was observed at an L-Arg concentration of 8.7 mM, which corresponds to a nitrite concentration of 1.8 nmol/mg of protein, similar to the result obtained previously with carp bile [22]. These data are in good agreement with the results in Figure 1 where the direct donors of NO (SNP and SNAP) inhibited the release of RDPase from the proximal tubules.

L-NAME nullifies the effect of L-Arg on the release of RDPase

Proximal tubules were incubated with the cell-permeable L-NAME, an NOS inhibitor, in order to examine whether L-Arg acts on the release of RDPase via NO generated by NOS. Proximal tubules were pretreated with L-NAME for 5 min to inhibit NOS and then incubated with 10 mM L-Arg for 30 min. NO production was increased by 2.6-fold with L-Arg treatment (1.96 ± 0.19 versus 0.75 ± 0.12 nmol/mg of protein for the control; $P < 0.05$; Figure 2B). L-NAME diminished the L-Arg-stimulated nitrite production in a concentration-dependent manner to control levels, and significantly restored the release of RDPase that was evident in the presence of 10 mM L-Arg. At 10 mM L-NAME the release of RDPase was restored to 80.9 ± 6.8 % ($P < 0.05$) of the control. Taken together, these data demonstrate that NO, which had been produced from L-Arg by NOS, decreased the release of RDPase from the proximal tubules.

Deferoxamine mesylate and superoxide dismutase do not restore the SNP-suppressed release of RDPase

A reduction in the release of RDPase could be a cytotoxic effect caused by peroxynitrite. To test this possibility, proximal tubules were incubated either with deferoxamine mesylate, which inhibits the oxidative action of peroxynitrite that is formed by the reaction between NO and superoxide radicals, or with superoxide dismutase, which converts superoxide into H_2O_2 and O_2 . Deferoxamine mesylate (1 and 2 mM) and superoxide dismutase (0.1 and 0.5 units) alone did not affect the release of RDPase from the proximal tubules and failed to restore the decrease in RDPase release caused by 0.25 mM SNP (results not shown), demonstrating that the effect of SNP did not result from oxidation of the proximal-tubular membrane by peroxynitrite.

Figure 1 Effects of SNP and SNAP on the release of RDPase and on the activity of purified RDPase

(A) Proximal tubules were incubated with various concentrations of either SNP or SNAP. Incubation of proximal tubules alone was used as control. After incubation for 30 min at 37 °C, the activity of the RDPase released into the incubation supernatant was measured by the spectrofluorometric method as described in the Materials and methods section. Results are expressed as a percentage (means $\pm$ S.D., $n = 3-7$) compared with the arbitrary fluorescence intensity of the control activity (* $P < 0.01$). (B) Proximal tubules were incubated for the indicated times at 37 °C alone (control) or with 0.5 mM SNP. The activity of the RDPase released from the proximal tubules is expressed as a percentage (means $\pm$ S.D., $n = 3$) compared with the control untreated proximal tubules for 60 min incubation (100%). (C) Purified RDPase (130 m-units/ml) was incubated with increasing concentrations of SNAP or SNP and its activity measured by the UV spectrophotometric method using glycyl-dehydrophenylalanine as a substrate. (D) Proximal tubules were pre-incubated for 5 min at 37 °C with 10 or 100 μ M 1,3-dimethyl-2-thiourea (DMTU), then supplemented with 0.5 mM SNP and further incubated for 30 min. Alternatively, proximal tubules were incubated in KRH buffer alone (Con) for 30 min prior to determination of the RDPase released into the incubation supernatants. The results are expressed as a percentage compared with the control (means $\pm$ S.D., $n = 3$; * $P < 0.01$ versus control and ** $P < 0.01$ versus SNP alone).

Dithiothreitol does not reverse the inhibitory effect of SNP on the release of RDPase

Dithiothreitol is known to remove NO groups from nitrosylated cysteine residues in proteins [32,33]. If the inhibitory effect of SNP on the release of RDPase is accomplished by S-nitrosylation, dithiothreitol would be expected to reverse the inhibitory effect. Although dithiothreitol appeared to reverse suppression of the release of RDPase ($78.0 \pm 3.6\%$ at 10 μ M dithiothreitol and $79.6 \pm 1.1\%$ at 100 μ M compared with $66.0 \pm 7.6\%$ for the control) by a low concentration of SNP (0.25 mM), it did not show a dose-dependent effect and did not affect the release of RDPase in either the presence or absence of 0.5 mM SNP (results not shown). In addition, the failure of SNP and SNAP to inhibit the activity of purified RDPase (Figure 1) also supports the conclusion that SNP does not induce S-nitrosylation of either RDPase itself or of any enzyme involved in its cleavage and release from the membrane.

