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Nitric Oxide Stimulates Chronic Ceramide Formation in Glomerular Endothelial Cells

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Exposure of glomerular endothelial cells for 24 h to compounds releasing NO, including spermine-NO, MAHMA-NO, and *S*-nitroso-glutathione, results in a dose-dependent and delayed (after 24 h) increase in the lipid signaling molecule ceramide. This NO-induced stimulation occurs in a cGMP-independent fashion since the membrane-permeant cGMP analogue dibutyryl cGMP has no effect on chronic ceramide production. Short-term incubation of endothelial cells for 20 min reveals that NO and dibutyryl cGMP fail to stimulate an acute ceramide increase, whereas TNF-α, a well-known activator of sphingomyelinases, is able to acutely increase ceramide formation. Interestingly, *N*-oleoylethanolamine, an acidic ceramidase inhibitor, potentiates NO-induced chronic ceramide production, indicating that ceramide generation rather than ceramide metabolism is modulated by NO. Furthermore, NO-induced delayed ceramide formation is partially inhibited by the thiol-specific inhibitor iodoacetamide and the radical scavenger α-tocopherol, suggesting a regulatory role of thiol-containing enzymes and the involvement of a redox-sensitive mechanism. In addition, NO causes an increased DNA fragmentation in glomerular endothelial cells which is further enhanced by *N*-oleoylethanolamine and can be mimicked by exogenous ceramide. In summary, these results imply that ceramide represents an important mediator of NO-triggered chronic cell responses like apoptosis. Inhibition of ceramide synthesis may provide a new therapeutic approach to the treatment of pathological conditions involving increased NO formation.

Nitric oxide (NO), a gas previously considered a potentially toxic chemical, has become established as a diffusible universal messenger mediating many biological functions in the cardiovascular, neuronal and immune system. Produced by NO synthases (NOS) in appropriate amounts in endothelial cells, neurons or macrophages, NO exerts several protective functions like causing vasodilatation and thereby improving tissue perfusion, inhibiting platelet aggregation and thus acting as an antithrombotic agent, inhibiting leukocyte adhesion to endothelial cells and thus the recruitment of inflammatory cells, and inhibiting smooth muscle cell proliferation and thereby preserving tissue and organ architecture.

On the other hand, 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 [1–4]. Obviously, it is the concentration of NO produced that determines whether it acts as a proinflammatory or an anti-inflammatory mediator. The careful control of this extremely reactive molecule is essential for the prevention of deleterious inflammatory reactions.

Whereas the activity of the constitutive brain and endothelial cell NO synthases are mainly regulated by cytoplasmic Ca^{2+} levels and phosphorylation, the inducible NOS is regulated primarily at a transcriptional level. Once induced the inducible NOS produces NO for long periods of hours and days [1, 5, 6].

Glomerular mesangial and endothelial cells are not only production sites of NO [7–9] but are also themselves targets for NO and undergo apoptotic cell death upon exposure to high concentrations of NO [10]. Apop-

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Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; TNFα, tumor necrosis factor α; DMEM, Dulbecco's modified Eagle medium; TLC, thin layer chromatography; BSA, bovine serum albumin; MAHMA-NO, (Z)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)-amino]}diazen-1-ium-1,2-diolate; spermine-NO, (Z)-1-{*N*-[3-Aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl] amino}-diazen-1-ium-1,2-diolate; GS-NO, S-nitrosoglutathione; SAPK, stress-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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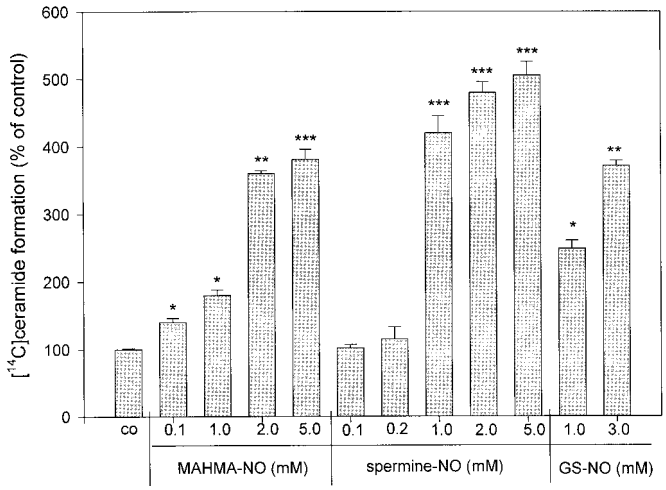

