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Nitric Oxide Induces Degradation of the Neutral Ceramidase in Rat Renal Mesangial Cells and Is Counterregulated by Protein Kinase C*

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Ceramide levels are strongly increased by stimulation of renal mesangial cells with nitric oxide (NO). This effect was shown previously to be due to a dual action of NO, comprising an activation of sphingomyelinases and an inhibition of ceramidase activity. In this study we show that the NO-triggered inhibition of neutral ceramidase activity is paralleled by a down-regulation at the protein level. A complete loss of neutral ceramidase protein is obtained after 24 h of stimulation. Whereas the selective proteasome inhibitor lactacystin blocked NO-evoked ceramidase degradation, several caspase inhibitors were ineffective. Moreover, the NO-induced degradation is reversed by the protein kinase C (PKC) activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), and also by the physiological PKC activators platelet-derived growth factor-BB (PDGF), angiotensin II and ATP, resulting in a normalization of neutral ceramidase protein as well as activity. *In vivo* phosphorylation studies using $^{32}P_i$-labeled mesangial cells revealed that TPA, PDGF, angiotensin II, and ATP trigger an increased phosphorylation of the neutral ceramidase, which is blocked by the broad spectrum PKC inhibitor Ro-31 8220 but not by CGP 41251, which has a preferential action on Ca^{2+}-dependent isoforms, thus suggesting the involvement of a Ca^{2+}-independent PKC isoform. *In vitro* phosphorylation assays using recombinant PKC isoenzymes and neutral ceramidase immunoprecipitated from unstimulated mesangial cells show that particularly the PKC-δ isoform and to a lesser extent the PKC-α isoform are efficient in directly phosphorylating neutral ceramidase. In summary, our data show that NO is able to induce degradation of neutral ceramidase, thereby promoting accumulation of ceramide in the cell. This effect is reversed by PKC activation, most probably by the PKC-δ isoenzyme, which can directly phosphorylate and thereby prevent neutral ceramidase degradation. These novel regulatory interactions will provide therapeutically valuable information to target neutral ceramidase stability and subsequent ceramide accumulation.

Sphingolipids exert important roles as signaling molecules under various physiological and pathophysiological conditions (1–4). Particularly ceramide has gained special recognition due to its potential involvement in regulation of programmed cell death, cell growth arrest, and differentiation (1–4). The regulatory mechanisms that determine the intracellular ceramide level are still largely unknown. Most studies have focused on the ceramide-generating enzymes, *i.e.* the acid and neutral sphingomyelinases. However, sphingomyelinases only depict one side of the regulation of ceramide level. Ceramide degradation, which is carried out by ceramidases, depicts the other equally important regulatory part. This key role of ceramidases in determining cellular levels of ceramide, which is a prototypic proapoptotic stimulus, demands a proper understanding of the regulation of these enzymes.

Besides an acidic form, which is localized in the lysosomes (5), a neutral ceramidase has been identified recently and cloned (6, 7). It is tempting to speculate that this latter enzyme plays a central role in signal transduction and is counterbalancing ceramide generation by the sphingomyelinases.

In recent years, nitric oxide (NO),[1] has become established as a diffusible universal messenger mediating cell-cell communication throughout the body. Excessive and uncontrolled production of NO is associated with severe diseases like septic shock, stroke, neurodegeneration, diabetes mellitus, arthritis, and other forms of acute and chronic inflammation (8–11). NO-induced apoptosis has been described for a variety of cell types, but it seems to be a matter of concentration whether NO acts pro- or anti-apoptotic (for review see Refs. 12 and 13). The early and rapid mechanisms of NO signaling depend primarily on post-translational modifications of pre-existing cellular proteins such as guanylate cyclase. However, the late phases that are required to accommodate the microenvironmental changes are mediated by alterations in gene expression (14, 15).

Previously, we showed (16, 17) that glomerular cells exposed to NO donors respond with a drastic increase in ceramide formation. This was due to a dual action of NO, on one side by activation of sphingomyelinases and on the other side by inhibition of ceramidase activities. In this study we investigated the mechanism by which NO reduces neutral ceramidase activity in mesangial cells. We show that NO-reduced activity of neutral ceramidase is due to proteasome-mediated degradation of the enzyme. Furthermore, we show that protein kinase C

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[1] The abbreviations used are: NO, nitric oxide; DMEM, Dulbecco's modified Eagle medium; Gadd45, growth arrest and DNA-damage-inducible gene 45; LC, liquid chromatography; MBP, myelin basic protein; MS, mass spectrometry; PDGF-BB, platelet-derived growth factor-BB; PBS, phosphate-buffered saline; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate, Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Z, benzyloxycarbonyl; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

(PKC) is able to prevent degradation of the enzyme via direct phosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals—[^{32}P]Orthophosphate, [γ-^{32}P]ATP (specific activity, >5000 Ci/mmol), protein A-Sepharose CL-4B, and CNBr-activated Sepharose were from Amersham Biosciences. [^{14}C]Ceramide (specific activity, 55 Ci/mol) was from ICN Biomedicals GmbH, Eschwege, Germany. (*Z*)-1-[*N*-[3-Aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino]-diazen-1-ium-1,2-diolate (spermine-NO) and Deta-NO were from Alexis Corp., Läufelfingen, Switzerland. ATPγS and myelin basic protein (MBP) were from Sigma. 10–20% Tris-Tricine ready gels were from Bio-Rad. 12-*O*-Tetradecanoylphorbol-13-acetate, Ro 318220, lactacystin, spermine, Z-VAD-FMK, and Z-DEVD-FMK were from Calbiochem-Novabiochem. Angiotensin II was from Bachem Biochemica GmbH, Heidelberg, Germany. CGP 41251 was kindly provided by Novartis Pharma Inc., Basel, Switzerland. PDGF-BB was kindly provided by Hoffmann-La Roche. All cell culture nutrients were from Invitrogen. 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 (18). β-Actin antibody was from Santa Cruz Biotechnology Inc., Heidelberg, Germany. PKC isoenzymes were expressed and purified as described previously (19).

Cell Culture—Rat mesangial cells were cultivated and characterized as described previously (20). In a second step, single cells were cloned by limited dilution on 96-well plates. Clones with apparent mesangial cell morphology were characterized 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.

