Nitric oxide induces neutral ceramidase degradation by the ubiquitin/proteasome complex in renal mesangial cell cultures

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Abstract The neutral ceramidase is a key enzyme in the regulation of cellular ceramide levels. Previously we have reported that stimulation of rat renal mesangial cells with nitric oxide (NO) donors leads to an inhibition of neutral ceramidase activity which is due to increased degradation of the enzyme. This and the concomitant activation of the sphingomyelinase results in an amplification of ceramide levels. Here, we show that the NO-triggered degradation of neutral ceramidase involves activation of the ubiquitin/proteasome complex. The specific proteasome inhibitor lactacystin completely reverses the NO-induced degradation of ceramidase protein and neutral ceramidase activity. As a consequence, the cellular amount of ceramide, which drastically increases by NO stimulation, is reduced in the presence of lactacystin. Furthermore, ubiquitinated neutral ceramidase accumulates after NO stimulation. In summary, our data clearly show that the ubiquitin/proteasome complex is an important determinant of neutral ceramidase activity and thereby regulates the availability of ceramide.

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Key words: Nitric oxide; Neutral ceramidase; Ceramide; Proteasome; Ubiquitination; Mesangial cell

1. Introduction

Nitric oxide (NO) has become established as a diffusible universal messenger mediating cell–cell communication. Renal mesangial cells exposed to proinflammatory cytokines express an inducible NO synthase and produce large amounts of NO which may contribute to certain forms of glomerulonephritis [1–3] and other forms of acute and chronic inflammation [3–6].

Previously, we have reported that in rat mesangial cells high levels of NO lead to high amounts of the lipid signaling compound ceramide which is due to a dual action of NO: on the one hand an activation of the ceramide-generating sphingomyelinases and on the other hand an inhibition of the ceramide-degrading enzymes, the ceramidases, which generate sphingosine and free fatty acids [7–9].

Besides the sphingomyelinases, more recently also the ceramide-degrading enzymes have attracted increasing interest. Although it has been shown that the NO-induced inhibition of neutral ceramidase is due to proteolytic degradation of the enzyme, the exact mechanism remains to be established [10]. Over the past years, it has become obvious that a major mechanism of selective protein degradation involves the post-translational modification of proteins by the small protein ubiquitin, and delivery of these proteins to the proteasome [11–13].

In this study we present evidence that the NO-evoked degradation of neutral ceramidase involves the ubiquitin/proteasome pathway.

2. Materials and methods

2.1. Materials

Protein A-Sepharose 4B CL was from Amersham Pharmacia Biotech Europe, Freiburg, Germany; [^{14}C]ceramide (specific activity 55 Ci/mol) was from ICN Biomedicals, Eschwege, Germany; (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropyl-ammonio)butyl]amino]-diazen-1-ium-1,2-diolate (spermine-NO) was from Alexis, Läufelfingen, Switzerland; the polyclonal rabbit anti-bovine ubiquitin antibody, clastolactacystin β-lactone and lactacystin were from Calbiochem-Novabiochem, Schwalbach, Germany; all cell culture nutrients were from Invitrogen/Life Technologies, Karlsruhe, Germany; for generation of a polyclonal anti-neutral ceramidase antibody a synthetic peptide (ENHKDSGNHWFSTC) based on the N-terminal sequence of the murine neutral ceramidase was synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. The detailed characterization is described elsewhere [14].

2.2. Cell culture

Rat mesangial cells were cultivated as described previously [15]. In a second step, single cells were cloned by limited dilution on 96-well plates. Clones with apparent mesangial cell morphology were characterized as reported [15] by positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, and negative staining for factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contaminations, respectively. For the experiments passages 8–20 were used.