FIG. 1. Effect of NO donors on chronic ceramide formation in glomerular endothelial cells. Glomerular endothelial cells were labeled with [^{14}C]serine and stimulated for 24 h with the indicated concentrations of MAHMA-NO, spermine-NO and GS-NO. Thereafter lipids were extracted, separated by TLC as described under Methods, and the spots corresponding to ceramide were quantitated on a TLC scanner. Data are expressed as percentage of control values and are means ± SD, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, statistically significant difference compared to the unstimulated control.

tosis is a controlled biological strategy to remove unwanted damaged cells from a given tissue and thus is involved in important physiological and pathophysiological processes [11, 12].

Another molecule that has attracted a lot of interest as a possible regulator of apoptosis is the sphingolipid ceramide (for review, see [13]). Ceramide is generated by sphingomyelinase-catalyzed sphingolipid turnover and has been characterized as an important intracellular mediator of stress signaling which may finally lead to apoptosis [13].

In this study we present for the first time evidence that NO donors induce a delayed upregulation of ceramide levels in glomerular endothelial cells which is accompanied by an enhanced rate of apoptosis.

MATERIALS AND METHODS

Chemicals. (Z)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)-amino]}diazen-1-ium-1,2-diolate (MAHMA-NO), (Z)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}-diazen-1-ium-1,2-diolate (Spermine-NO) and S-nitrosoglutathione (GS-NO) were from Alexis Corp., Läufelfingen, Switzerland; [^{14}C]serine (sp act 53 Ci/mol) was from Amersham; dibutyryl-cyclic GMP, *N*-oleoylethanolamine and iodoacetamide were from Sigma (St. Louis, MO); iodoacetamide was from Fluka, Buchs, Switzerland; all cell culture nutrients were from Gibco-BRL, Breda, the Netherlands; TNFα was a gift of Knoll AG, Ludwigshafen, Germany; α-tocopherole was kindly provided by Hoffmann–La Roche Ltd., Basel, Switzerland.

Cell culture. Bovine glomerular endothelial cells were cultivated as described [14]. Individual clones of endothelial cells were characterized by positive staining for Factor VIII-related antigen and uniform uptake of fluorescent acetylated low-density lipoproteins. Negative staining for smooth muscle actin and cytokeratin excluded mesangial cell and epithelial cell contaminations. Cells were utilized at passages 5–15.

Lipid extraction and ceramide quantitation. Confluent mesangial cells in 30-mm-diameter dishes were labeled for 24 h with [^{14}C]serine (0.2 μCi/ml) and stimulated as indicated. Lipids were extracted and ceramide was analyzed as previously described [15].

Apoptosis assay. Confluent mesangial cells in 60-mm-diameter dishes were incubated with the indicated stimuli in DMEM containing 0.1 mg/ml of fatty acid-free BSA for the indicated time periods. Thereafter, oligonucleosomal DNA fragmentation, a characteristic biochemical feature of apoptotic cell death, was measured using a nucleosome DNA ELISA (Boehringer-Mannheim), which quantitatively records histone-associated DNA fragments.

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

RESULTS

Incubation of [^{14}C]serine-labeled glomerular endothelial cells with high concentrations of NO donors for 24 h results in a drastic increase of ceramide formation. Figure 1 shows that (Z)-1-{*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate (MAHMA-NO), spermine-NO and *N*-nitrosogutathione (GS-NO) all cause a concentration-dependent increase in radioactive ceramide levels after 24 h of stimulation.