Western Blot Analysis—Confluent mesangial cells in 60-mm diameter dishes were stimulated for the indicated times in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg/ml of fatty acid-free bovine serum albumin. 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, 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 $\times$ *g* and the supernatant taken for protein determination. 100 μg of protein were separated on SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed as described previously (21) using a polyclonal antibody against the neutral ceramidase at a dilution of 1:500 and a monoclonal antibody against β-actin at a dilution of 1:1000.

Lipid Extraction and Ceramide Quantification—Confluent mesangial cells in 30-mm diameter dishes were stimulated as indicated. Lipids were extracted (22), and ceramide was quantitated by liquid chromatography (LC)/MS/MS spectrometry exactly as described previously (18).

Neutral Ceramidase Activity Assay—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 for the neutral ceramidase. Activity assays were performed according to Mitsutake *et al.* (23) with some modifications as described previously (16, 18).

In Vivo Phosphorylation Studies and Immunoprecipitation—Confluent mesangial cells in 100-mm diameter dishes were washed twice with PBS and incubated for 48 h in DMEM containing 0.1 mg of bovine serum albumin/ml. The cells were washed three times with phosphate-free DMEM in order to remove all phosphate. Afterwards, the cells were metabolically labeled for 4 h with [^{32}P]orthophosphate (0.5 mCi/plate). After labeling, cells were stimulated at 37 °C for the indicated times with various agents. To stop the reaction, the medium was removed, and the cells were washed twice with ice-cold buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl. Cells were then scraped directly into 1.0 ml of ice-cold lysis buffer and homogenized by 10 passes through a 26-gauge needle fitted to a 1-ml syringe. The homogenate was centrifuged for 15 min at 14,000 $\times$ *g*, and the supernatant was taken for immunoprecipitation.

The supernatants (containing 2.5 $\times$ 10^6 cpm of labeled proteins) were incubated overnight at 4 °C with a polyclonal antiserum against the neutral ceramidase 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 CL-4B in PBS was added, and the mixture was rotated for 1 h at room temperature. After centrifugation for 5 min at 3000 $\times$ *g,* immunocomplexes were washed 3 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), 3 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. After fixing in 25% isopropyl alcohol, 10% acetic acid, the gels were dried and exposed to an Imaging System (Fuji).

In Vitro Phosphorylation Studies—Cell lysate of unstimulated mesangial cells was used for immunoprecipitation of the neutral ceramidase with a polyclonal antiserum at a dilution of 1:100 as described above. The immunocomplexes were tested for direct phosphorylation by using 1 μg of recombinant PKC isoenzymes (19) in the presence or absence of calcium. The beads were incubated with 1 μg of the partially purified enzymes in a total volume of 40 μl containing 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 μM Na_2ATP, 8 μCi of [γ-^{32}P]ATP, 250 μg/ml phosphatidylserine, 25 μg/ml diolein, 1 μM TPA, and either 100 μM $CaCl_2$ or 100 μM EDTA for 15 min at 32 °C. To show the activities of the PKC isoenzymes, 5 μg of MBP, a well known substrate for PKC (19), was included. Thereafter, SDS buffer was added to stop the reaction, and the samples were separated on an 8% SDS-PAGE for neutral ceramidase and 13% for MBP. Phosphorylated bands corresponding to neutral ceramidase were analyzed on an Imaging system (Fuji).

Tryptic Digest—^{32}P-Phosphorylated neutral ceramidase that was affinity-purified on an anti-ceramidase-Sepharose column was incubated with or without trypsin (100 μg/ml) in a final volume of 50 μl for 3 h at 37 °C. Thereafter, undigested samples were separated on a Tris-glycine SDS-PAGE (7% acrylamide gel), and trypsin-digested samples were separated on a Tris-Tricine SDS-PAGE (10–20% acrylamide gel). Phosphorylated bands were analyzed on an Imaging system.

Statistical Analysis—Statistical analysis was performed by one-way analysis of variance. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

RESULTS

Nitric Oxide Stimulation Leads to a Decrease in Neutral Ceramidase Activity and Protein Level—Previously, we have shown (16) that high levels of nitric oxide caused an increased ceramide formation with subsequent apoptosis of mesangial cells which was due to enhanced sphingomyelinase activity and reduced ceramidase activity. We have now extended these studies and investigated the mechanism by which NO inhibits ceramidase activity. Stimulation of renal mesangial cells with the nitric oxide donor spermine-NO leads to a delayed and time-dependent reduction of neutral ceramidase activity (Fig. 1*A*) with a first significant effect after 8 h of treatment. This reduction of activity is paralleled by a decrease of neutral ceramidase protein amount as shown by a Western blot analysis (Fig. 1*B*). The effect on neutral ceramidase activity (Fig. 2*A*) and protein level (Fig. 2*B*) occurs in a concentration-dependent manner. A maximal reduction to ~60% of control level is obtained with 0.3 mM spermine-NO, which is not further reduced by increasing the concentration of the NO donor. Interestingly, the protein level of neutral ceramidase is almost completely reduced at 0.3 mM of NO. The total amount of protein was not changed upon NO treatment as shown by staining for the housekeeping enzyme β-actin (Fig. 1*B* and 2*B*, *lower panels*). To verify that the observed effect of spermine-NO is indeed mediated by released NO and not by an unspecific effect, spermine was used as a control substance. As seen in Fig. 2*C*, spermine has no effect on neutral ceramidase protein levels nor on activity (data not shown). Furthermore, Deta-NO, another NO donor which possesses a much longer half-life than spermine-NO, shows the same effect on ceramidase protein degradation (Fig. 2*C*).