2.3. Cell stimulation, immunoprecipitation and Western blot analysis

Confluent mesangial cells in 60 mm diameter dishes were kept serum-free for 20 h in Dulbecco's modified Eagle's medium containing 0.1 mg/ml of fatty acid-free bovine serum albumin. Thereafter, the cells were pretreated for 2 h with lactacystin where indicated, before stimulation with the indicated concentrations or time periods with NO donors in the absence or presence of lactacystin. To stop the reaction, the medium was removed and the cells were washed with ice-cold phosphate-buffered saline (PBS). Cells were then scraped directly into lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100,

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Abbreviations: NO, nitric oxide; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; spermine-NO, (*Z*)-1-[*N*-[3-aminopropyl]-*N*-[4-(3-aminopropyl-ammonio)butyl]amino]-diazen-1-ium-1,2-diolate; TNFα, tumor necrosis factor α

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20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and homogenized by 10 passes through a 26-gauge needle fitted to a 1-ml syringe. The homogenate was centrifuged for 10 min at 14 000 × *g* and the supernatant taken for protein determination. 100 μg of protein was either taken for immunoprecipitation and following Western blot analysis or immediately separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane as previously described [16] using a polyclonal antibody against the neutral ceramidase at a dilution of 1:500 or against ubiquitin at a dilution of 1:1000.

For immunoprecipitation the proteins were incubated overnight at 4°C with a polyclonal antiserum against the neutral ceramidase or against ubiquitin at a dilution of 1:100 and 5% fetal calf serum in lysis buffer. Then 100 μl of a 50% slurry of protein A-Sepharose 4B CL in PBS was added and the mixture was rotated for 1 h at room temperature. After centrifugation for 5 min at 3000 × *g* immunocomplexes were washed three times with a low salt buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS), three times with a high salt buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS) and once with 10 mM Tris. Pellets were boiled for 5 min in Laemmli dissociation buffer and subjected to SDS–PAGE with following Western blot analysis.

