Activation of Endothelial Nitric-Oxide Synthase by Tumor Necrosis Factor-α: A Novel Pathway Involving Sequential Activation of Neutral Sphingomyelinase, Phosphatidylinositol-3′ kinase, and Akt

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ABSTRACT

Activation of endothelial nitric-oxide synthase (eNOS) has been shown to occur through various pathways involving increases in the cytosolic Ca^{2+} concentration, activation of the phosphatidylinositol-3′ kinase/Akt pathway, as well as regulation by other kinases and by protein-protein interactions. We have recently reported that eNOS, expressed in an inducible HeLa Tet-off cell line, is activated by tumor necrosis factor-α (TNF-α) in a previously undescribed pathway that involves the lipid messenger ceramide. We have now characterized this pathway. We report here that eNOS activation in response to TNF-α correlated with phosphorylation of Akt at Ser 473 and of eNOS itself at Ser 1179. Akt and eNOS phosphorylation, as well as eNOS activation, were blocked by inhibitors of both phosphatidylinositol-3′ kinase and neutral sphingomyelinase. In contrast, although acid sphingomyelinase was also stimulated by TNF-α, its inhibition was without effect. The activation of neutral sphingomyelinase triggered by TNF-α was insensitive to phosphatidylinositol-3′ kinase inhibitors. Taken together, these results indicate that eNOS activation by TNF-α occurs through sequential activation of neutral sphingomyelinase and of the phosphatidylinositol-3′ kinase/Akt pathway. The time course of eNOS activation induced through this pathway was markedly different from that triggered by ATP and epidermal growth factor, which activate eNOS through an increase in intracellular Ca^{2+} concentration and through a sphingomyelinase-independent stimulation of the phosphatidylinositol-3′ kinase/Akt pathway, respectively. The novel pathway of activation of eNOS described here may have broad biological relevance because neutral sphingomyelinase is activated not only by TNF-α but also by a variety of other physiological and pathological stimuli.

The nitric-oxide synthase originally described in the endothelium (eNOS) is now known to be expressed in a variety of other cells and to play a role not only in the cardiovascular system but also in the regulation of the nervous and immune systems, in muscle function, and in tissue and organ development (see, e.g., Dinerman et al., 1994; Sciorati et al., 1997; Ignarro et al., 1999; Yamashita et al., 2000; Aguirre et al., 2001; Hefler et al., 2001). In the last few years, attention has focused on the mechanisms by which eNOS is activated. Most eNOS-activating stimuli act via increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which lead to binding of the Ca^{2+}-calmodulin complex to eNOS via the phosphatidyl-inositol 3′ kinase (PI3K)-dependent activation of Akt, which phosphorylates eNOS at its Ser 1177/1179 (human/bovine sequences) residue, or via a combination of these signaling pathways. In addition, eNOS activity may be regulated through phosphorylation at various sites by other kinases, as well as by protein-protein interaction with caveolin-1, 90-kDa heat shock protein, dynamin-2, and NOSIP (for review, see Fulton et al., 2001).

We have recently developed a HeLa Tet-off cell line that expresses eNOS only when doxycycline is removed (Bulotta

ABBREVIATIONS: eNOS, endothelial nitric oxide synthase; PI3K, phosphatidyl-inositol 3′ kinase; TNF-α, tumor necrosis factor-α; TNF-RI, 55-kDa tumor necrosis factor-α receptor; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; SMase, sphingomyelinase; Ab, antibody; PDMP, DL-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; D609, tricyclodecan-9-yl xanthate; A-SMase, acid sphingomyelinase; N-SMase, neutral sphingomyelinase; LY 294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; L-NAME, N^{ω}-nitro-L-arginine methyl ester; EGF, epidermal growth factor; Fum B1, fumonisin B1.

et al., 2001). Using these eNOS-Tet off cells, we recently discovered that tumor necrosis factor α (TNF-α) leads to eNOS activation and generation of bioavailable nitric oxide (NO) (Bulotta et al., 2001). TNF-α is a cytokine involved in the regulation of a variety of biological events, including cell death. Most of the actions exerted by TNF-α are through the stimulation of its p55 type I receptor (TNF-RI) and involve, among other signals, generation of the lipid messenger ceramide (for review, see Baud and Karin, 2001; Hannun and Obeid, 2002). Our recent results showed that an early production of ceramide is involved in the pathway leading to eNOS activation by TNF-α and that NO thus generated is cytoprotective (Bulotta et al., 2001). However, the intracellular signaling events involved in ceramide-dependent eNOS activation, and the relationship between this and the other eNOS activating signaling pathways were not known.

We have now investigated in detail the mechanism of eNOS activation in response to TNF-α. We focused on the enzymes acid sphingomyelinase (A-SMase) and neutral sphingomyelinase (N-SMase) because they are activated after initial signaling events triggered by the TNF-α/TNF-RI interaction (Wiegmann et al., 1999; Ségui et al., 2001) and account for the initial wave of ceramide generation (Wiegmann et al., 1994; Bourteele et al., 1998; Schütze et al., 1999). We found that eNOS activation occurs through a sequence of events involving activation of N-SMase and then of PI3K/Akt. The time course and mechanism of activation of eNOS via this pathway differ markedly from those observed after stimuli that either increase $[Ca^{2+}]_c$ or activate PI3K/Akt in an SMase-independent way.

Materials and Methods

Materials. The following reagents were purchased as indicated: [N-methyl-^{14}C]sphingomyelin, L-[^{3}H]arginine, and the Enhanced Chemiluminescence Plus kit from Amersham Biosciences (Little Chalfont, U.K.); recombinant human TNF-α from Alexis Italia (Florence, Italy); anti-eNOS monoclonal antibody (Ab) from BD Transduction Laboratories (Lexington, KY); anti-phospho-eNOS (Ser 1177), anti-Akt and anti-phospho-Akt (Ser 473) polyclonal Abs from Cell Signaling Technology (Beverly, MA); DL-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) from Calbiochem (Bad Soden, Germany), cell culture media from Invitrogen (Paisley, UK) except for the Tet System-Approved fetal bovine serum and G418, obtained from BD Biosciences Clontech (Palo Alto, CA) and Roche Diagnostics (Mannhein, Germany), respectively. Fumonisin B1 (Fum B1), wortmannin, LY 294002, and all other chemicals were from Sigma (St.Louis, MO).

Of the compounds used to inhibit SMases, imipramine and manumycin A were obtained from Sigma and tricyclodecan-9-yl xanthate (D609) from Calbiochem, whereas scyphostatin was a kind gift of Dr. T. Ogita (Sankyo Ltd., Tokyo, Japan). Concentrations of use for these compounds were selected in preliminary experiments by measuring concentration-dependent inhibition of both A- and N-SMase.