Activation of PKC Prevents NO-induced Down-regulation of Neutral Ceramidase Activity and Protein—When mesangial

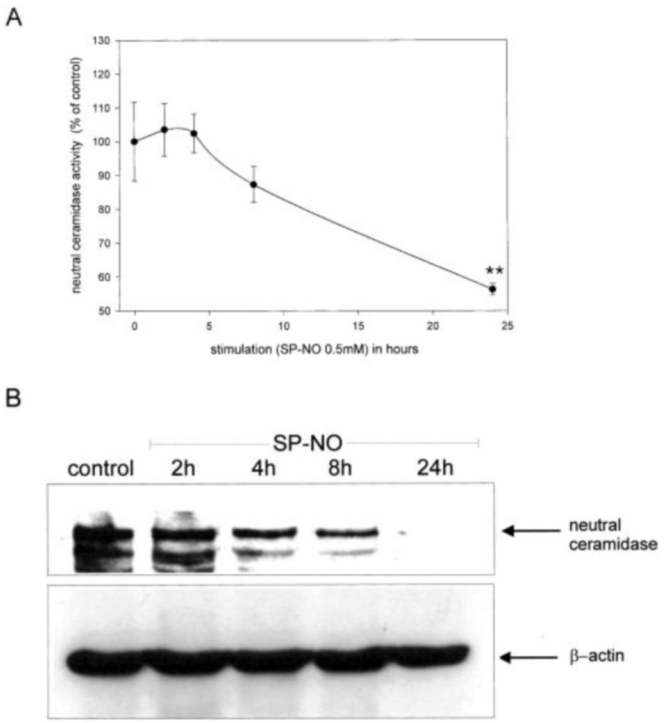

FIG. 1. **Time-dependent effect of spermine-NO on neutral ceramidase activity (*A*) and protein (*B*) in rat mesangial cells.** Confluent rat mesangial cells were stimulated for the indicated times with spermine-NO (*SP-NO*; 0.5 mM). Thereafter, cell lysates containing 100 μg of protein were taken for a neutral ceramidase activity assay (*A*) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (*B*) as described under "Experimental Procedures." The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on a Imaging System (Fuji). Results in *A* are expressed as % of control values and are means ± S.D. (n = 3–4). Neutral ceramidase activity in control cells was 21.5 ± 2.5 pmol/mg/h. **, $p < 0.01$, statistically significant difference compared with the unstimulated control. Data in *B* are representative of at least three independent experiments giving similar results.

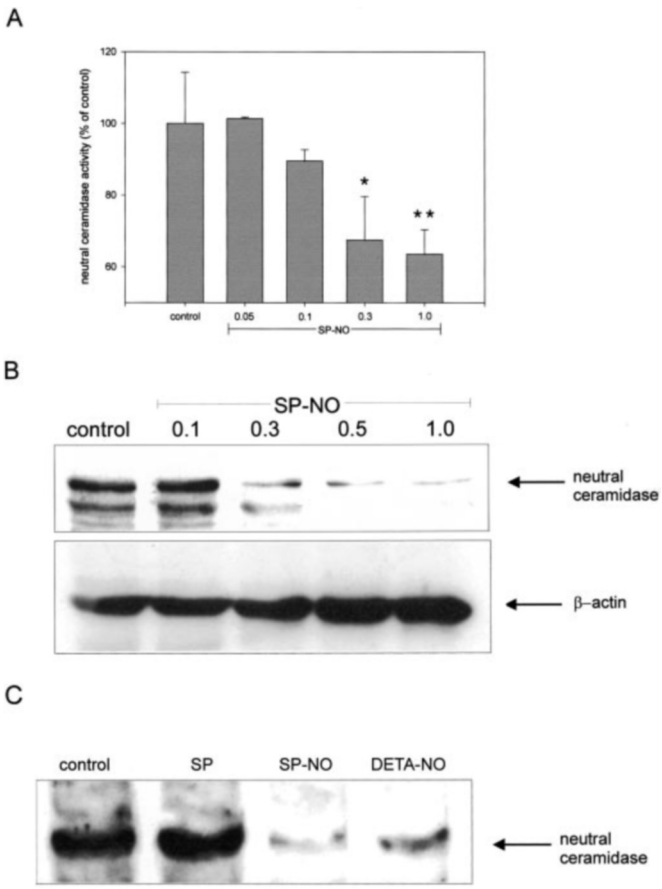

FIG. 2. Concentration-dependent effect of spermine-NO on neutral ceramidase activity (*A*) and protein level (*B*) in rat mesangial cells. Quiescent rat mesangial cells were stimulated for 24 h with the indicated concentrations of spermine-NO (*SP-NO*; in mM) (*A* and *B*); spermine (SP; 0.5 mM) (*C*) or DETA-NO (0.5 mM) (*C*). Thereafter, cell lysates containing 100 μg of protein were taken for a neutral ceramidase activity assay (*A*) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (*B* and *C*) as described under "Experimental Procedures." The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on a Imaging System (Fuji). Data in *A* are expressed as % of control values and are means ± S.D. (n = 3–4). Neutral ceramidase activity in control cells was 20.8 ± 3.0 pmol/mg/h. *, $p < 0.05$; **, $p < 0.01$, statistically significant difference compared with the unstimulated control. Data in *B* are representative of three independent experiments giving similar results.

cells were exposed to NO donors in the presence of phorbol esters which directly activate PKC, both ceramide accumulation and DNA fragmentation were completely blocked (16). We therefore investigated the effect of PKC on the NO-induced down-regulation of neutral ceramidase. Treatment of mesangial cells with spermine-NO in the presence of TPA dose-dependently reversed the inhibitory action of NO on neutral ceramidase activity (Fig. 3*A*). In parallel, the protein level of neutral ceramidase also recovered in the presence of TPA (Fig. 3*B*). Neither short term nor long term stimulation with TPA alone has any effect on neural ceramidase activity or protein level (data not shown).

Furthermore, we tested the physiological activators of PKC, like platelet-derived growth factor (PDGF-BB), angiotensin II, and the stable ATP analog ATPγS, which all evoke phosphoinositide hydrolysis and generation of 1,2-diacylglycerol, the endogenous activator of PKC (24–26). All these substances mimicked the effect of TPA and reversed the NO-mediated inhibition of neutral ceramidase activity (Fig. 4*A*), as well as neutral ceramidase protein level (Fig. 4*B*). Consistent with the observed increase of neutral ceramidase protein by all PKC activators, we found a decrease of NO-induced ceramide formation by these substances in intact mesangial cells when performing mass spectrometry of extracted lipids (Fig. 4*C*).

NO Promotes Degradation of Neutral Ceramidase—To investigate how NO down-regulates the protein amount of neutral ceramidase, we used the protein synthesis inhibitor cycloheximide (27). As seen in Fig. 5*A*, the presence of cycloheximide does not abrogate the NO-mediated reduction of neutral ceramide protein nor does it affect the reversal by the PKC activators TPA, angiotensin II, or ATPγS, thus suggesting that NO modulates the degradation of neutral ceramidase rather than its *de novo* synthesis. To investigate whether one of the caspases is responsible for the NO-induced degradation of neutral ceramidase, the caspase family inhibitor Z-VAD-FMK as well as the specific caspase-3 inhibitor Z-DEVD-FMK were tested. However, none of these two inhibitors is able to block NO-induced enzyme degradation at the recommended concentration of 2 μM (data not shown). In contrast, the highly selective proteasome inhibitor lactacystin inhibited the NO-induced degradation of neutral ceramidase (Fig. 5*B*).

