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Peptides modified by myristoylation activate eNOS in endothelial cells through Akt phosphorylation

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1 Myristoylated pseudosubstrate of PKCζ (mPS) – a synthetic myristoylated peptide with a sequence (13 amino acids) mimicking the endogenous PKCζ pseudosubstrate region – is considered a selective cell-permeable inhibitor of PKCζ. We present strong evidence that in endothelial cells the action of mPS is not limited to inhibition of PKC activity and that myristoylation of certain peptides can activate eNOS (endothelial nitric oxide synthase) through Akt phosphorylation.

2 mPS at micromolar concentrations (1–10 μM) induced profound phosphorylation of eNOS, Akt, ERK 1/2, and p38 MAPK in cultured pulmonary artery endothelial cells (PAEC). The same changes were observed after treatment of PAEC with a myristoylated scrambled version of mPS (mScr), whereas a cell-permeable version of PKCζ pseudosubstrate fused to the HIV-TAT membrane-translocating peptide did not induce analogous changes, suggesting that myristoylation confers new properties on the peptides consisting of activation of different signaling pathways in endothelial cells.

3 In addition to mPS and mScr, a number of other myristoylated peptides induced phosphorylation of eNOS suggesting that myristoylation of peptides can activate eNOS by mechanisms unrelated to inhibition of PKC. All active myristoylated peptides contained basic amino acids motif and were longer than six amino acids.

4 Activation of eNOS by myristoylated peptides was dependent on the PI3K/Akt pathway and the rise of intracellular calcium and was associated with an elevation of cGMP levels in PAEC and with relaxation of precontracted isolated pulmonary artery segments.

5 Myristoylated peptides can be considered a new class of activators of NO production in endothelial cells and that using mPS as a specific inhibitor of PKCζ should be done with caution, especially in endothelial cells.
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Abbreviations: Akt, protein kinase B; AoEC, aortic endothelial cells; eNOS, endothelial nitric oxide synthase; ERK 1/2, extracellular signal-regulated kinases; HBSS, Hanks' balanced salt solution; mPS, myristoylated pseudosubstrate of PKCζ; mScr, scrambled version of mPS; p38 MAPK, mitogen-activated protein kinase; PAEC, pulmonary artery endothelial cells; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PS, pseudosubsrate; PSMC, pulmonary artery smooth muscle cells; SOD, superoxide dismutase

Introduction

Nitric oxide (NO) produced by endothelial cells is an important regulator of vascular homeostasis. Endothelial-derived NO affects a number of biological processes in the vessel wall – vessel dilation, aggregation of blood platelets, adhesion of leukocytes to the vessel wall, and migration of vascular smooth muscle cells (Huang *et al*., 1995; Hingorani *et al*., 1999). A decrease in endothelial nitric oxide synthase (eNOS)-dependent NO production is thought to contribute to the pathogenesis of various cardiovascular disorders such as hypertension and atherosclerosis (Arnal *et al*., 1999; Huang, 2003; Barbato & Tzeng, 2004).

Endothelial-derived NO is synthesized from the cationic amino-acid L-arginine in a reaction catalyzed by eNOS, and the level of NO production is dependent on eNOS activity and availability of L-arginine (Knowles & Moncada, 1994). The mechanisms controlling eNOS activity involve multiple regulatory steps including gene expression, co- and post-translational modification, intracellular localization and protein–protein interactions (Vallance & Hingorani, 1999; Fulton *et al*., 2001; Govers & Rabelink, 2001; Fleming & Busse, 2003).

To evaluate the role of protein kinase C (PKC), an intracellular serine/threonine kinase, in the regulation of eNOS, we used as a PKCζ inhibitor a myristoylated peptide with the sequence of pseudosubstrate of PKCζ. The PKC family is comprised of three subfamilies, classical (α, β_1, β_2, γ), novel (ε, δ, η, θ), and atypical (λ/ι and ζ) (Nishizuka, 2001). All members of the PKC family are inactive in the absence of activating agents owing to an intramolecular interaction between a short substrate-like peptide at the N-terminus, called the pseudosubstrate, and substrate binding region of