The main intracellular receptor for NO is the enzyme guanylate cyclase [5, 16] which mediates most of the physiological functions of NO, whereas pathophysiological functions of NO are mainly independent of guanylate cyclase but involve other reactions, like in-

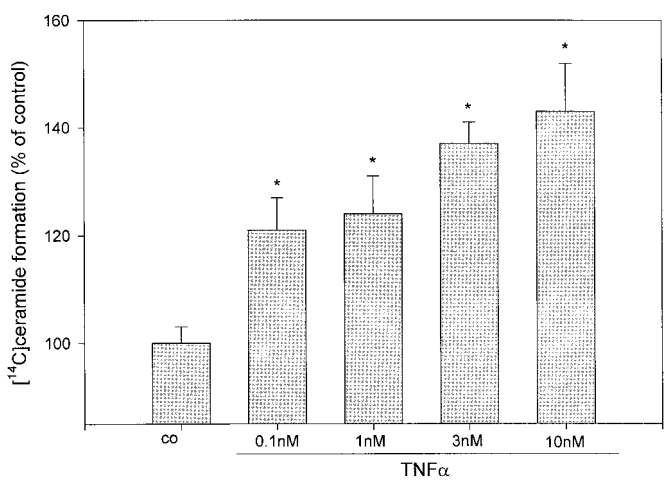

FIG. 2. Effect of TNF-α on chronic ceramide formation in glomerular endothelial cells. Glomerular endothelial cells were labeled with [^{14}C]serine and stimulated for 24 h with the indicated concentrations of TNF-α. Thereafter lipids were extracted, separated by TLC as described under Methods, and the spots corresponding to ceramide were quantitated on a TLC scanner. Data are expressed as percentage of control values and are means ± SD, $n = 3$. *$p < 0.05$, statistically significant difference compared to the unstimulated control.

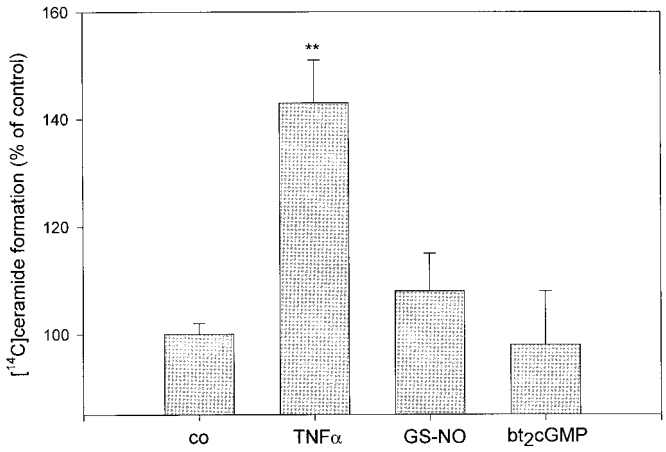

FIG. 3. Effect of short-term stimulation with TNF-α, GS-NO and dibutyryl cGMP on ceramide formation in glomerular endothelial cells. Glomerular endothelial cells were labeled with [^{14}C]serine and stimulated for 20 min with either vehicle (co), TNF-α (0.1 nM), GS-NO (1 mM) or dibutyryl cGMP (bt_2cGMP; 1 mM). Thereafter lipids were extracted, separated by TLC as described under Methods, and the spots corresponding to ceramide were quantitated on a TLC scanner. Data are expressed as percentage of control values and are the means ± SD of three independent experiments; **p $<$ 0.01, statistically significant difference compared to the unstimulated control.

teractions of NO with iron sulfur centers or protein thiols in various important cellular molecules [17].

In glomerular endothelial cells, we found that the NO-induced chronic ceramide production is independent of cGMP formation as the membrane-permeant cGMP analog dibutyryl cGMP (10 μM–1 mM) does not cause an increased ceramide generation (data not shown). In contrast, TNF-α, a potent activator of sphingomyelinases in many cell systems, leads to a concentration-dependent enhancement of ceramide production as seen in Fig. 2.

Short-term treatment of endothelial cells for 20 min reveals that only TNF-α is able to stimulate a acute burst of ceramide formation whereas spermine-NO and dibutyryl-cGMP fail to do so (Fig. 3).

To further determine the molecular mechanism by which NO triggers the delayed ceramide formation, cells were stimulated in the presence of an acidic ceramidase inhibitor, *N*-oleoylethanolamine, which prevents the degradation of ceramide to sphingosine [18]. As seen in Fig. 4A, *N*-oleoylethanolamine potentiated NO-induced ceramide formation without affecting basal ceramide levels.

Since PKC activation modulates NO-induced cell responses in a variety of cell types [19], we tested the effect of the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA) on NO-induced ceramide formation in endothelial cells. However, as seen in Fig. 4A, TPA does not significantly affect NO-stimulated ceramide generation in glomerular endothelial cells.