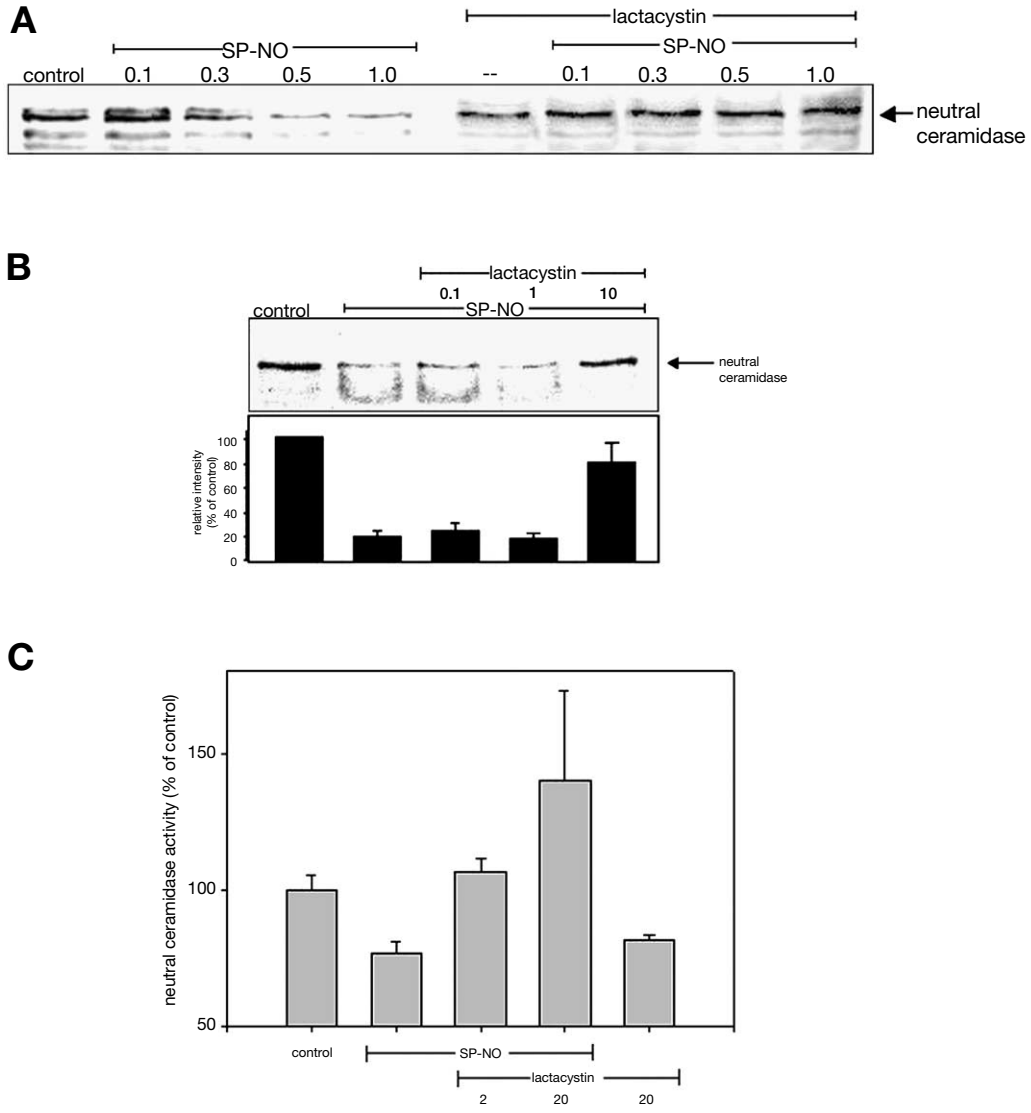

Fig. 1. Effect of the proteasome inhibitor lactacystin on NO-mediated neutral ceramidase degradation and activity in renal mesangial cells. Quiescent mesangial cells were incubated with a proteasome inhibitor prior to NO treatment and the protein level of neutral ceramidase was detected. A: Cells were stimulated for 24 h with either vehicle (control) or spermine-NO (SP-NO; in mM) in the presence of the proteasome inhibitor lactacystin (20 μM; 2 h pretreated) where indicated. B: Cells were incubated for 24 h with either vehicle (control) or spermine-NO (SP-NO; 0.5 mM) in the presence of the indicated concentrations of lactacystin (in μM; 2 h pretreated). Thereafter, cell lysates containing 100 μg of protein were subjected to SDS–PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analyses were performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. The upper panel shows one representative blot. The lower panel shows the densitometric evaluation of two independent experiments. Data are means ± variance. C: Cells were incubated for 24 h with either vehicle (control) or spermine-NO (SP-NO; 0.5 mM) in the presence of the indicated concentrations of lactacystin (in μM; 2 h pretreated). Thereafter, cell lysates containing 100 μg of protein were taken for a neutral ceramidase activity assay as described in Section 2. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on a Imaging System (Fuji). Results are expressed as percent of control values and are means ± S.D. ($n = 4$). Neutral ceramidase activity in control cells was 24.0 ± 1.5 pmol/mg/h.

2.4. Lipid extraction and ceramide quantification

Confluent mesangial cells in 30 mm diameter dishes were stimulated as indicated in the figure legends. Lipids were extracted [17] and ceramide was quantitated by liquid chromatography tandem mass spectrometry exactly as previously described [14].

2.5. Neutral ceramidase activity assays

Confluent mesangial cells were stimulated as described above and homogenized in lysis buffer containing 50 mM Tris, pH 7.5, 0.5% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM D-galactonic acid γ-lactone. Neutral ceramidase activity assays were performed according to Mitsutake et al. [18] with some modifications as previously described [14].

3. Results

To test whether the degradation of neutral ceramidase induced by nitric oxide is mediated through the ubiquitin/proteasome system, we applied lactacystin, a highly selective and cell-permeable inhibitor of the ubiquitin/proteasome complex. Analyzing neutral ceramidase protein levels by immunodetection of mesangial cell lysates reveals that the NO donor spermine-NO leads to a concentration-dependent downregulation of ceramidase protein, thus confirming our previous observations [10]. This depletion is completely prevented by pretreatment of renal mesangial cells with lactacystin (Fig. 1A) in a concentration-dependent manner (Fig. 1B). The lower immunoreactive band in Fig. 1A is most probably due to unspecific interaction of the antiserum. To fully block the NO-mediated effect a concentration of 10 μM lactacystin is required. In parallel, neutral ceramidase activity measurements reveal that the NO-mediated reduction of activity is also reverted by lactacystin (Fig. 1C). A similar effect is obtained with clasto-lactacystin β-lactone [19], which is the active metabolite of the natural product lactacystin (data not shown). Consistent with the observed increase of ceramidase protein and activity in lactacystin-treated mesangial cells, we found a decrease of NO-induced ceramide formation in the cells as analyzed by mass spectrometry of extracted lipids (Fig. 2).