Cell Culture and Preparation. The characterization of the eNOS Tet-off cells used in this study has been reported elsewhere (Bulotta et al., 2001). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% Tet system-approved fetal bovine serum, 50 UI/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 100 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml doxycycline and maintained at 37°C under a 5% CO_2 atmosphere. To induce expression of eNOS, cells were plated in the absence of doxycycline. The experiments were carried out on cells cultured for 72 h without the antibiotic. Incubations with D609 (25 μg/ml), scyphostatin (1 μM), manumycin A (5 μM), wortmannin (100 nM), LY 294002 (5 μM), or N^ω-nitro-L-arginine methyl ester (L-NAME; 2 mM) were for 15 min; those with imipramine (20 μM) were for 60 min; those with PDMP or Fum B1 for 3 h in the culture medium before administration of ATP (100 μM), epidermal growth factor (EGF; 100 ng/ml), or TNF-α (50 ng/ml). Cells were incubated in the presence of the various compounds throughout the experiments.

Measurement of SMase Activities. SMase activities were assayed essentially as reported previously (Wiegmann et al., 1994). Cells were detached by trypsinization and, after preincubation with the various drugs, cell suspensions (2×10^6 cells in 1 ml) were treated for 10 min at 37°C with TNF-α, ATP, or EGF. The incubations were terminated by rapid immersion of reaction tubes in a methanol/dry ice bath. The suspensions were then centrifuged (1000g for 2 min at 4°C), and the pellet was washed once with ice-cold phosphate-buffered saline. To assay A-SMase activity, cell pellets were resuspended in 0.2 ml of 0.1% Triton X-100 at 4°C for 15 min and subsequently homogenized by repeated squeezing through an 18-gauge needle. Homogenates were then centrifuged (1000g for 5 min at 4°C) to remove nuclei and insoluble material and 100 μl of the supernatant was supplemented with an equal volume of a solution containing: 500 mM Na acetate, 2 mM EDTA, [N-methyl-^{14}C]-sphingomyelin (55 mCi/mmol; 50000 dpm/assay), pH 5.5, and then incubated for 2 h at 37°C. To assay N-SMase activity, cell pellets were resuspended in 0.2 ml of a buffer consisting of 0.2% Triton X-100, 20 mM HEPES, 1 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na₃VO₄, 30 mM p-nitrophenylphosphate, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin, 5 mg/ml soybean trypsin inhibitor, and 2 mg/ml aprotinin, pH 7.4. After a 5-min incubation on ice, cells were homogenized and centrifuged as above. One hundred microliters of the supernatant were then supplemented with an equal volume of a solution containing 20 mM HEPES, 1 mM MgCl₂, and [N-methyl-^{14}C]-sphingomyelin (55 mCi/mmol; 50,000 dpm/assay), pH 7.4, and then incubated for 2 h at 37°C. Reactions for both A- and N-SMase were stopped by addition of 250 μl of ice-cold CHCl₃/MeOH (2:1, v/v) and 250 μl of ice-cold H_2O. Phosphorylcholine thus produced was separated from sphingomyelin by sequential washing with 800 μl of ice-cold CHCl₃/MeOH (2:1, v/v), 500 μl of CHCl₃, and 750 μl of CHCl₃/MeOH/H_2O (2:47:48, v/v/v). Aqueous and organic phases were collected separately, and radioactive phosphorylcholine in the aqueous phase was counted by liquid scintillation in a Beckman β-counter. Nonhydrolyzed, radioactive sphingomyelin in the organic phase was routinely measured as an internal control. Samples containing either purified A- or N-SMase (Sigma) were assayed in parallel as positive controls. Results were normalized to the protein content evaluated by the bicinchoninic acid procedure (Perbio, Bezons, France).

Assay of NOS Activity. NOS activity was assayed in intact cells by measuring the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline. After preincubation in the presence or absence of various inhibitors for the times indicated above (see *Cell Culture and Preparation*), cell monolayers were washed and then incubated for 20 min at 37°C in a reaction buffer containing, in addition to the inhibitors (when used), 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM glucose, 1 mM CaCl₂, and 10 mM HEPES, pH 7.4. In the experiments measuring eNOS activation by TNF-α, 2.5 μCi/ml of L-[^{3}H]arginine was added 1 min before stimulation with the cytokine. The reaction was stopped 10 min later by washing the monolayers with 2 ml of ice-cold phosphate-buffered saline, pH 7.4, supplemented with L-arginine (5 mM) and EDTA (4 mM). Before a final addition of 2 ml of 20 mM HEPES, pH 6.0, 0.5 ml of 100% cold ethanol was added to the dishes and left to evaporate. In the experiments aimed at comparing the time course of eNOS activation by TNF-α, ATP, or EGF, we used the same protocol except that 2.5 μCi/ml of L-[^{3}H]arginine was added at various time-points after administration of each eNOS activating compound, and the reaction was stopped after 2 min. Nonstimulated cells were run in parallel. Separation of L-[^{3}H]citrulline from

L-[^{3}H]arginine was carried out by DOWEX 50 × 8-400 chromatography (Sigma) as described previously (Bulotta et al., 2001). Data are presented without background correction. L-[^{3}H]citrulline formed was normalized to protein content (bicinchoninic acid assay).

Protein Extraction and Immunoblot Analysis. After the various treatments, cell monolayers were washed free of medium, solubilized by direct addition of a preheated (to 80°C) denaturing buffer containing 50 mM Tris-Cl, pH 6.8, 2% SDS, and a protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany), and immediately boiled for 2 min as described previously (Bulotta et al., 2001). After addition of 0.05% bromphenol blue, 10% glycerol, and 2% β-mercaptoethanol, samples were boiled again and loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). Antibodies specific for phospho-

eNOS or phospho-Akt or those recognizing both the phosphorylated and nonphosphorylated forms of the enzymes were used to reveal the respective antigens. After incubation with appropriate secondary Abs, blots were developed with the enhanced chemiluminescence procedure.