Iodoacetamide has been reported as a thiol-specific inhibitor by its ability to carboxymethylate accessible cysteine residues in proteins [20]. In the presence of iodoacetamide NO-induced ceramide generation is partially reduced, reaching maximally 56 ± 12% inhibition (Fig. 4B), thus indicating that enzymes containing cysteines in their active center play an important role in the mechanism leading to delayed ceramide accumulation. A similar dose-dependent reduction was observed with α-tocopherol (Fig. 4B), a potent radical scavenger that prevents oxidative damage [21] which suggests that ceramide production is regulated in a redox-sensitive manner.

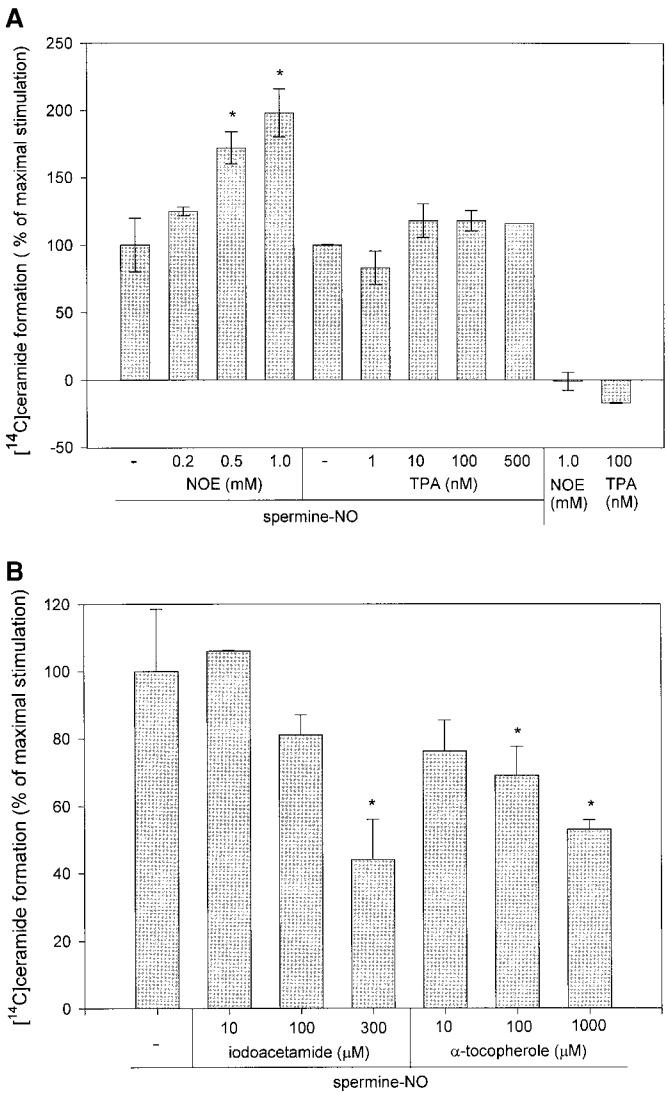

FIG. 4. Regulation of NO- induced ceramide formation in endothelial cells. Glomerular endothelial cells were labeled with [^{14}C]serine and stimulated for 24 h with either vehicle (co) or spermine-NO (1 mM) in the absence (−) or presence of the indicated concentrations of *N*-oleoylethanolamine (NOE, A), iodoacetamide(B) and α-tocopherol (B), or TPA (C). Thereafter lipids were extracted, separated by TLC as described under Methods, and the spots corresponding to ceramide were quantitated on a TLC scanner. Data are expressed as percentage of control values and are the means ± SD, n = 3–4. *p $<$ 0.05, statistically significant difference compared to the spermine-NO-stimulated control.