NO inhibits the GPI-PLC that cleaves and releases RDPase from the membrane

We investigated whether the GPI-PLC, known to hydrolyse the GPI anchor of RDPase [23,24], was involved in the observed inhibitory effect of NO on the release of RDPase. Cleavage of the GPI anchor on RDPase by the GPI-PLC results in the formation of the inositol-1,2-cyclic monophosphate CRD epitope on the released protein [23,28]. Aliquots (140 μ g) of the incubation supernatants recovered from proximal tubules incubated with increasing concentrations of SNP were subjected to cilastatin-affinity chromatography on a mini scale and the purified released RDPase was analysed by Western blotting using a polyclonal anti-RDPase serum (Figures 3A and 3B) or an anti-CRD serum (Figures 3C and 3D). The anti-RDPase antibody recognized a single band of 47 kDa, with bacterial PI-PLC-solubilized RDPase used as a positive control (Figures 3A and 3B, lanes 1). As expected, the anti-CRD antibody recognized the bacterial PI-

Figure 2 Effects of L-Arg and L-NAME on NO production and on the release of RDPase

(A) Proximal tubules were incubated for 30 min at 37 °C either alone or with various concentrations of L-Arg (1, 5, 10 or 20 mM) followed by centrifugation (18000 *g*, 5 min). The nitrite concentration in the incubation supernatants was determined with the Griess reagent using a 96-well microplate reader at 540 nm, and the activity of the released RDPase was measured using the spectrofluorometric method as described in the Materials and methods section (means $\pm$ S.D., $n = 3$). The nitrite concentration giving half-maximal inhibition of RDPase release was determined by finding the L-Arg concentration at this inhibition and correlating it with the nitrite production at that L-Arg concentration, as indicated by the arrow. (B) Proximal tubules were incubated for 5 min at 37 °C with increasing concentrations of L-NAME (0, 1, 5 or 10 mM) and further incubated for 30 min in the absence (con) or presence of 10 mM L-Arg. The nitrite concentration in the incubation supernatants was determined with the Griess reagent (means $\pm$ S.D., $n = 3$; * $P < 0.05$ versus control and ** $P < 0.05$ versus L-Arg alone). The activity of the released RDPase is expressed as a percentage compared with the arbitrary fluorescence intensity of the control ($n = 3$; * $P < 0.05$ versus control and ** $P < 0.05$ versus L-Arg alone).

PLC-solubilized RDPase (Figures 3C and 3D, lanes 1). The intensity of the bands in the incubation supernatants recognized by the anti-RDPase and anti-CRD sera gradually decreased with increasing concentrations of SNP (Figure 3A, lanes 2–5, and Figure 3C, lanes 2–5, respectively), in correlation with the released RDPase activity profile (Figure 1). Treatment of the samples prior to electrophoresis with 1 M HCl, which selectively destroys the inositol-1,2-cyclic monophosphate epitope [28], failed to prevent recognition by the anti-RDPase antiserum (Figure 3B, lanes 2–5) but completely prevented binding of the anti-CRD antiserum (Figure 3D, lanes 2–5). The control, not treated with HCl, was recognized by the anti-CRD antiserum (Figure 3D, lane 1). These results demonstrate that NO probably diminishes

Figure 3 Detection of the CRD epitope on the released RDPase by Western-blot analysis

The incubation supernatants (140 μ g) obtained from the SNP-treated proximal tubules as described in Figure 1(A) were subjected to cilastatin–Sepharose 4B affinity chromatography, which was carried out using a 100 μ l bed volume of resin in a 1 ml syringe. Following extensive washing the bound RDPase was eluted with 40 μ l of cilastatin solution (0.5 mg/ml) as described in the Materials and methods section. Samples eluted with cilastatin were mixed with SDS sample buffer and loaded on to a 12% polyacrylamide/SDS gel. Lane 1 (con), purified RDPase solubilized by bacterial PI-PLC (2 μ g); lanes 2–5, RDPase purified from the incubation supernatants of proximal tubules incubated with the indicated concentrations of SNP. Samples were untreated (A and C) or treated (B and D), except lane 1 (con), with 1 M HCl prior to electrophoresis. (A and B) RDPase was detected with the polyclonal anti-RDPase serum. (C and D) The CRD was detected with the anti-CRD serum.

the release of RDPase through inhibition of the GPI-PLC that cleaves the GPI anchor of RDPase.

SNP does not act on the release of RDPase via cGMP

As a NO donor, SNP stimulates cGMP synthesis via a soluble guanylate cyclase in various cells and tissues, suggesting a NO/cGMP signal-transduction pathway [13,17]. Thus to investigate the involvement of cGMP in the release of RDPase, proximal tubules were incubated for 30 min at 37 °C with lipophilic cyclic nucleotides such as 8-Br-cGMP (100 and 1000 μ M), dibutyl-cGMP (100 and 1000 μ M) and 8-Br-cAMP (100 and 300 μ M). However, they did not demonstrate any significant effect on RDPase release from its GPI anchor (results not shown).