Measurement of [Ca^{2+}]$_c$. Cells were detached by trypsinization, washed in a Krebs/Ringer/HEPES buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 6 mM glucose, and 25 mM HEPES-NaOH, pH 7.4, and loaded in the same buffer with the Ca^{2+}-sensitive dye fura-2 (4.5 μM), administered as acetoxymethyl ester for 30 min at 25°C. Cell preparations were then diluted (5 × 10^6 cells) and [Ca^{2+}]$_c$ was analyzed, before and after challenge with either ATP, EGF, or TNF-α, in an LS-50B fluorimeter (PerkinElmer, Boston, MA) as described previously (Clementi et al., 1995).
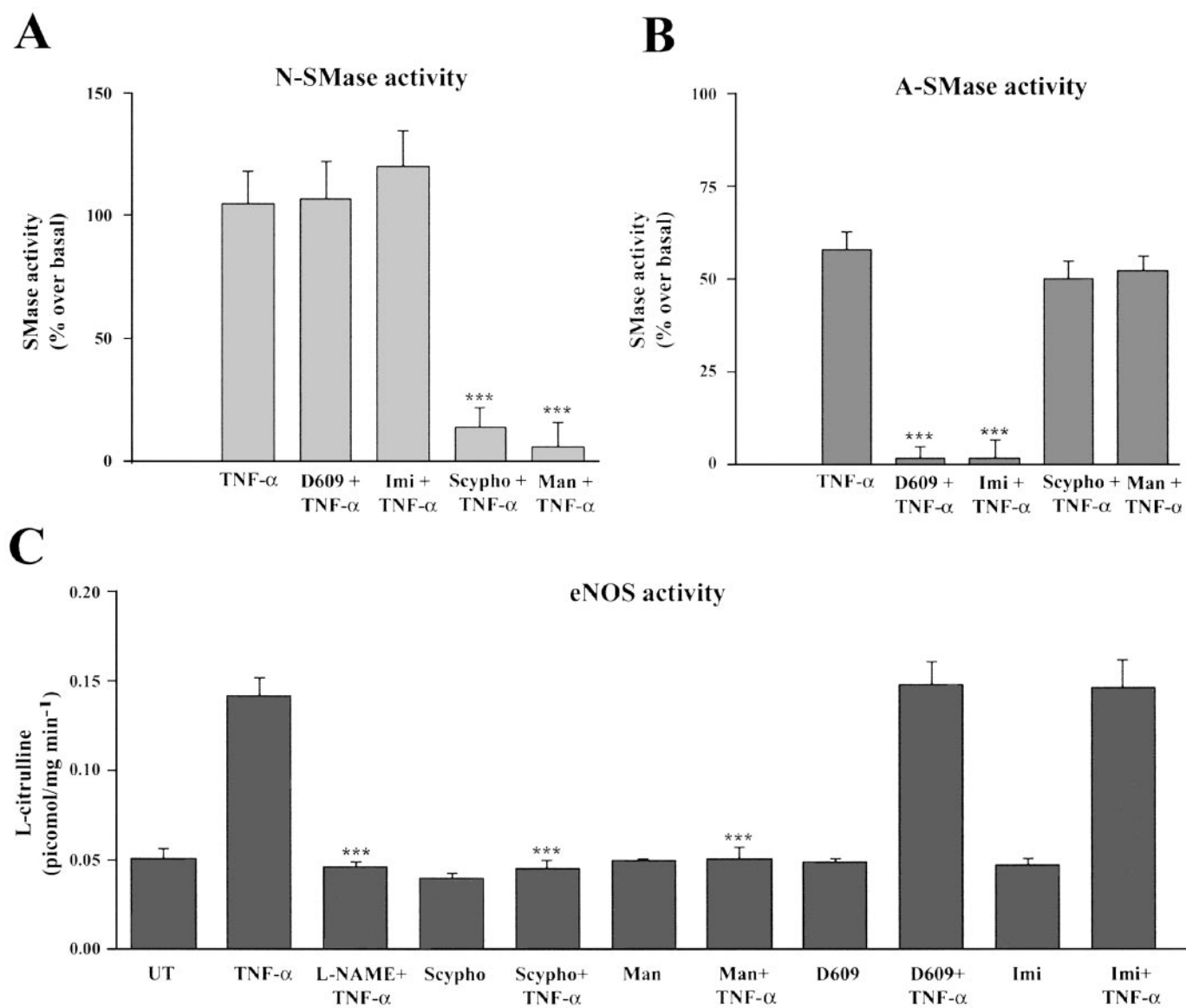

Fig. 1. TNF-α activates eNOS through N-SMase. eNOS-Tet off cells were treated for 10 min with TNF-α (50 ng/ml) with or without either D609 (25 μg/ml), imipramine (Imi; 20 μM), scyphostatin (Scypho; 1 μM), manumycin A (Man; 5 μM) or L-NAME (2 mM), as indicated. A and B, specificity of action of the various SMase inhibitors on TNF-α–triggered N-SMase (A) and A-SMase (B) activities, which were determined in cell lysates by measuring [N-methyl-^{14}C]-sphingomyelin hydrolysis to phosphorylcholine at pH 7.4 (for N-SMase activity) and 5.5 (for A-SMase activity). Values are expressed as percentage ± S.E.M. over SMase activity measured in untreated controls (0.61 ± 0.3 and 1.33 ± 0.6 nmol/mg/h for N-SMase and A-SMase, respectively) (n = 5). C, effects of L-NAME and the various SMase inhibitors on basal and TNF-α–triggered eNOS activity. eNOS activity was estimated as picomoles per minute of L-[^{3}H]citrulline formed in the reaction, normalized to protein content. UT shows L-[^{3}H]citrulline formed in untreated controls. Results are expressed as mean ± S.E.M. (n = 8). Statistical probability is indicated by the asterisks and calculated versus cells treated with only TNF-α.

Statistical Analysis. The results are expressed as means ± S.E.M.; n represents the number of individual experiments. Statistical analysis was carried out using the Student's t test for unpaired variables (two-tailed). The symbol *** in the figure refers to statistical probability (P) of <0.001 as detailed in the legends to figures.

Results

Our previous work showed that increased ceramide levels and NO generation in eNOS-Tet off cells are detected at 10 to 15 min after challenge with TNF-α (Bulotta et al., 2001). To investigate the mechanism of ceramide generation, eNOS-Tet off cells were treated with TNF-α (50 ng/ml) for 10 min and SMase activity was measured in cell lysates at the optimum pH for each enzyme (pH 5.5 and 7.4 for A- and N-SMase, respectively). As shown in Fig. 1, A and B, TNF-α activated both N- and A-SMase.

To elucidate the role of each SMase in eNOS activation, we used a range of compounds known to inhibit, directly or indirectly, either the acid or the neutral enzymatic activity. To inhibit A-Smase, we used imipramine, which induces proteolysis of the enzyme (Hurwitz et al., 1994; Grassmé et al., 1997; Jensen et al., 1999), and D609, a potent inhibitor of the phosphatidylcholine-specific phospholipase C, an enzyme known to be involved in A-SMase activation by TNF-α, and other stimuli, through generation of diacylglycerol (Müller-Decker, 1989; Schütze et al., 1992; Wiegmann et al., 1994; Grassmé et al., 1997). Inhibition of N-SMase was obtained using two specific, direct inhibitors (i.e., scyphostatin and manumycin A) (Tanaka et al., 1997; Arenz et al., 2001). We tested the specificity of these compounds in our cell model. When administered alone, none of the drugs had any effect on the basal level of sphingomyelin hydrolysis (not shown). At the concentrations used, however, scyphostatin (1 μM) and manumycin A (5 μM) were found to inhibit N-SMase, but not A-SMase activation by TNF-α. In contrast, D609 (25 μg/ml) and imipramine (20 μM) inhibited the A-SMase activity, without any effect on N-SMase (Fig. 1, A and B). These data confirm the specificity of these compounds in terms of SMase inhibition; the results with D609 also confirm that A-SMase activation by TNF-α involves activation of phosphatidylcholine-specific phospholipase C.