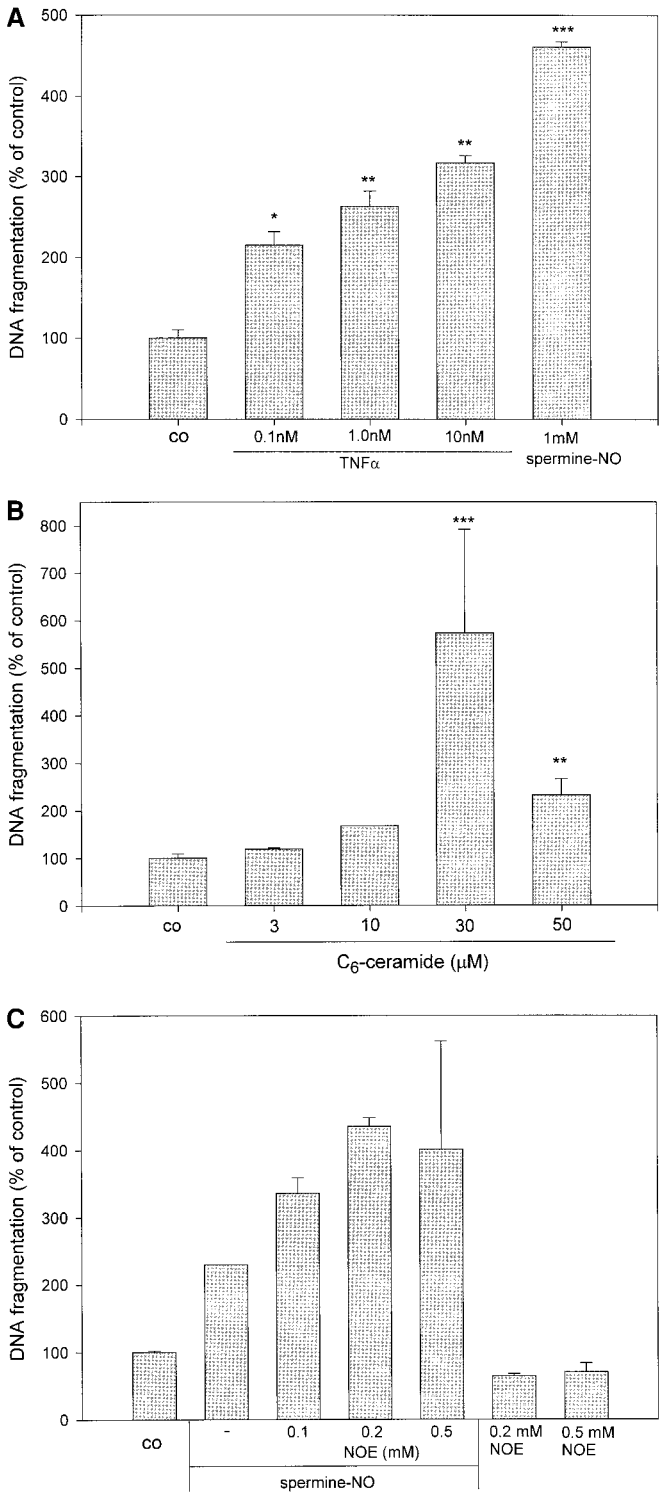

FIG. 5. Effect of TNF-α, NO and exogenous ceramide on DNA fragmentation of glomerular endothelial cells. Confluent endothelial cells were stimulated for 24 h with either vehicle (control) or the indicated concentrations of TNF-α (A) and spermine-NO (A), or C6-ceramide (B). (C) Endothelial cells were stimulated for 24 h with 0.5 mM spermine-NO in the absence (−) or presence of the indicated concentrations of *N*-oleoylethanolamine (NOE). Thereafter DNA fragmentation was measured as described under Methods. Data are expressed as percentage of control values and are the means ± SD, $n = 2$–10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, statistically significant difference compared to the unstimulated control.

To see whether the delayed NO-induced ceramide production is associated with increased cell death, we measured DNA fragmentation of glomerular endothelial cells. Figure 5A demonstrates that both stimuli, TNFα as well as NO induce DNA fragmentation, thus confirming our previously obtained data. This stimulatory effect on DNA fragmentation can be mimicked by addition of an exogenous short chain analogue of ceramide (Fig. 5B). In addition, *N*-oleoylethanolamine further significantly increased NO-stimulated DNA fragmentation (Fig. 5C) without affecting DNA fragmentation per se (Fig. 5C). These results suggest that there is a correlation between the two events, i.e., delayed ceramide generation and onset of apoptosis.