A G-protein-coupled PI-PLC is involved in NO generation and the subsequent inhibition of RDPase release

Incubation of the proximal tubules with AlF_4^- (40 μ M AlCl_3 and 10 mM NaF), a G-protein activator, in the presence of 1 mM L-Arg resulted in a 1.9-fold increase in NO generation (0.71 ± 0.02 versus 0.37 ± 0.06 nmol/mg of protein for control; $P < 0.01$; Figure 4) and a concomitant inhibition of the release of RDPase (78.7 ± 3.1 versus 100 % for control; $P < 0.05$). U73122, which is known to specifically inhibit intracellular PI-PLC [34], diminished the nitrite concentration (Figure 4) and restored the release of RDPase completely, suggesting the involvement of a G-protein-regulated PI-PLC in NO generation and the resulting inhibition of RDPase release. These results obtained with AlF_4^- and U73122

Figure 4 Effects of AIF_4^- and U73122 on the release of RDPase in the presence of L-Arg

Proximal tubules were resuspended in 10 vol. of KRH buffer containing 1 mM L-Arg. Aliquots were pretreated for 5 min at 37 °C with U73122 (10 or 20 μM), and incubated further for 30 min in the absence (Con) or presence of AIF_4^- (40 μM AlCl_3 and 10 mM NaF). The nitrite concentration in the incubation supernatant was determined with the Griess reagent as described in the Materials and methods section and expressed as nmol/mg of protein (means ± S.D., $n = 4$; * $P < 0.05$ versus control and ** $P < 0.05$ versus AIF_4^- alone). The RDPase released was also assayed by the spectrofluorometric method as described in the Materials and methods section. The activity is expressed as a percentage compared with the arbitrary fluorescence intensity of the control (means ± S.D., $n = 4$; * $P < 0.05$ versus control and ** $P < 0.05$ versus AIF_4^- alone).

correlate well with our previous observations that carp bile led to the generation of NO via an intracellular PI-PLC [22].

DISCUSSION

It has been observed that the level of soluble dipeptidase in the urine of acute renal-failure patients is lower than in healthy individuals [25,26]. The underlying mechanism for the shedding of the GPI-anchored RDPase from the membrane of the kidney proximal tubules involves an endogenous GPI-PLC based on the identification of the inositol-1,2-cyclic monophosphate CRD on both RDPase released *in vitro* [23] and on the protein in the urine of pigs and humans *in vivo* [24]. A reduction in the release of RDPase from porcine proximal tubules treated with carp bile, which is known to cause acute renal failure, has been shown to be caused by NO generation, although the reduction was not observed with eel bile [22]. These observations correlate with reports that NO is elevated in conditions of renal disease [7,8,17]. This raised the possibility that NO may be involved in regulating the release of RDPase from proximal tubules.

We examined the effects of NO-producing agents on the release of RDPase and, interestingly, observed that the release of RDPase from the proximal tubules was decreased by treatment with SNP and SNAP and that it was not affected by cyanide, which is released from SNP prior to NO generation. The inhibition of RDPase release by these compounds, which are not only NOS substrates but also direct donors of NO, is a significant finding because it is the first time that a relationship between NO generation and the shedding of a GPI-anchored protein has been shown. The half-maximal inhibition of the release of RDPase was accomplished with a corresponding nitrite concentration of approx. 1.8 nmol/mg of protein (Figure 2A), in good agreement with a nitrite concentration of 1.6 nmol/mg of protein correlating with the half-maximal inhibition of RDPase release by carp bile treatment [22]. These data imply that NO may down-regulate

the release of RDPase quantitatively. The reversal of the SNP effect on the release of RDPase by the NO-scavenging agent 1,3-dimethyl-2-thiourea and of the L-Arg effect by the NOS inhibitor L-NAME provide direct evidence that NO is involved in inhibiting the release of RDPase from the proximal tubules.

The possibility of the inhibitory effect of NO on the release of RDPase being due to cytotoxicity involving peroxynitrite [36,37] was examined. Involvement of peroxynitrite in cytotoxicity has been implicated previously by detection of 3-nitrotyrosine, an allegedly characteristic protein-oxidation product, in various inflamed tissues. However, removal of peroxynitrite with deferoxamine mesylate or superoxide dismutase did not elicit any alteration in the release of RDPase after SNP treatment in our system. Fricker et al. [38] demonstrated that the tumour-cell-killing effect of NO was not due to peroxynitrite because superoxide dismutase had no effect on cell killing by activated macrophages that were producing NO. NO, under aerobic conditions in the absence of superoxide, and the peroxynitrite formed from NO at alkaline pH cannot produce detectable concentrations of 3-nitrotyrosine [39], supporting our data because the reaction was performed at pH 8.5 without any superoxide donor. Together with these reports, our data indicate that NO does not inhibit the release of RDPase by membrane disruption through peroxidation.