We then used these compounds to evaluate the relative contribution of each SMase to eNOS activation by TNF-α. As shown in Fig. 1C, treatment with TNF-α for 10 min resulted
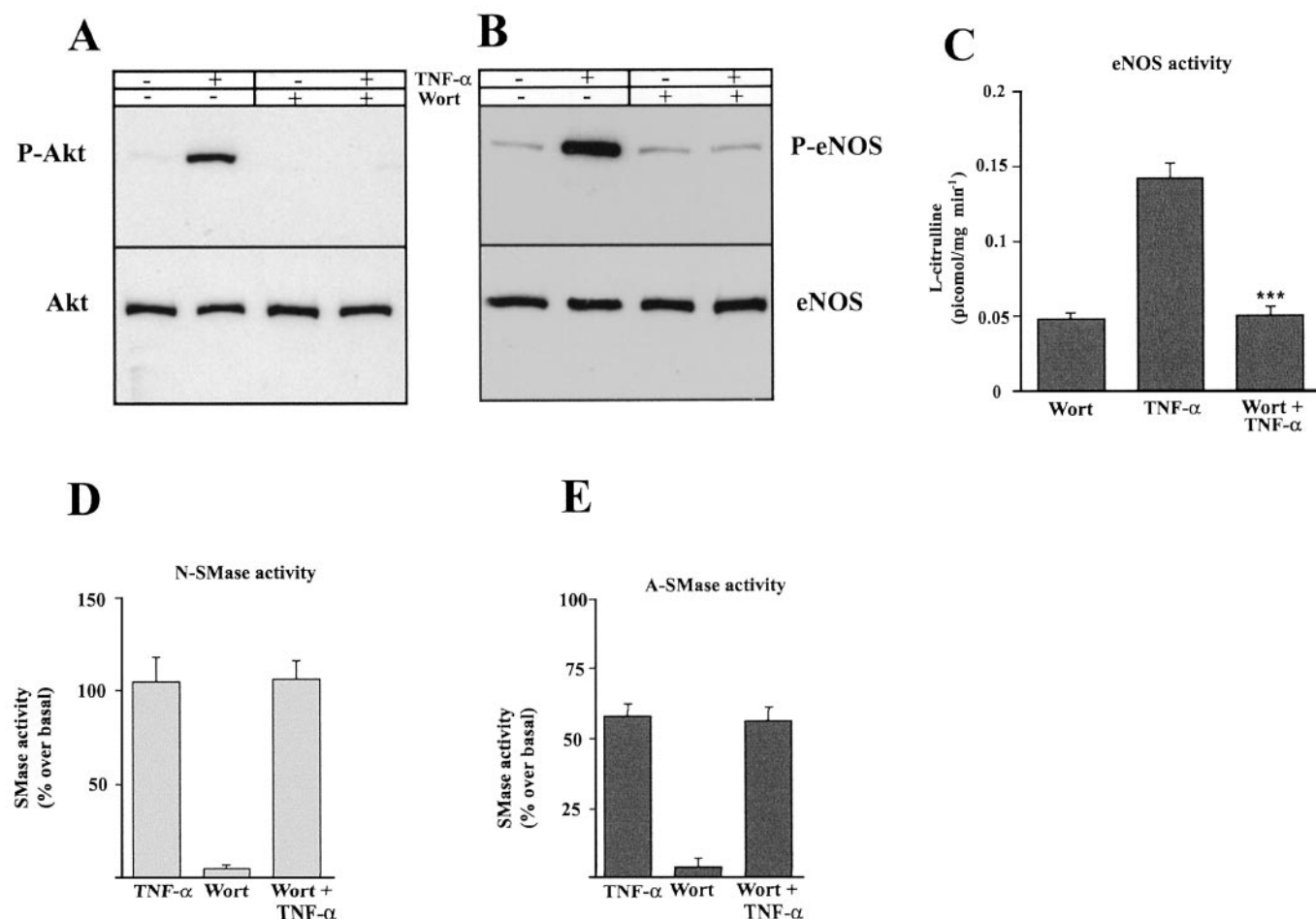

Fig. 2. eNOS activation by TNF-α involves PI3K. eNOS-Tet off cells were treated for 10 min with TNF-α (50 ng/ml) with or without wortmannin (Wort; 100 nM) as indicated in the key. A and B, cells were lysed and samples (40 μg) analyzed by Western blotting with Abs recognizing specifically either active, phosphorylated (Ser 473) Akt (P-Akt), or active, phosphorylated (Ser 1179) eNOS (P-eNOS). Control analysis of the amount of total, active, and inactive enzymes was carried out in parallel, with Abs recognizing both phosphorylated and nonphosphorylated enzymes (Akt and eNOS). The data shown are representative of four reproducible experiments. Analyses of eNOS (C), N-SMase (D), and A-SMase (E) activities were carried out and reported as described in the legend to Fig. 1 ($n = 6$). Statistical probability is indicated by the asterisks and calculated versus cells treated with only TNF-α.

in NOS activation, measured as conversion of L-arginine into L-citrulline in intact cells. This enzymatic activity, which was inhibited by the NOS inhibitor L-NAME (2 mM), is caused by activation of the transfected eNOS because it is not detectable when eNOS expression is suppressed by doxycycline (Bulotta et al., 2001). eNOS activation was prevented by coincubation with scyphostatin or manumycin A, whereas D609 and imipramine had no effect (Fig. 1 C), suggesting that N-SMase mediates eNOS activation triggered by TNF-α and that A-SMase is not involved.

Because activation of the PI3K/Akt pathway represents a major mechanism involved in eNOS activation (Fulton et al., 2001), we investigated its involvement in the N-SMase-dependent stimulation of eNOS by TNF-α. To this end, we analyzed first whether TNF-α stimulated phosphorylation of Akt on its Ser 473 residue, a hallmark of enzyme activation (Alessi et al., 1996), and whether this resulted in the activating phosphorylation of eNOS on its Ser 1179 (bovine sequence) residue (Dimmeler et al., 1999; Fulton et al., 1999). This analysis was carried out by Western blotting using antibodies selectively recognizing Akt and eNOS phosphorylated at these specific residues. Treatment with TNF-α for 10 min was found to induce phosphorylation of both Akt and eNOS (Fig. 2, A and B). Phosphorylation of these enzymes was prevented by coincubation with the PI3K inhibitor wortmannin (100 nM). None of these treatments modified the content of total Akt and eNOS protein, detected using antibodies recognizing both the phosphorylated and nonphospho-

rylated enzymes. Wortmannin also inhibited stimulation by TNF-α of eNOS activity (Fig. 2 C), whereas it had no effect on the activation by the cytokine of either N- or A-SMase (Fig. 2, D and E). Similar results were obtained with the PI3K inhibitor LY 294002 (5 μM) (not shown). Thus, activation of eNOS by TNF-α requires both PI3K/Akt- and N-SMase-dependent steps. The relationship between these two pathways was investigated further. As shown in Fig. 3, phosphorylation of both Akt and eNOS induced by treatment with TNF-α for 10 min was inhibited by coincubation with scyphostatin and manumycin A. By contrast, imipramine and D609 were without any effect. Thus, activation of eNOS by TNF-α occurs through the sequential activation of the N-SMase and the PI3K/Akt pathway.