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the catalytic domain. Peptides based on the pseudosubstrate sequence of PKC isoenzymes have been reported to be good and selective inhibitors of specific isoforms of PKC (Eichholtz *et al*., 1993; Persaud, 1997). However, like most peptides, they are plasma membrane impermeable. Several methods have been developed to overcome this problem. (1) The pseudosubstrate sequence can be expressed in cells (Harrington *et al*., 2000). (2) The passage of pseudosubstrate into cells can be achieved using transient permeabilization of the plasma membrane by reagents such as saponin (Johnson *et al*., 1996). (3) Pseudosubstrate peptides can be fused with membrane-translocated peptides derived from Antennapedia Protein or TAT protein from human immunodeficiency virus for delivery into cells (Disatnik *et al*., 2002). (4) Pseudosubstrate peptide can be modified by attachment of a fatty acid (usually myristate) which makes the peptide membrane-permeable (Eichholtz *et al*., 1993; Thiam *et al*., 1999; Harishchandran & Nagaraj, 2005). With all these methods, it is assumed that modification itself does not have an influence on cellular processes. Herein we demonstrate that modification of peptides by myristoylation confers new properties to them beyond their original function. We show that myristoylated pseudosubstrate of PKCζ, its scrambled version and a number of other myristoylated peptides are able to activate a variety of enzymes, including eNOS, that are related to regulation of NO synthesis in endothelial cells.

Methods

Materials

Unless specified, reagents were obtained from Sigma Chemical (St Louis, MO, U.S.A.). Antibodies to Akt and phospho-Akt (Ser^{473}) were purchased from Cell Signaling (Danvers, MA, U.S.A.). Antibodies to eNOS and p38 MAPK were obtained from BD Biosciences (San Diego, CA, U.S.A.), to phospho-eNOS (Ser^{1177}) and to ERK1/2 from Upstate (Charlottesville, VA, U.S.A.), and to phospho-ERK1/2 and to phospho-p38 MAPK from Calbiochem (San Diego, CA, U.S.A.). Wortmannin, *N*-nitro-L-arginine methyl ester (L-NAME), PD 98059, SB 203580, protease inhibitor cocktail (Set III), and phosphatase inhibitor cocktail (Set II) were obtained from Calbiochem. L-[^{3}H]-arginine (specific activity 35–70 μCi/mmol) was obtained from Amersham (Piscataway, NJ, U.S.A.). Peptides: myristoylated pseudosubstrate of PKCζ (mPS) – Myr-SIYRRGARRWRKL – was purchased from BioSource International, Inc. (Camarillo, CA, U.S.A.), the non-myristoylated version (PS) was obtained from Calbiochem. The scrambled version of mPS – Myr-RLYRKRIWRSAGR (mScr), and peptides mRLYRKRIW (m1), Myr-RSAGR (m2), mRVHLKDRRAI (m3), SNYLKDHQAI (m4), and Myr-VLFKDHWRI (m5), were synthesized by SynPep Corp. (Dublin, CA, U.S.A.). Pseudosubstrate of PKCζ fused with the membrane translocating peptide of HIV-TAT protein (MTP-PS) – H-GYGRKKRRQRRR-G-SIYRRGARRWRKL – was synthesized by AnaSpec Inc. (San Jose, CA, U.S.A.). All other peptides were purchased from Calbiochem.

Cell culture

Pulmonary artery endothelial cells (PAEC) were isolated from the main pulmonary artery and aortic endothelial cells (AoEC) were isolated from the descending aorta of 6- to 7-month-old pigs by collagenase treatment as previously reported (Block *et al*., 1986). Pulmonary artery smooth muscle cells (PSMC) were obtained from the pulmonary arteries of 6-to-7-month-old pigs by the explant method (Frid *et al*., 1997). The pulmonary arteries were denuded of endothelium via scraping, and explants of the central tissue of the vessels were dissected out and plated. Cultures were subcultured when heavy growth surrounding explants was observed. Third to sixth passage cells in monolayer culture were maintained in RPMI-1640 medium containing 4% fetal bovine serum and antibiotics (10 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 20 μg ml^{-1} gentamicin, and 2 μg ml^{-1} Fungizone) and were used 2 or 3 days after confluence. Human embryonic kidney-293 cells (HEK 293) and 3T3-L1 preadipocytes were maintained in DMEM containing 5 or 10% fetal bovine serum, respectively, and antibiotics.