PKC Activation Induces Phosphorylation of Neutral Ceramidase—To study whether PKC exerts its effect on neutral ceramidase via phosphorylation of the enzyme either directly or indirectly we performed *in vivo* phosphorylation assays. Mesangial cells were labeled with [^{32}P]orthophosphate before stimulation. Thereafter, neutral ceramidase was immunoprecipitated, and incorporated ^{32}P was analyzed. As seen in Fig. 6*A*, stimulation of cells for 10 min with TPA causes a dose-de-

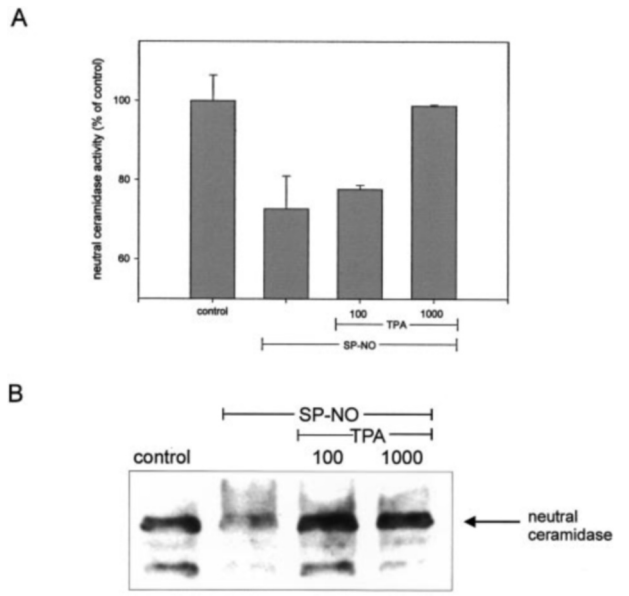

FIG. 3. **Effect of protein kinase C on NO-reduced neutral ceramidase activity (*A*) and neutral ceramidase protein (*B*) in rat mesangial cells.** Confluent rat mesangial cells were stimulated for 24 h with vehicle (*control*) or spermine-NO (*SP-NO*; 1 mM) in the absence or presence of the indicated concentrations of the phorbol ester TPA (in nM). Thereafter, cell lysates containing 100 μg of protein were taken for a neutral ceramidase activity assay (*A*) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (*B*) as described under "Experimental Procedures." The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on a Imaging System (Fuji). Results in *A* are expressed as % of control values and are means ± S.D. (n = 3–4). Neutral ceramidase activity in control cells was 24.5 ± 2.1 pmol/mg/h. Data in *B* are representative of two independent experiments giving similar results.

pendent increase of phosphorylated neutral ceramidase. Similarly, PDGF-BB (Fig. 6*A*) and angiotensin II (Fig. 6*B*) also induce phosphorylation of the enzyme. The TPA-induced phosphorylation is inhibited by the potent PKC inhibitor Ro 318220 (28) but not by CGP 41251 (29), an inhibitor that preferentially blocks Ca^{2+}-dependent PKC isoenzymes (30, 31) (Fig. 7).

Moreover, we investigated whether PKC directly phosphorylates the neutral ceramidase in an *in vitro* system. Neutral ceramidase was enriched by immunoprecipitating the enzyme from unstimulated mesangial cell lysates and was then incubated with recombinant PKC of the four subtypes that have been identified in mesangial cells, *i.e.* PKC-α, -δ, -ϵ, and -ζ (32–34). As seen in Fig. 8, *upper panel*, the PKC-δ is the most efficient isoform to phosphorylate neutral ceramidase, which appears as a double band at ~120 kDa. PKC-α and PKC-ζ induce only minor phosphorylation of the neutral ceramidase. In contrast, PKC-ϵ has no effect. To verify that all PKC isoenzymes used are active enzymes, phosphorylation of MBP, which is a well accepted substrate for PKC isoenzymes (19), is shown (Fig. 8, *lower panel*). We further investigated whether the phosphorylation of neutral ceramidase by PKC *in vitro* occurs at the same phosphorylation sites as in intact cells. For this the phosphorylation pattern of *in vivo* phosphorylated neutral ceramidase was compared with *in vitro* phosphorylated ceramidase after trypsin digestion. As seen in Fig. 9*A*, ^{32}P-phosphorylated ceramidase from *in vivo* and *in vitro* assays runs at 120 kDa on a 7% Tris-glycine gel system. Upon trypsin digestion, enzyme preparations are both fragmented into an identical pattern of polypeptides. By using a 10–20% gradient Tris-Tricine gel system to allow a better separation of small polypeptides, two radioactive fragments between 15 and 20 kDa can be detected (Fig. 9*B*).