SMase and ceramide-dependent activation of Akt had been described in some cell types (Ibitayo et al., 1998; Hanna et al., 1999; Huang et al., 2001; Monick et al., 2001). Reports in other systems, however, indicate that ceramide can also inhibit Akt activation (see, for example, Schubert et al., 2000; Stratford et al., 2001; Bourbon et al., 2002). To investigate further the role of ceramide generation by SMases in the Akt-dependent activation of eNOS by TNF-α, we incubated eNOS Tet-off cells for 3 h with either the ceramide synthase inhibitor Fum B1 (10 μM) or the glucosyl ceramide synthase inhibitor PDMP (50 μM) before administration of TNF-α. As we reported previously (Bulotta et al., 2001), in our cells, incubations with these compounds reduce and increase, respectively, the concentration of ceramide and the activation
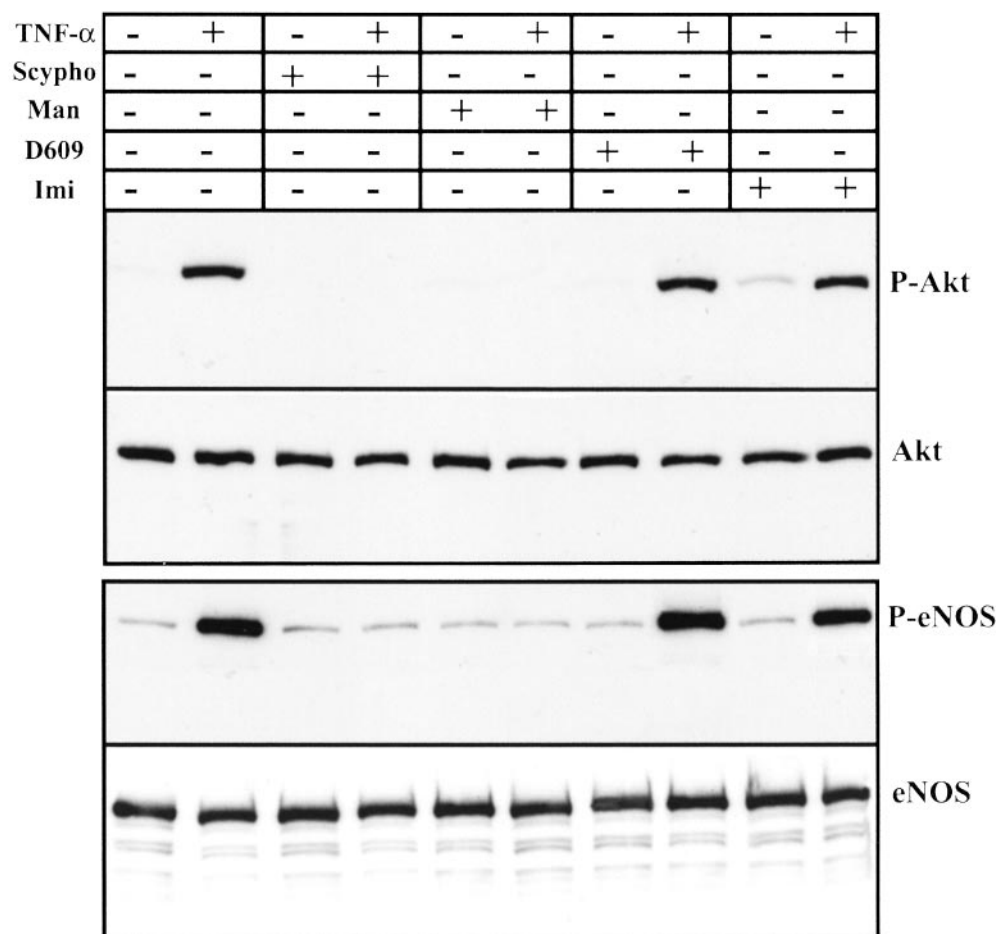
TNF-α	-	+	-	+	-	+	-	+	-	+
Scypho	-	-	+	+	-	-	-	-	-	-
Man	-	-	-	-	+	+	-	-	-	-
D609	-	-	-	-	-	-	+	+	-	-
Imi	-	-	-	-	-	-	-	-	+	+

P-Akt

Akt

P-eNOS

eNOS

Fig. 3. PI3K-dependent phosphorylation of Akt and eNOS by TNF-α is downstream to N-SMase. eNOS-Tet off cells were treated for 10 min with TNF-α (50 ng/ml) with or without either scyphostatin (Scypho; 1 μM), manumycin A (Man; 5 μM), D609 (25 μg/ml), or imipramine (Imi; 20 μM), as indicated in the key. Analysis of active, phosphorylated Akt (P-Akt) and eNOS (P-eNOS), versus total phosphorylated and nonphosphorylated enzymes, was performed by Western blotting, carried out as described in the legend to Fig. 2. The data shown are representative of four reproducible experiments.
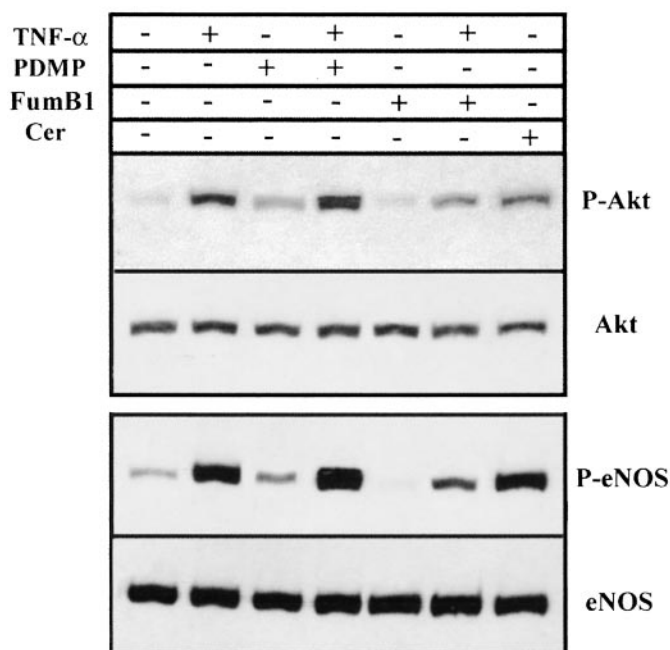
Fig. 4. Effects of varying ceramide concentration on the phosphorylation of Akt and eNOS induced by TNF-α. eNOS-Tet off cells were incubated for 3 h in the presence or absence of Fum B1 (10 μM) or PDMP (50 μM) and then treated for 10 min with either TNF-α (50 ng/ml) or C_2 ceramide (Cer; 20 μM) as indicated in the key. Analysis of active, phosphorylated Akt (P-Akt) and eNOS (P-eNOS), versus total phosphorylated and nonphosphorylated enzymes, was performed by Western blotting, carried out as described in the legend to Fig. 2. The data shown are representative of three reproducible experiments.

of eNOS observed after TNF-α treatment, presumably by altering substrate availability to SMases (Rani et al., 1995; Laouar et al., 1999; De Nadai et al., 2000; Bulotta et al., 2001). Preincubations with Fum B1 and PDMP reduced and increased, respectively, the phosphorylation of Akt and eNOS induced by TNF-α (Fig. 4). Furthermore, treatment with the membrane-permeant short-chain C_2 ceramide (20 μM) mimicked the effect of TNF-α on Akt and eNOS activation. These results confirm the role of ceramide generation in the PI3K/Akt-dependent phosphorylation of eNOS triggered by TNF-α through N-SMase.