DISCUSSION

Many inflammatory diseases are accompanied by a dramatically increased NO production which is centrally involved in blood vessel permeability in the inflamed tissue, the generation of highly reactive radicals like peroxynitrite, and the initiation of proapoptotic processes [10, 22]. In this study we demonstrate for the first time that NO triggers ceramide formation in glomerular endothelial cells in a cyclic GMP-independent manner.

The sphingomyelin pathway is an ubiquitous signaling pathway that is used by an variety of cell surface receptors and environmental stress factors and includes the generation of ceramide by sphingomyelinases [23, 24]. Depending on the cell type ceramide has been connected to various cell responses such as cell growth and differentiation, inflammation, aging and programmed cell death [23, 24].

Our data clearly suggest a cross-talk between the NO signaling system and the sphingolipid signaling pathways. It is tempting to speculate that NO affects one of the ceramide producing or metabolizing enzymes, i.e., acidic or neutral sphingomyelinases or ceramidases. Work is in progress to elucidate possible direct effects of NO on enzymes in the ceramide signaling cascade. Potential molecular targets of NO include iron sulfur centers, protein thiols and Fe or Cu containing proteins in various important cellular molecules [25]. Another important reaction partner of NO is superoxide and the subsequent generation of peroxynitrite, which, in the presence of a metal catalyst (like Fe^{3+}) can be converted to nitronium ion that readily nitrates tyrosine residues in proteins, and thus may block critical phosphorylation reactions in signal transduction cascades [26].

The number of newly discovered targets of NO is steadily increasing and NO was shown to affect crucial intracellular signaling pathways. In Jurkat T cells, human umbilical vein endothelial cells and in mesangial cells NO was found to activate distinct subgroups of mitogen-activated protein kinases including the stress-activated protein kinases (SAPK) and p38 kinase [10, 27, 28].

Interestingly, iodoacetamide, a potent carboxymethylating reagent that serves as a thiol-specific inhibitor, partially blocks NO-induced ceramide formation, thus indicating an essential role of cysteines in the reaction mechanism. It may be speculated that one of the ceramide-producing or metabolizing enzymes contains a critical cysteine residue in its active center.

Furthermore, the potent antioxidant and radical scavenger α-tocopherol blocks NO-induced ceramide formation clearly suggesting the involvement of a redox-sensitive pathway. α-Tocopherol has been reported to inhibit the mutagenic DNA-damaging action of NO in bacteria [29], the NO-induced cytotoxicity in pancreatic islet cells [18] and to block NO-mediated lipid peroxidation in rat brain homogenates [30]. However, the detailed mechanism by which α-tocopherol inhibits NO action still remains unclear.

PKC activation has been proposed to negatively regulates NO-induced cell responses in a variety of cell types, including RAW 264.7 macrophages [31], thymocytes [32], and glomerular mesangial cells [33], whereas a potentiation of NO-mediated effects by PKC is observed in HL60-promyelocytic leukemia cells [34] and smooth muscle cells [35].

Here we describe that in glomerular endothelial cells neither NO-mediated ceramide formation (Fig. 4C) nor DNA fragmentation (data not shown) are negatively regulated by PKC, but rather positively influenced since a slight increase in NO-induced ceramide generation (Fig. 4A) and apoptosis (data not shown) occurs. One possible explanation may be that glomerular endothelial cells express a constitutive NOS which is per se activated by phorbol esters to produce increased amounts of endogenous NO [36]. Alternatively, the negative regulation of NO-mediated effects may be mediated by a specific PKC isoenzyme which is differentially expressed and activated in the different cell types investigated [37, 38].

The fact that not only TNF-α and NO-donors, but also exogenous ceramide is able to induce apoptosis in glomerular endothelial cells further provides evidence that ceramide is a key player in cytokine- and NO-stimulated cell death. Additional support for a possible causal relationship between ceramide formation and increased endothelial cell apoptosis [this study and 10, 24] is given by the fact that an inhibitor of the acidic ceramidase, *N*-oleoylethanolamine [20] enhances both NO-stimulated ceramide formation (Fig. 4A) and NO-induced apoptosis (Fig. 5C).

It is however noteworthy that not the acute formation of ceramide, but rather the prolonged time period of the increased levels of ceramide, may be essential for initiating programmed cell death.

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