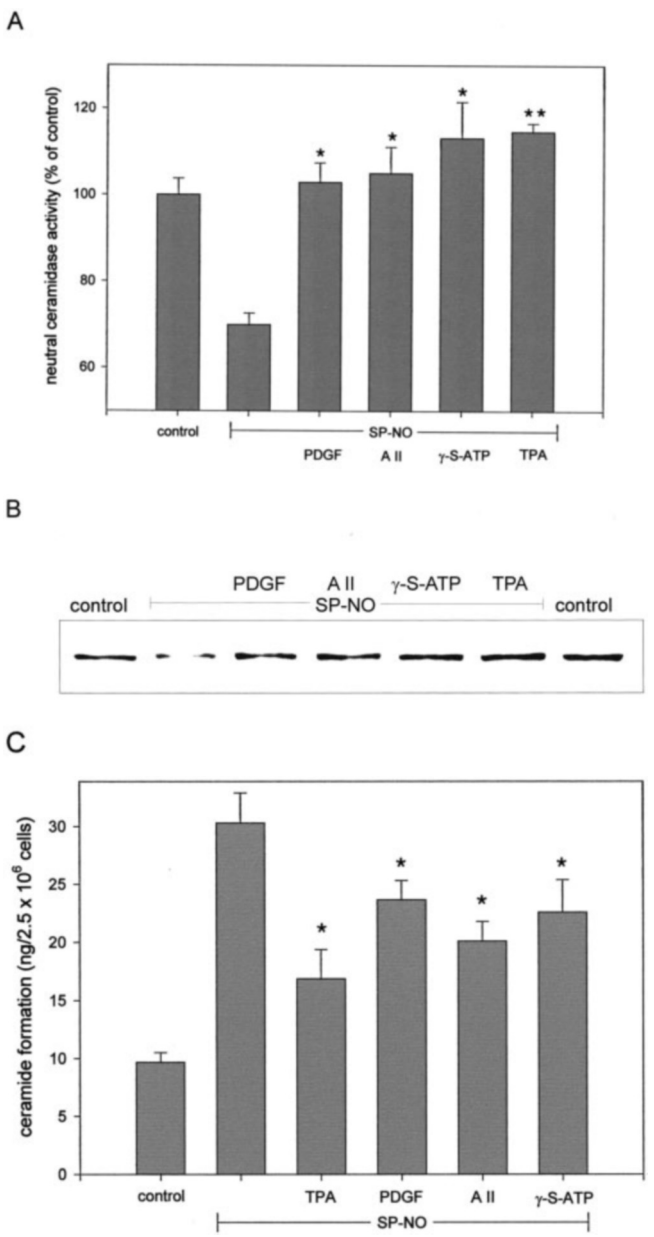

FIG. 4. **Effect of PDGF, angiotensin II, and ATPγS on NO-reduced neutral ceramidase activity (*A*), protein level (*B*), and ceramide formation (*C*).** Quiescent rat mesangial cells were stimulated for 24 h with either vehicle (*control*) or spermine-NO (*SP-NO*; 1 mM) in the absence or presence of TPA (1 μM), PDGF-BB (3 ng/ml), angiotensin II (100 nM), or ATPγS (γ-*S-ATP*) (100 μM) as indicated. *A*, cell lysates containing 100 μg of protein were taken for a neutral ceramidase activity assay as described under "Experimental Procedures." The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on a Imaging System (Fuji). Results are expressed as % of control values and are means ± S.D. (n = 4). Neutral ceramidase activity in control cells was 27.3 ± 1.0 pmol/mg/h. *B*, cell lysates containing 100 μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of two independent experiments giving similar results. *C*, lipids were extracted, and ceramide was analyzed by LC/MS/MS spectrometry as described under "Experimental Procedures." Results are means ± S.D. (n = 4). *, $p < 0.05$; **, $p < 0.01$, statistically significant difference compared with the NO-stimulated control.

DISCUSSION

Mesangial cells have been shown to respond to high amounts of NO with an increased generation of the sphingolipid molecule ceramide, which involves a dual mechanism, the activation

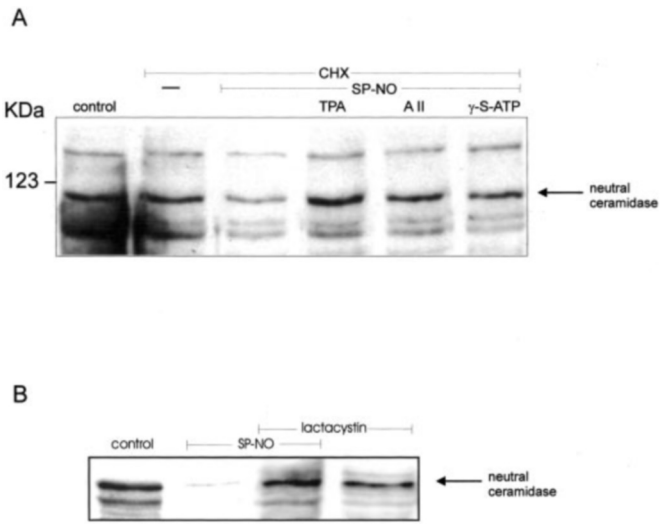

FIG. 5. **Effect of cycloheximide and lactacystin on NO-reduced neutral ceramidase protein level in mesangial cells.** *A*, quiescent mesangial cells were stimulated for 24 h in the absence or presence of cycloheximide (*CHX*; 10 μM) with either vehicle (−) or spermine-NO (*SP-NO*; 1 mM) in the absence or presence of TPA (1 μM), PDGF-BB (3 ng/ml), angiotensin II (100 nM), or ATPγS (*γ-S-ATP*) (100 μM). *B*, cells were stimulated for 24 h in the absence or presence of lactacystine (20 μM; pretreated for 2 h) with either vehicle (*control*) or spermine-NO (*SP-NO*; 1 mM). Thereafter, cell lysates containing 100 μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 2–3 independent experiments giving similar results.

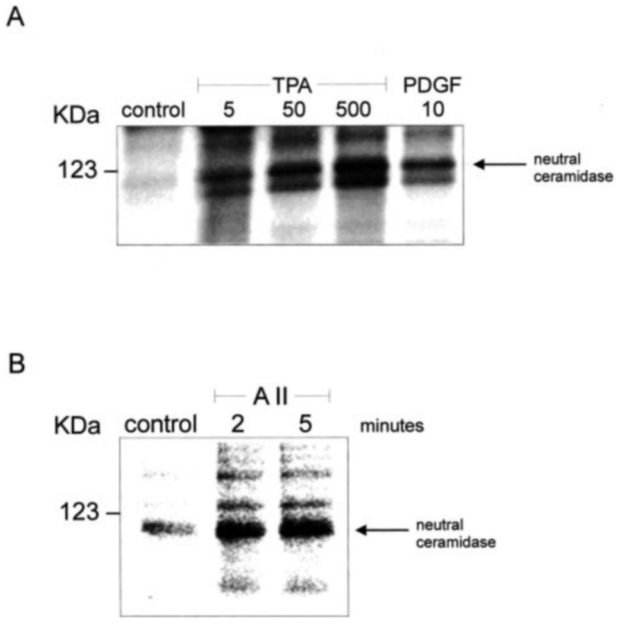

FIG. 6. **Effect of PKC activators on phosphorylation of neutral ceramidase in intact mesangial cells.** ^{32}P-Labeled mesangial cells were stimulated for 15 min with either vehicle (*control*) or the indicated concentrations of TPA (*A*; in nM) and PDGF-BB (*A*; in ng/ml) or for the indicated times with angiotensin II (A II) (*B*; 100 nM). Cells were then lysed, and neutral ceramidase was immunoprecipitated with a polyclonal antibody at a dilution of 1:100, as described under "Experimental Procedures." The immunoprecipitates were separated by SDS-PAGE (7% acrylamide gels) and visualized on an Imaging System (Fuji). Data are representative of three independent experiments giving similar results.