Because several growth factors are known to operate through the PI3K/Akt pathway, we decided to compare the effects of TNF-α with those of EGF, a growth factor known to activate this signal transduction pathway in HeLa cells (Wang et al., 1998). In these experiments, we also evaluated the involvement of Ca^{2+} in eNOS activation by TNF-α, because this messenger has been reported to stimulate eNOS activation in many conditions, sometimes in concert with PI3K/Akt (for review, see Fulton et al., 2001). To this end, we analyzed the effects of ATP, an agonist that induces $[Ca^{2+}]_c$ increases via activation of the P_{2y} receptor in HeLa cells (Bastianutto et al., 1995). eNOS activity was determined at different times after challenge with the three agonists. ATP triggered a transient eNOS activation that peaked at 2 min and then diminished, returning to basal values after 6 min (Fig. 5A). This pattern resembled that of the $[Ca^{2+}]_c$ increase induced by this compound (Fig. 5B). TNF-α gave rise to a slower wave of eNOS activation. In contrast, EGF treatment resulted in a rapid onset of activation, and the enzyme activity remained well above basal values for nearly 30 min (Fig.

5A). Activation by both TNF-α and EGF occurred without a concomitant rise in $[Ca^{2+}]_c$, ruling out a major involvement of the cation in the activation of eNOS by these stimuli (Fig. 5B).

We then investigated how stimulation of the PI3K/Akt pathway correlated with the time course of eNOS activation. As shown in Fig. 5C, both TNF-α and EGF induced phosphorylation of Akt (at Ser 473) and eNOS (at Ser 1179), whereas ATP caused phosphorylation of neither enzyme. Notably, however, the time-courses of phosphorylation induced by TNF-α and EGF were different. Consistent with the different patterns of eNOS activation, phosphorylations of both Akt and eNOS induced by TNF-α occurred after a time lag compared with those induced by EGF. In addition, Akt phosphorylation induced by TNF-α persisted well beyond the period of eNOS phosphorylation and activity, whereas this dissociation between the effects on Akt and eNOS was not observed with EGF.

The results of Fig. 5 confirm that eNOS activation by both EGF and TNF-α involves phosphorylation by Akt; the difference in the time course of activation, however, also suggests that the pathway leading to Akt activation could be different for the two agonists. In agreement with this, we found that Akt and eNOS phosphorylation, as well as eNOS activation by EGF, were inhibited by wortmannin but not by SMase inhibitors (Fig. 6). Furthermore, EGF did not trigger activation of SMases (not shown), indicating that ceramide is not involved in signaling by the growth factor.

Discussion

Our results show that eNOS activation by TNF-α occurs by phosphorylation of Ser 1179 and requires activation of both N-SMase and Akt. The data indicate that the involvement of the two enzymes is sequential, because inhibition of N-SMase abolished the phosphorylation of Akt induced by TNF-α. Changes in $[Ca^{2+}]_c$ seem to play no major role in this signaling pathway. These findings establish a link between N-SMase and eNOS and indicate that activation of eNOS by TNF-α occurs through a pathway different from those activated by any other stimulus described so far. Indeed a variety of stimuli as diverse as shear stress, estrogen, vascular endothelial growth factor, insulin, corticosteroids, sphingosine 1-phosphate, lysophosphatidic acid, and possibly bradykinin have been shown to activate eNOS through a PI3K/Akt-dependent pathway (Fulton et al., 1999; Dimmeler et al., 1999; Haynes et al., 2000; Fleming et al., 2001; Harris et al., 2001; Igarashi et al., 2001; Montagnani et al., 2001; Morales-Ruiz et al., 2001; Kou et al., 2002); none of these stimuli, however, seems to involve activation of N-SMase as an intermediate step toward eNOS activation.

We have not characterized which N-SMase, among those described so far, is the one involved in eNOS activation by TNF-α, also because the molecular identity of these enzymes is still debated (Levade and Jaffrézou, 1999; Tomiuk et al., 2000; Hannun and Obeid, 2002); the involvement of an N-SMase, however, seems to have relevant consequences in the PI3K/Akt-dependent eNOS activation by TNF-α. The difference between the TNF-α–driven pathway and those elicited by other PI3K/Akt-dependent agonists was clearly illustrated in the comparison between TNF-α- and EGF-induced eNOS activation. We observed significant differences in the

pattern of both activation and inactivation of the enzyme. EGF, like the Ca^{2+}-mobilizing agonist ATP, triggered a rapid activation of eNOS, already detectable after 2 min, consistent with its ability to induce a rapid increase in Akt phosphorylation. In contrast, activation of eNOS by TNF-α occurred after a delay of 4 to 6 min; this was paralleled by delayed phosphorylation of both Akt and eNOS. SMase and ceramide have been reported to activate both PI3K and Akt in various cell systems, including epidermal cells, lymphoblasts, macrophages, and smooth muscle cells. Interestingly, in all these studies, activation of PI3K and Akt was shown to be initiated 4 to 5 min after administration of ceramide or activation of SMase (Ibitayo et al., 1998; Hanna et al., 1999; Huang et al., 2001; Monick et al., 2001). These results suggest that the delayed activation of eNOS observed with TNF-α depends on the involvement of N-SMase.

At variance with the observations reported above, in other

cell types, ceramide has been shown to inhibit Akt phosphorylation on Ser 473 and thus its activity (see, for example, Schubert et al., 2000; Stratford et al., 2001; Bourbon et al., 2002). Notably, in these cells, ceramide exerted its inhibitory effect downstream to PI3K, because PI3K activity was unaffected by the lipid. Differences in sensitivity to ceramide of the three Akt forms identified so far, or of the protein kinases responsible for their activation once recruited to the plasma membrane, only some of which have been identified (Brazil and Hemmings, 2001), may contribute to explain the difference in the Akt response to ceramide in the various cell types. Studies with cell systems other than the one employed here will thus provide further insight into the role of ceramide as second messenger after TNF-RI stimulation.