To further confirm the degradation of ceramidase by the ubiquitin/proteasome complex in response to nitric oxide, we

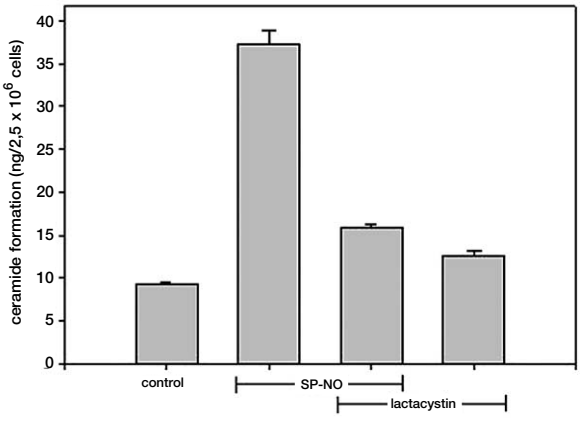

Fig. 2. Effect of lactacystin on NO-induced ceramide formation in mesangial cells. Quiescent mesangial cells were stimulated for 24 h with either vehicle (control) or spermine-NO (0.5 mM) in the absence or presence of the indicated concentrations of the proteasome inhibitor lactacystin (in μM; 2 h pretreated). Thereafter, lipids were extracted and ceramide was analyzed by tandem mass spectrometry as described in Section 2. Results are indicated as ng ceramide per 2.5×10^6 cells and are means ± S.D. ($n = 4$).

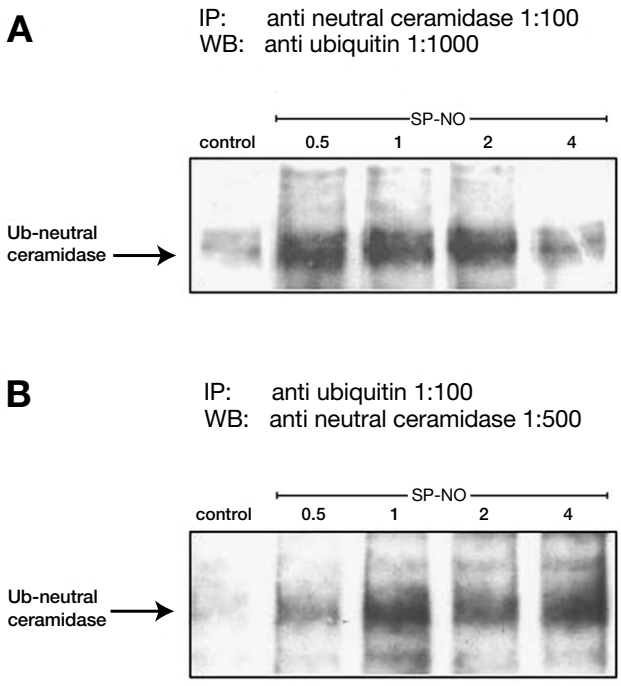

Fig. 3. NO induces ubiquitination of neutral ceramidase in mesangial cells. Quiescent mesangial cells were pretreated for 2 h with 20 μM lactacystin in order to block the proteasome, and then either directly lysed (control) or treated with 1 mM spermine-NO (SP-NO) for either 0.5, 1, 2 or 4 h before cell lysis. Thereafter, cells lysates were taken for immunoprecipitation (IP) using either a polyclonal antibody against neutral ceramidase followed by Western blot analysis with an anti-ubiquitin antibody (A) or an anti-ubiquitin antibody followed by Western blot (WB) analysis with neutral ceramidase antibody (B). Data are representative of two independent experiments giving similar results.

investigated a potential ubiquitination of the enzyme in response to NO. For this purpose cells were pretreated with lactacystin in order to block degradation and thereafter stimulated for various time periods with spermine-NO. As shown in Fig. 3A, immunoprecipitated ceramidase shows a time-dependent increase of ubiquitination when Western blot analysis was performed using an anti-ubiquitin antibody. Maximal ubiquitination occurs at 30–120 min. Similar data were obtained when cell extracts were subjected to immunoprecipitation with an anti-ubiquitin antibody followed by Western blot detection using the anti-ceramidase antibody (Fig. 3B).