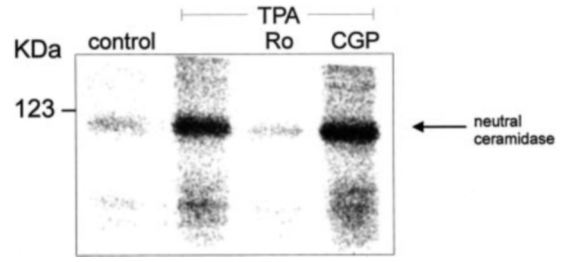

FIG. 7. **Effect of PKC inhibitors on TPA-induced phosphorylation of neutral ceramidase in mesangial cells.** ^{32}P-Labeled mesangial cells were preincubated for 20 min with either Ro 318220 (1 μM) or CGP 41251 (1 μM) as indicated before stimulation with TPA (in nM) for 10 min. Cells were then lysed, and neutral ceramidase was immunoprecipitated with a polyclonal antibody at a dilution of 1:100, as described under the "Experimental Procedures." The immunoprecipitates were separated by SDS-PAGE (7% acrylamide gels) and visualized on a Imaging System (Fuji). Data are representative of two independent experiments giving similar results.

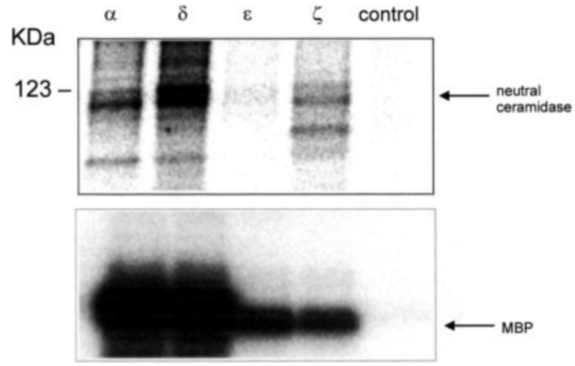

FIG. 8. **Effect of recombinant PKC isoenzymes on neutral ceramidase phosphorylation *in vitro*.** 1 μg of recombinant PKC-α, -δ, -ϵ, and -ζ isoenzymes or buffer (control) were incubated for 15 min with neutral ceramidase that was immunoprecipitated from unstimulated mesangial cells (*upper panel*) or with myelin basic protein (*lower panel*), as described under "Experimental Procedures." Thereafter, samples were separated on SDS-PAGE (7% acrylamide gel for neutral ceramidase and 13% for MBP) and analyzed on a Imaging System (Fuji). Data are representative of two independent experiments giving similar results.

of sphingomyelinases, and the concomitant inhibition of ceramidase activities (16).

In this study, we have investigated the mechanism by which NO reduces neutral ceramidase activity. Our data clearly show that the NO-mediated reduction of neutral ceramidase activity is paralleled by a decrease of the ceramidase protein level. This suggests that either the *de novo* synthesis of neutral ceramidase is blocked by NO or that NO induces degradation of the enzyme. The experiments performed in the presence of the protein synthesis inhibitor cycloheximide, which does not affect the NO-triggered down-regulation nor the basal level of neutral ceramidase (Fig. 5), rather argues for an increased degradation of the enzyme. This notion is further substantiated by the observation that a specific proteasome inhibitor blocked NO-induced ceramidase degradation, whereas several caspase inhibitors were without effect. Thus ceramidase is targeted by the ubiquitin proteasome machinery in mesangial cells. Interestingly, the NO-mediated degradation of ceramidase can be reversed by PKC-activating agents, like the phorbol ester TPA, and also the physiologically important ligands PDGF, angiotensin II, and ATP. The mechanisms by which PKC prevents degradation are still unclear, but it is tempting to speculate that phosphorylation events play an important role.

Phosphorylation is a well known mechanism for regulating protein stability. Thus many proteins are subjected to phosphorylation and as a consequence are degraded more rapidly, like the inhibitor of κB (35), the transcription factor RelB, which is a member of the Rel/NF-κB family of transcription factors (36), or the tumor suppressor protein p53 (37). On the contrary, phosphorylation may also stabilize certain proteins like the

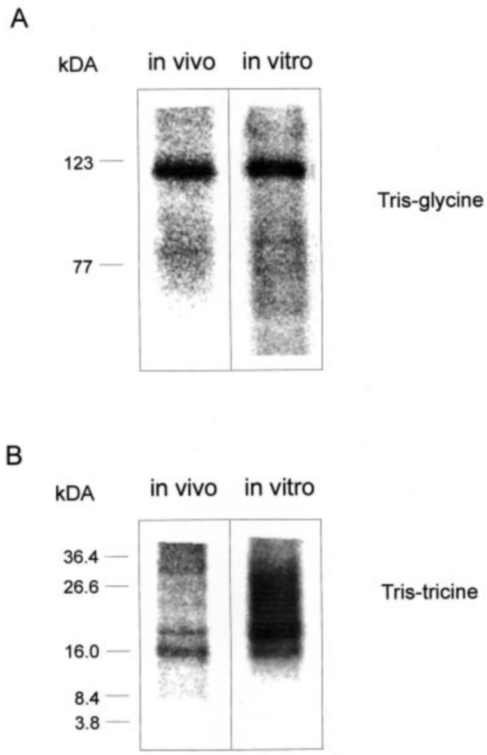

FIG. 9. **Phosphopeptide mapping of trypsin-digested phosphorylated neutral ceramidase.** Neutral ceramidase, either purified from mesangial cells and subjected to *in vitro* phosphorylation by PKC-δ (*A*, *right lane*) or affinity-purified from *in vivo* P_i-labeled TPA-stimulated mesangial cells (*A*, *left lane*), was digested with trypsin as described under "Experimental Procedures." Undigested control samples were separated on a Tris-glycine SDS-PAGE (7% acrylamide gel) (*A*), whereas trypsin-digested samples were separated on a Tris-Tricine SDS-PAGE (10–20% gradient) (*B*). Gels were analyzed on an Imaging system (Fuji) for radioactive fragments.