The direct S-nitrosylation of thiol groups of target enzymes, as demonstrated in c-Jun N-terminal kinase and nuclear factor-κB [40,41], has been suggested as an alternative possibility for the action of NO. However, neither SNP nor SNAP inhibited the activity of purified RDPase, thus failing to demonstrate any structural modification of the active site of the enzyme that might have led to a reduction in enzyme activity. Furthermore, dithiothreitol, as the denitrosylating agent, did not reverse the SNP-mediated inhibition of the release of RDPase, indicating that NO suppresses RDPase release independently of S-nitrosylation.

The third possibility is that the effect of NO on the release of RDPase is through a cellular signal-transduction pathway with NO acting as a signalling molecule. We observed previously [23] that the soluble RDPase released from porcine proximal tubules had been cleaved from its GPI anchor by a GPI-PLC, as confirmed through the identification of the inositol-1,2-cyclic monophosphate CRD, a critical determinant of the action of a GPI-PLC [28,42,43]. The susceptibility of the GPI-PLC to inhibition by NO was assessed by analysing the released RDPase for the CRD determinant. The parallel decrease, in an SNP-concentration-dependent manner, in the level of soluble RDPase and the CRD on the protein as determined by Western-blot analyses (Figure 3) demonstrate that NO inhibits the GPI-PLC, resulting in a reduction in the release of RDPase. The notion that NO can inhibit the GPI-PLC suggests that NO may be a key regulator of the release of other GPI-anchored proteins. Thus we propose that the cleavage and release of RDPase from its GPI anchor by a GPI-PLC is the last stage of a signalling pathway involving NO.

Some of the possible molecules downstream and upstream of NO in the signal-transduction pathway were investigated. Many effects of NO have been shown to be mediated by activation of soluble guanylate cyclase yielding cGMP and subsequent activation of cGMP-dependent protein kinase. In glomeruli of rats treated with the nephrotoxic antibiotic gentamicin, NO synthesis was increased along with cGMP, and induced renal failure [44]. However, we couldn't find definitive evidence of cGMP involvement in RDPase release from its GPI anchor. The lipophilic cGMP analogues 8-Br-cGMP and dibutyl-1-cGMP failed to demonstrate significant release of RDPase.

Sands et al. [45] reported that NO synthesis in murine macrophages, activated by interferon- γ and lipopolysaccharide, involved the activation of a phosphatidylcholine-specific PLC, not a PI-PLC or phospholipase D, which then stimulated inducible NOS expression and NO release. However, the inhibition of the release of RDPase by NO appears to be achieved via the constitutive NOS, which is the Ca^{2+} /calmodulin-dependent endothelial isoform, since the inhibition by L-Arg and AlF_4^- was rapid (within 30 min). Wu et al. [46] demonstrated that vascular endothelial growth factor increased tyrosine phosphorylation of PLC γ and serine phosphorylation of endothelial NOS, and that the effect was blocked by U73122 but not by a protein kinase C inhibitor. NO production mediated by sphingosine 1-phosphate was further suppressed by the addition of pertussis toxin, an inhibitor of G_i proteins, U73122 and a Ca^{2+} chelator in human umbilical vein endothelial cells, suggesting that the activation of endothelial constitutive NOS activity occurs mainly through the G_i /PLC/ Ca^{2+} signalling pathway [47]. In our previous report, the carp bile-stimulated generation of NO appeared to be PI-PLC-mediated due to the reversal of the effect by U73122 [22]. In this study, the L-Arg-mimetic effect of AlF_4^- and its reversal by U73122 suggest the involvement of a G-protein and PI-PLC in NO synthesis via constitutive NOS, although the exact NOS isoforms involved were not defined.

In 3T3-L1 adipocytes, guanosine 5'-[β -thio]diphosphate, as well as activators of G-proteins, including pertussis toxin and AlF_4^- , did not alter the insulin-stimulated shedding of RDPase by a GPI-PLC [43,48]. Together these results support the concepts that (i) a G-protein-coupled intracellular PI-PLC is involved in regulating the synthesis of NO, rather than in the actual cleavage of RDPase, and (ii) the cleavage and release of RDPase from the proximal tubules is regulated by an extracellular GPI-PLC [23], whose activity is modulated by NO.

The low amount of soluble RDPase in the urine of acute renal-failure patients [25] is consistent with the inhibition of RDPase release by carp bile, which causes acute renal failure, being exerted through the generation of NO [22]. Taken together, the results of the present study on the relationship between NO, pathological conditions and the release of RDPase suggest strongly that NO acts as a signalling molecule regulating the shedding of the GPI-anchored RDPase.

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