Concerning the duration of eNOS activation, EGF gave rise to a prolonged activation of the enzyme, paralleled by sustained Akt phosphorylation. In contrast, eNOS phosphor-
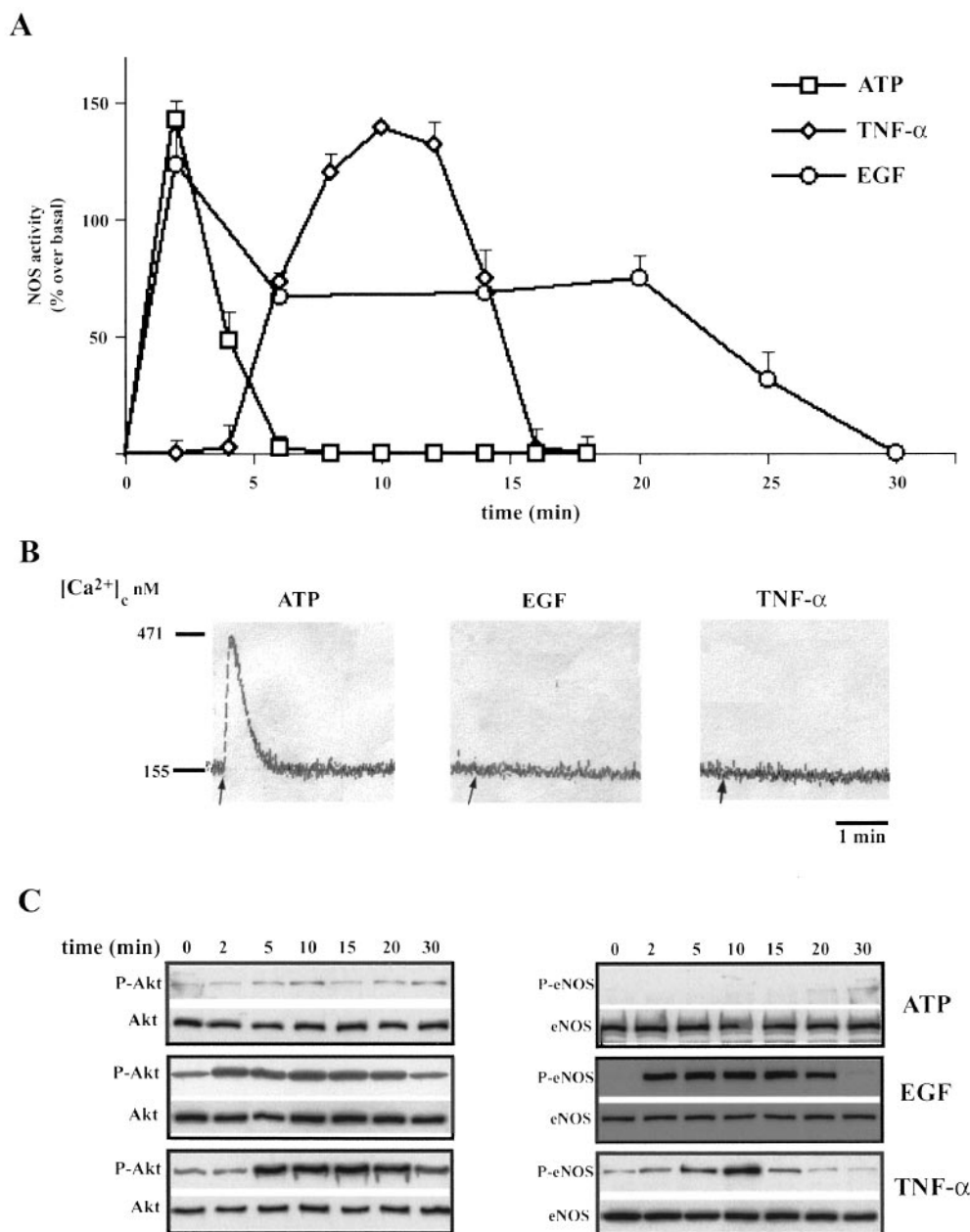
Fig. 5. Effects of different pathways of eNOS stimulation on the pattern of activity of this enzyme. A, eNOS-Tet off cells were treated with ATP (100 μM), EGF (100 ng/ml) or TNF-α (50 ng/ml) at time 0. L-[^{3}H]arginine was added at the indicated time-points and the reaction stopped after 2 min. eNOS activity was estimated as picomoles per minute of L-[^{3}H]citrulline formed in the 2-min reaction and normalized to protein content. Results are expressed as mean ± S.E.M of the percentage increase over basal L-[^{3}H]citrulline formation, measured for 2 min before administration of the various eNOS-activating stimuli (n = 6). B, [Ca^{2+}]$_c$ increases induced by ATP, EGF, or TNF-α in eNOS-Tet off cells loaded with the Ca^{2+}-sensitive dye fura-2. Stimuli were added when indicated by the arrows. [Ca^{2+}]$_c$ values are reported on the left hand side. Traces are representative of five reproducible experiments. C, pattern of Akt and eNOS phosphorylation. eNOS-Tet off cells were treated with ATP, EGF, or TNF-α, and the reactions were stopped at the indicated time points. Western blot analysis of active, phosphorylated Akt (P-Akt) and eNOS (P-eNOS), versus total phosphorylated and nonphosphorylated enzymes, was carried out as described in the legend to Fig. 2. The data shown are representative of four reproducible experiments.

ylation and activity triggered by TNF-α were of a distinctly shorter duration, despite the fact that Akt phosphorylation induced by the cytokine was sustained. This suggests that the TNF-α/N-SMase mode of activation includes switching on of signal transduction pathway(s) capable of selectively dephosphorylating, and thus inactivating, eNOS. SMases have been reported to activate various protein kinases, namely the ERK 1/2, JNK and p38 members of the mitogen- activated protein kinase family, protein kinase C-ζ, and the kinase suppressor of Ras, as well as protein phosphatases 1 and 2A (Hannun and Obeid, 2002). In principle, all of these enzymes might be involved in eNOS dephosphorylation, either directly or through intermediate proteins. A likely candidate, however, is protein phosphatase 2A, because this enzyme has been proposed to play a role in dephosphorylating eNOS at Ser 1179 (Fleming et al., 2001). Future studies will address this question, as well as investigate the consequences in terms of cellular signaling of these marked differences in the pattern of eNOS activity.