Western blot analysis

PAEC were grown in 60 mm Petri dishes until confluence. Before each experiment, confluent PAEC were 'starved' in serum-free DMEM overnight. Cells were treated as indicated in the legends to the figures, washed twice with ice-cold PBS, and scraped in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) containing protease inhibitor cocktail and phophatase inhibitor cocktail. The lysates were clarified, and samples (20–30 μg of protein) were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Membranes were blotted with phosphospecific antibodies to eNOS, Akt, ERK1/2, or p38 MAPK. To normalize phospho-signal to total protein level, the second gel was run in parallel, and a second membrane was blotted with antibodies to eNOS, Akt, Erk1/2, or p38 MAPK, respectively. In some cases, after detection of the phosphospecific signal, the membranes were stripped with Blot Restore Membrane Rejuvenation Kit (Chemicon Int., Temecula, CA, U.S.A.) and then blotted with antibodies corresponding to the relevant enzyme to determine the total level of enzyme protein.

Determination of eNOS activity in total membrane fractions

eNOS activity was determined by measurement of formation of L-[^{3}H]-citrulline from L-[^{3}H]-arginine as previously described (Patel & Block, 1995). Briefly, confluent PAEC were scraped and homogenized in buffer A (50 mM Tris-HCl, pH 7.4, containing 0.1 mM each of EDTA and EGTA, 1 mM phenylmethylsulfonyl fluoride, 1.0 μg ml^{-1} leupeptin, and 10 μM calpain inhibitor-1). Homogenates were centrifuged at 100,000 × *g* for 60 min at 4°C, and total membrane pellets were resuspended in buffer C (buffer A + 2.5 mM $CaCl_2$). Total membranes (100–200 μg of protein) were incubated in buffer C containing 1 mM NADPH, 100 nM calmodulin, 10 μM tetrahydrobiopterin (BH_4), and 5 μM combined L-arginine and purified L-[^{3}H]-arginine (0.6 μCi) (control) or with addition of 10 μM mPS, 10 μM mScr, or 100 μM L-NAME for 30 min at 37°C. L-[^{3}H]-citrulline was separated from L-[^{3}H]-arginine by cationic exchange chromatography (Dowex AG50W-X8; Na^+ form; Bio-Rad Laboratories (Hercules, CA, U.S.A.). The amount of the eluted L-[^{3}H]-citrulline was determined by liquid

scintillation counting. The specific activity of eNOS is expressed as L-citrulline formed (pmol min^{-1} mg^{-1} of protein).

cGMP measurements

To determine whether mPS- or mScr-induced activation of eNOS leads to an increase in intracellular cGMP, measurements of cGMP levels in PAEC were performed using a cGMP Enzymeimmunoassay Biotrak (EIA) system kit (Amersham, Arlington Heights, IL, U.S.A.). In brief, PAEC grown in six-well tissue culture plates until confluence were 'starved' in serum-free DMEM overnight. PAEC were pretreated with 20 Uml^{-1} SOD plus 300 μM isobutylmethylxanthine (IBMX) for 30 min, and then cells were incubated with different concentrations of myristoylated peptides for 30 min. In some experiments, before treatment with SOD and IBMX, PAEC were preincubated with 100 μM L-NAME, an inhibitor of eNOS, for 1 h. After treatment with the peptides, PAEC were washed twice with HBSS, scraped in ice-cold 65% ethanol, and centrifuged. The supernatants were collected, dried under vacuum at 60°C, and dissolved in the assay buffer. cGMP contents in the cell extract samples were quantitated by acetylation assay as described by the manufacturer.

Vasorelaxation of mouse pulmonary artery segments

Vasorelaxation of pulmonary artery segments (PAS) was evaluated as we have described earlier (Hu *et al*., 2004). Briefly, PAS (0.5 mm diameter, 1.5–2 mm length) were isolated from 2- to 3-month old mice. PAS were suspended in individual organ chambers (Radnoti Four-Unit Tissue Bath System) with 5 ml of Krebs buffer and oxygenated with 95% O_2 and 5% CO_2 at 37°C. After equilibration at a resting force of 0.5 *g*, 0.5 μM U-46619 (a thromboxane mimetic) was used to obtain a stable contraction after which acetylcholine (ACh, 5 μM) was added to assess the endothelium-dependent vasorelaxation response to agonist-stimulated NO production. After determination of the endothelium-dependent response, the segments were washed several times and contracted again using U-46619. After stable contraction, the segments were incubated with mPS, mScr, ACh (5 μM), or without drugs for 30 min. The vascular tensions were continuously monitored with an isometric force transducer (Harvard Apparatus, Holliston, MA, U.S.A.). To standardize the data, the vascular tone of U-46619-contracted vessels was set equal to 0% relaxation.