epidermal growth factor receptor (38), the growth arrest and DNA damage-inducible gene *gadd45* (39), or the human proto-oncogene *ETS-2* (40) and thereby lead to their accumulation. In the case of neutral ceramidase, it seems that phosphorylation of the enzyme by PKC directly or indirectly by alteration of another kinase or phosphatase activity leads to reduced protein degradation. Our data further suggest that in mesangial cells the PKC-δ isoenzyme is the most likely candidate for a direct stabilizing effect. This finding is corroborated by the fact that (i) only the broad spectrum PKC inhibitor Ro-31 8220, but not the inhibitor of the Ca^{2+}-dependent PKC isoenzymes, CGP 41251, is able to block TPA-stimulated phosphorylation of neutral ceramidase and that (ii) recombinant PKC-δ is the most effective isoform to phosphorylate neutral ceramidase in an *in vitro* system. In accordance with our data, Leung *et al.* (39) reported that PKC-δ plays an important role in protein stabilization of *Gadd45* in A431 cells. Mechanistically, they showed that epidermal growth factor, by activating PKC-δ, decreased ubiquitination of *Gadd45*, an effect that was blocked by the PKC-δ-selective inhibitor rottlerin. It is tempting to speculate that PKC-δ also decreases ubiquitination of neutral ceramidase and thus stabilizes the enzyme in mesangial cells.

By sequence analysis of neutral ceramidase, nine putative PKC and several casein kinase II consensus phosphorylation sites can be found in the primary sequence (7). However, site-directed mutagenesis studies of these phosphorylation sites will be required in order to identify which of these putative PKC phosphorylation sites, if any, are indeed relevant for ceramidase stability.

Remarkably, phosphorylation of neutral ceramidase *per se*, either by PKC or another kinase, seems not to be sufficient for activation, since short term stimulation with either TPA, PDGF-BB, or angiotensin II did not change the activity of the enzyme (data not shown). This contrasts to a report by Coroneos *et al.* (41), who found that PDGF activated an alkaline ceramidase in mesangial cells. This activation was under the regulation of a tyrosine phosphatase because activity increased in the presence of vanadate. Whether this alkaline ceramidase is identical to the neutral ceramidase investigated in this study is not clear, since no biochemical characterization of the alkaline enzyme has been reported. Moreover, when we added vanadate to mesangial cells, it had no effect on neutral ceramidase activity (data not shown).

One obvious cellular consequence of neutral ceramidase stabilization by PKC is a more efficient elimination of ceramide which in turn may protect the cell from the proapoptotic action of this lipid mediator. There is increasing evidence that PKC can act as a cytoprotective enzyme preventing stress-induced programmed cell death (apoptosis) in various cell types, including mesangial cells (16, 42). Furthermore, Jun *et al.* (43) described that overexpression of PKC-βII, -δ, and -η isoenzymes protected 264.7 macrophages from nitric oxide-induced apoptosis by blocking nitric oxide-activating effects on c-Jun N-terminal kinase/stress-activated protein kinase and p38 kinase. Moreover, overexpression of PKC-α in 32D myeloid progenitor cells leads to an increased activation of the protein kinase B/Akt which promotes cell survival (44). Obviously, there is a cell type-specific involvement of PKC isoenzymes in cell protection. From our data, we conclude that especially the PKC-δ is a prime candidate for exerting an anti-apoptotic effect in mesangial cells via stabilizing neutral ceramidase. This fits with our previous findings and hypothesis (45) that PKC-δ mediates mitogen-activated protein kinase activation in mesangial cells and promotes proliferation in response to extracellular nucleotides.

In this context it is worth noting that ceramide is able to bind directly to PKC-δ in mesangial cells (46). Such a binding leads to a decreased autophosphorylation of PKC-δ probably reflecting an inhibitory effect on the activity of the enzyme. This may constitute an intriguing positive feedback loop, with increased ceramide levels causing inhibition of PKC-δ, which in turn leads to an increased degradation of neutral ceramidase and amplified ceramide accumulation. Powerful negatively acting regulatory pathways are required to terminate amplification loops such as the one suggested here. Noteworthy, ceramide also binds to and triggers PKC-α-mediated feedback inhibition of cytokine-induced ceramide formation (46–48). Can these seemingly disparate results be reconciled? We suggest that ceramide acts as a negative feedback regulator to control its own synthesis in response to cytokine stimulation via PKC-α activation. But as soon as a critical threshold of activation is reached and high amounts of NO are produced by a delayed cytokine-induced expression of the inducible NO synthase, ceramide then functions as a positive feedback modulator to amplify its production via ceramidase destabilization due to inhibition of PKC-δ. This may operate in a switch-like mechanism (49) and initiate programmed cell death which is a common phenomenon in acute inflammation as well as resolution of disease (50).

Taken together the results of this study provide evidence that NO blocks neutral ceramidase activity by inducing degradation of the enzyme and that this effect can be reversed by activating PKC, especially the δ−isoform. The neutral ceramidase may thus represent a novel attractive target to interfere with cellular stress response and to modulate programmed cell

death which is a typical feature of many inflammatory diseases.