4. Discussion

The availability of the signaling molecule ceramide is fine-tuned by the rate of generation involving sphingomyelinases and de novo synthesis and the rate of degradation catalyzed by ceramidases [20]. Whereas sphingomyelinases have attracted interest due to their immediate regulation by an increasing list of ligands such as vitamin D_3, UV light, γ-irradiation, Fas ligand or doxorubicin, more recently, the neutral ceramidase has turned out to be a key enzyme in the regulation of intracellular free ceramide concentration particularly in response to the stress mediator NO and NO-inducing cytokines [7,8,21]. In terms of neutral ceramidase regulation, NO was found to inhibit neutral ceramidase activity in mesangial cells [7]. This effect is due to downregulation of the

protein by increased degradation [10]. In this study we show that the degradation of neutral ceramidase by NO depends on the ubiquitin/proteasome pathway. Activation of the proteasome is a well known, fundamental mechanism for protein turnover, cell cycle control and signal transduction [11–13]. Conceptually, proteolysis by the ubiquitin/proteasome system is considered to comprise three steps: identification of the protein to be degraded, marking of that protein by attachment of ubiquitin to lysine residues, and delivering it to the proteasome, a multienzyme protease complex specific for multiubiquitinated substrates [12] that will degrade it and recycle ubiquitin. How NO mechanistically activates the proteasome system is presently unknown. In principle, NO can mediate, especially under inflammatory conditions when large amounts of reactive oxygen species are also generated, protein nitration, nitrosation and oxidation. In addition, it can form nitrosyl complexes with metalloproteins [22].

It could be speculated that one ubiquitinating enzyme or a component of the 26S proteasome complex is modified at a critical site by NO. Alternatively, it may be possible that NO stimulation leads to nitration or nitrosation of neutral ceramidase itself, which subsequently may facilitate faster degradation of the enzyme by the ubiquitin/proteasome. In this context, it is interesting to note that Souza et al. [23] reported an increased degradation of nitrated proteins by the proteasome which thus guarantees the removal of nitrated proteins in vivo. Furthermore, others have shown that oxidative stress leading to the generation of peroxynitrite increases the degradation of various proteins, including aconitase, by the proteasome pathway [24]. Peroxynitrite is thought to cause nitration of target proteins at tyrosine residues [22].

A proposed activation of the proteasome by NO, as suggested by our data from renal mesangial cells and other studies in K562 leukemia cells and pheochromocytoma PC12 cells, is contrasted by the finding that in the mouse macrophage cell line RAW 264.7 NO inhibited the proteasome [25]. That study showed that lactacystin mimicked the effect of NO to induce accumulation of the tumor suppressor p53. Furthermore, NO was shown to trigger deubiquitination of p53.

It is worth noting that RAW 264.7 macrophages and renal mesangial cells respond differentially to NO stimulation most likely due to their different capacities to handle reactive nitrogen and oxygen species determined by differences in their glutathione redox system [9,26,27]. Thus macrophages and endothelial cells are very sensitive to endogenous NO production and readily undergo apoptosis after stimulation with tumor necrosis factor α (TNFα) [28], whereas mesangial cells are completely resistant to TNFα stimulation, although endogenous NO production takes place [28]. This mesangial cell protection correlates with high levels of reduced glutathione.

By sequence analysis of neutral ceramidase, several lysine residues as necessary targets for ubiquitin attachment can be found in the primary sequence [29]. Further obvious sequence characteristics, such as a destruction box (D box) composed of the sequence R-X-X-L-X-X-X-X-N found in the N-terminus of proteins [30] or a KEN box composed of the sequence K-E-N-X-X-X-N/D located in the N- or C-terminus of the proteins [31] which are targets for one special class of ubiquitin ligase, the anaphase-promoting complex, are missing. However, several other ubiquitin ligase complexes have been identified.

Further studies have to be done to characterize the involved ligase complex in detail.

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