Although ceramide generation induced by TNF-α seems to be caused by activation of both A- and N-SMase, only the latter enzyme is involved in eNOS activation. This finding was not surprising, because a functional dichotomy between A- and N-SMase in mediating biological effects had already been documented in previous studies, and may be due to the different ways of interaction of these enzymes with TNF-RI (see, for example, Wiegmann et al., 1994; Zhang et al., 1997; Barsacchi et al., 2002). Recruitment of SMases to TNF-RI

occurs via different adapters (FADD for A-SMase and FAN for N-Smase) (Wiegmann et al., 1999; Ségui et al., 2001). In addition, A-SMase resides in intracellular pool(s) and may require translocation to the plasma membrane (Grassmé et al., 2001), or interaction with TNF-RI after internalization of the latter (Schütze et al., 1999). By contrast, N-SMase is localized to caveolae/rafts at the plasma membrane (i.e., the same structure where TNF-RI and eNOS reside) (Garcia-Cardeña et al., 1996; Shaul et al., 1996; Ko et al., 1999; Sowa et al., 2001; Veldman et al., 2001). Thus, the functional link we demonstrate here may be dependent on the spatial proximity between these molecules, which might lead to localized generation of ceramide. Another possibility that remains to be analyzed is whether the diverse modes of activation of SMases result in different levels of endogenous ceramide production that would activate specific effector molecules endowed with different thresholds of sensitivity to the lipid messenger.

Experimental evidence suggests that TNF-RI, N-SMase, and eNOS may interact to constitute a physiological signaling complex. SMases have been shown to participate in TNF-α–induced apoptosis in various cell systems through generation of ceramide (see, for example, Cai et al., 1997; Liu et al., 1998; Schütze et al., 1999; Ségui et al., 2001) and their inhibition by NO seems to be one of the mechanisms through which this messenger protects cells from apoptosis (De Nadai et al., 2000; Barsacchi et al., 2002). We now show that generation of NO by TNF-α occurs through an N-SMase/cer-
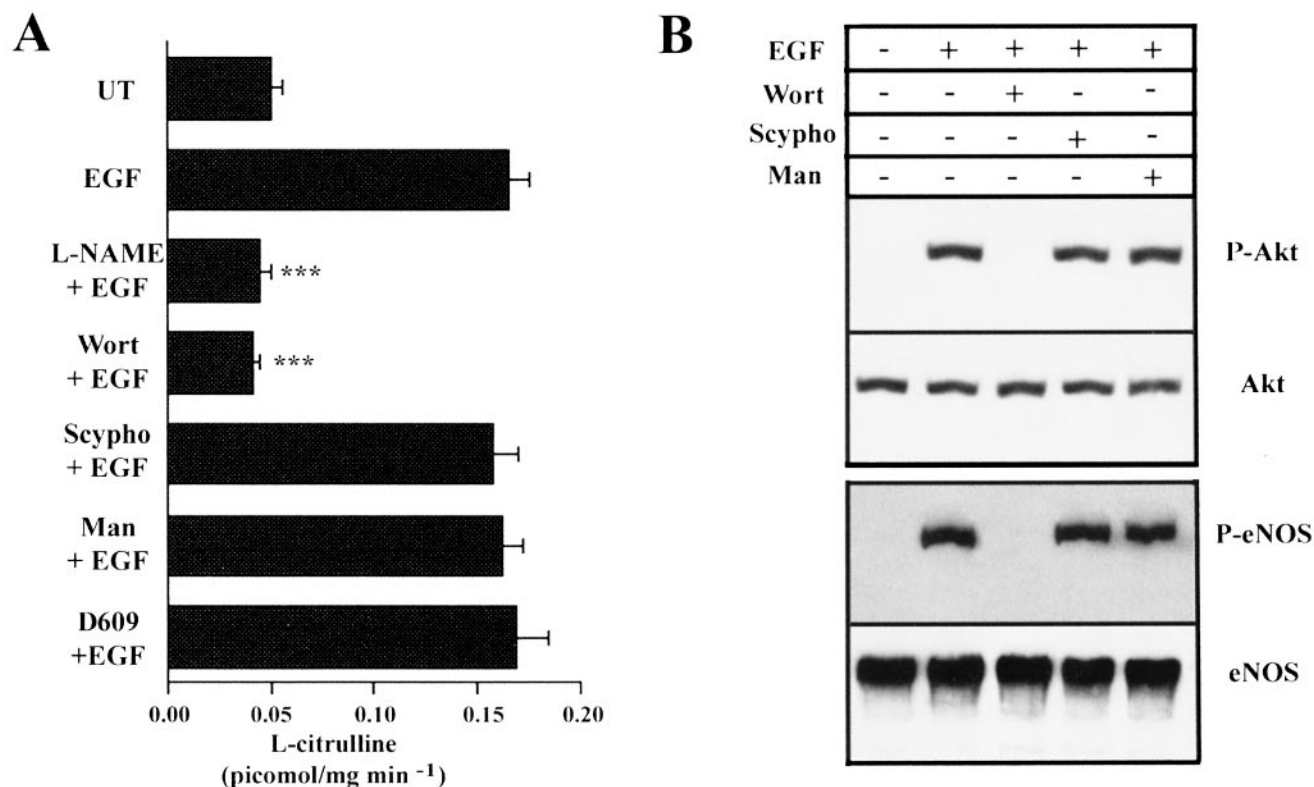
Fig. 6. eNOS activation by EGF involves PI3K but not SMases. eNOS-Tet off cells were treated for 10 min with EGF (100 ng/ml) with or without either wortmannin (Wort; 100 nM), L-NAME (2 mM), scyphostatin (Scypho; 1 μM), or D609 (25 μg/ml), as indicated. A, eNOS activity was estimated as pmol/min of L-[^{3}H]citrulline formed in the reaction, normalized to protein content, as detailed in Fig. 1. UT shows L-[^{3}H]citrulline formed in untreated controls. Results are expressed as mean ± S.E.M. ($n = 4$). Statistical probability is indicated by the asterisks and calculated versus cells treated with only EGF. B, pattern of Akt and eNOS phosphorylation, assessed by Western blot analysis of active, phosphorylated Akt (P-Akt) and eNOS (P-eNOS), versus total phosphorylated and nonphosphorylated enzymes, carried out as described in the legend to Fig. 2. The data shown are representative of three reproducible experiments.

amide-dependent pathway. Thus, eNOS and SMases seem to generate an NO/ceramide-based two-messenger system, which TNF-α triggers to regulate bidirectionally the initial steps of its own signaling pathway. Current evidence indicates that the NO-dependent protective effect may turn into a pro-apoptotic one if cell exposure to NO is prolonged (Beltràn et al., 2000). Therefore, the brief duration of eNOS activation demonstrated here could be a crucial factor in determining a protective, rather than a pro-apoptotic, effect of NO.

The link we have established between TNF-α, N-Smase, and eNOS might have broad significance. A recent report in a rat model of portal hypertensive gastropathy indicates that TNF-α also activates eNOS in endothelial cells (Kawanaka et al., 2002). In addition, N-SMase is involved not only in apoptosis but also in regulation of the cell cycle and of cell differentiation, and is activated by many stimuli other than TNF-α, among which are cytokines, growth factors, hormones, stress-inducing agents, and anticancer drugs (Levade and Jaffrézou, 1999). It is conceivable, therefore, that our findings might shed light on molecular mechanisms presiding over various pathophysiological conditions to which TNF-α, SMases, and eNOS substantially contribute.

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