REFERENCES

1. Riboni, L., Viani, P., Bassi, R., Prinetti, A., and Tettamanti, G. (1997) *Prog. Lipid Res.* **36,** 153–195
2. Perry, D. K., and Hannun, Y. A. (1998) *Biochim. Biophys. Acta* **1436,** 233–243
3. Levade, T., and Jaffrézou, J. P. (1999) *Biochim. Biophys. Acta* **1438,** 1–17
4. Huwiler, A., Kolter, T., Pfeilschifter, J., and Sandhoff, K. (2000) *Biochim. Biophys. Acta* **1485,** 63–99
5. Li, C. M., Hong, S. B., Kopal, G., He, X., Linke, T., Hou, W. S., Koch, J., Gatt, S., Sandhoff, K., and Schuchman, E. H. (1998) *Genomics* **50,** 267–274
6. Tani, M., Okino, N., Mori, K., Tanigawa, T., Izu, H., and Ito, M. (2000) *J. Biol. Chem.* **275,** 11229–11234
7. Mitsutake, S., Tani, M., Okino, N., Mori, K., Ichinose, S., Omori, A., Iida, H., Nakamura, T., and Ito, M. (2001) *J. Biol. Chem.* **276,** 26249–26259
8. Nathan, C. (1992) *FASEB J.* **6,** 3051–3064
9. Knowles, R. G., and Moncada, S. (1994) *Biochem. J.* **298,** 249–258
10. Krönke, K.-D., Fehsel, K., and Kolb-Bachofen, V. (1995) *Biol. Chem. Hoppe-Seyler* **376,** 327–343
11. Pfeilschifter, J. (1995) *Kidney Int.* **51,** S50–S60
12. Brüne, B., von Knethen, A., and Sandau, K. B. (1998) *Eur. J. Pharmacol.* **351,** 261–272
13. Beck, K. F., Eberhardt, W., Frank, S., Huwiler, A., Messmer, U. K., Mühl, H., and Pfeilschifter, J. (1999) *J. Exp. Biol.* **202,** 645–653
14. Pfeilschifter, J., Eberhardt, W., and Beck, K. F. (2001) *Pfluegers Arch.* **442,** 479–486
15. Pfeilschifter, J., Eberhardt, W., and Huwiler, A. (2001) *Eur. J. Pharmacol.* **429,** 279–286
16. Huwiler, A., Pfeilschifter, J., and van den Bosch, H. (1999) *J. Biol. Chem.* **274,** 7190–7195
17. Huwiler, A., Dorsch, S., Briner, V. A., van den Bosch, H., and Pfeilschifter, J. (1999) *Biochem. Biophys. Res. Commun.* **258,** 60–65
18. Franzen, R., Pautz, A., Bräutigam, L., Geisslinger, G., Pfeilschifter, J., and Huwiler, A. (2001) *J. Biol. Chem.* **276,** 35382–35389
19. Geiges, D., Meyer, T., Marte, B., Vanek, M., Weissgerber, G., Stabel, S., Pfeilschifter, J., Fabbro, D., and Huwiler, A. (1997) *Biochem. Pharmacol.* **53,** 865–875
20. Pfeilschifter, J. (1990) *Biochem. J.* **272,** 469–472
21. Huwiler, A., Wartmann, M., van den Bosch, H., and Pfeilschifter, J. (2000) *Br. J. Pharmacol.* **129,** 612–618
22. Bligh, E. G., and Dyer, W. J. (1953) *Can. J. Biochem. Physiol.* **37,** 911–917
23. Mitsutake, S., Kita, K., Okino, N., and Ito, M. (1997) *Anal. Biochem.* **247,** 52–57
24. Pfeilschifter, J., and Hosang, M. (1991) *Cell. Signal.* **3,** 413–424
25. Pfeilschifter, J. (1986) *FEBS Lett.* **203,** 262–266
26. Pfeilschifter, J. (1990) *Cell. Signal.* **2,** 129–138
27. Ch'ih, J. J. Faulkner, L. S., and Devin, T. M. (1979) *Biochem. Pharmacol.* **28,** 2404–2407
28. Wilkinson, S. E., Parker, P. J., Nixon, J. S. (1993) *Biochem. J.* **294,** 335–337
29. Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rösel, J., Müller, M., Caravatti, G., Matter, A. (1989) *Int. J. Cancer* **43,** 851–856
30. Ochsner, M., Huwiler, A., Fleck, T., Pfeilschifter, J. (1993) *Eur. J. Pharmacol. Mol. Pharmacol. Sec.* **245,** 15–21
31. Marte, B. M., Meyer, T., Stabel, S., Standke, G. J. R., Jaken, S., Fabbro, D., Hynes, N. E. (1994) *Cell Growth Differ.* **5,** 239–247
32. Huwiler, A., Fabbro, D., and Pfeilschifter, J. (1991) *Biochem. J.* **279,** 441–445
33. Huwiler, A., Fabbro, D., Stabel, S., and Pfeilschifter, J. (1992) *FEBS Lett.* **300,** 259–262
34. Huwiler, A., Schulze-Lohoff, E., Fabbro, D., and Pfeilschifter, J. (1993) *Exp. Nephrol.* **1,** 19–25
35. Steffan, N. M., Bren, G. D., Frantz, B., Tocci, M. J., O'Neill, E. A., and Paya, C. V. (1995) *J. Immunol.* **155,** 4685–4691
36. Marienfeld, R., Berberich-Siebelt, F., Berberich, I., Denk, A., Serfling, E., and Neumann, M. (2001) *Oncogene* **20,** 8142–8147
37. Chernov, M. V., Bean, L. J., Lerner, N., and Stark, G. R. (2001) *J. Biol. Chem.* **276,** 31819–31824
38. Bao, J., Alroy, I., Waterman, H., Schejter, E. D., Brodie, C., Gruenberg, J., and Yarden, Y. (2000) *J. Biol. Chem.* **275,** 26178–26186
39. Leung, C. H., Lam, W., Zhuang, W. J., Wong, N. S., Yang, M. S., and Fong, W. F. (2001) *Biochem. Biophys. Res. Commun.* **285,** 283–288
40. Fujiwara, S., Fisher, R. J., Bhat, N. K., Diaz de la Espina, S. M., and Papas, T. S. (1988) *Mol. Cell. Biol.* **8,** 4700–4706
41. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) *J. Biol. Chem.* **270,** 23305–23309
42. Meinhardt, G., Roth, J., and Totok, G. (2000) *Eur. J. Cell Biol.* **79,** 824–833
43. Jun, C. D., Oh, C. D., Kwak, H. J., Pae, H. O., Yoo, J. C., Choi, B. M., Chun, J. S., Park, R. K., Chung, H. T. J. (1999) *J. Immunol.* **162,** 3395–3401
44. Li, W., Zhang, J., Flechner, L., Hyun, T., Yam, A., Franke, T. F., and Pierce, J. H. (1999) *Oncogene* **18,** 6564–6572
45. Huwiler, A., and Pfeilschifter, J. (1994) *Br. J. Pharmacol.* **113,** 1455–1463
46. Huwiler, A., Fabbro, D., and Pfeilschifter, J. (1998) *Biochemistry* **37,** 14556–14562
47. Kaszkin, M., Huwiler, A., Scholz, K., van den Bosch, H., and Pfeilschifter, J. (1998) *FEBS Lett.* **440,** 163–166
48. Peilschifter, J., and Huwiler, A. (1999) *News Physiol. Sci.* **15,** 11–15
49. Pfeilschifter, J., Beck, K.-F., Eberhardt, W., and Huwiler, A. (2002) *Kidney Int.* **61,** 809–815
50. Savill, J., and Johnson, R. J. (1995) *Exp. Nephrol.* **3,** 149–158