Statistics

All results are expressed as the mean ± s.e. Statistical analysis was performed using the two-tailed Student's *t*-test, and $P<0.05$ was considered statistically significant.

Results

mPS and mScr activate phosphorylation of eNOS, Akt, ERK 1/2 and p38MAPK in PAEC

The treatment of PAEC with mPS induced a concentration-dependent (1–10 μM) increase in phosphorylation of eNOS at Ser^{1177} (Figure 1a) whereas phosphorylation of eNOS at Ser^{116} and Thr^{413} did not change with the mPS treatment (data not shown). Therefore, further reference to phosphorylation of eNOS in this paper will mean phosphorylation at Ser^{1177}. Phosphorylation was time-dependent and maximal phosphorylation was observed after 15–30 min treatment with 10 μM mPS, and returned to the basal level within a few hours (Figure 1b). Another cell-permeable version of PKCζ pseudosubstrate fused to the HIV-TAT membrane-translocating peptide (MTP-PS) did not have an effect on phosphorylation of eNOS whereas mScr had the same effects as mPS (Figure 1c). Non-myristoylated and, hence, non-cell-permeable PKCζ pseudosubstrate (PS) did not induce eNOS phosphorylation (Figure 1c). Because only myristoylated peptides induced changes in eNOS phosphorylation status, we tested the possibility that myristic acid might induce the phosphorylation of eNOS. However, myristic acid did not induce changes in phosphorylation levels of eNOS comparable to the myristoylated peptides (Figure 1d and e).

We further assessed the phosphorylation status of Akt kinase, which is known to phosphorylate eNOS at Ser^{1177} (Fulton *et al*., 1999) as well as the phosphorylation status of ERK1/2 and p38 MAP kinase after incubation with peptides or myristic acid (Figure 2). Both myristoylated peptides mPS and mScr induced phosphorylation of Akt, ERK1/2, and p38 MAPK in PAEC whereas MTP-PS and myristic acid did not. Considering that only the myristoylated peptides – mPS and mScr – induced phosphorylation of eNOS, Akt, ERK1/2 and p38 MAPK in PAEC, we suggest that myristoylation confers new properties to peptides making them able to activate different signal molecules.

To determine whether these properties were unique to mPS and mScr or common to all myristoylated peptides, we checked the capability of other available and newly synthesized myristoylated peptides for inducing the phosphorylation of eNOS and other kinases in PAEC. The results are summarized in Table 1. We found that some peptides were more active than the others and that some did not have any effects on eNOS. Therefore, the phosphorylation of eNOS is not specific to mPS and mScr. The active peptides contained several positively charged amino acids and were at least six amino acids in length. To facilitate the further studies, we used mPS and mScr only.

mPS and mScr increase cGMP production in PAEC and induce vasorelaxation of pulmonary artery segments

It is generally accepted that phosphorylation of eNOS at Ser^{1177} leads to activation of eNOS and, as a result, to elevated cGMP production (Fleming & Busse, 2003). The levels of cGMP were measured in PAEC after 30 min incubation with mPS and mScr. Both peptides induced concentration-dependent increases in cGMP levels: 5 μM mPS increased cGMP level from 0.33 pmol $dish^{-1}$ (control cells) to 1.09 pmol $dish^{-1}$, and 5 μM mScr from 0.22 pmol $dish^{-1}$ (control cells) to 0.84 pmol $dish^{-1}$ (Figure 3). L-NAME (100 μM), an inhibitor of eNOS, blocked cGMP production in control cells and cells treated with peptides suggesting NO dependence of cGMP production.

The main physiological response to elevated NO/cGMP production is vasorelaxation. Therefore, activation of eNOS and NO production in response to incubation with myristoylated peptides were further studied in experiments using PAS. mPS caused vasorelaxation of U-46619-contracted PAS, which

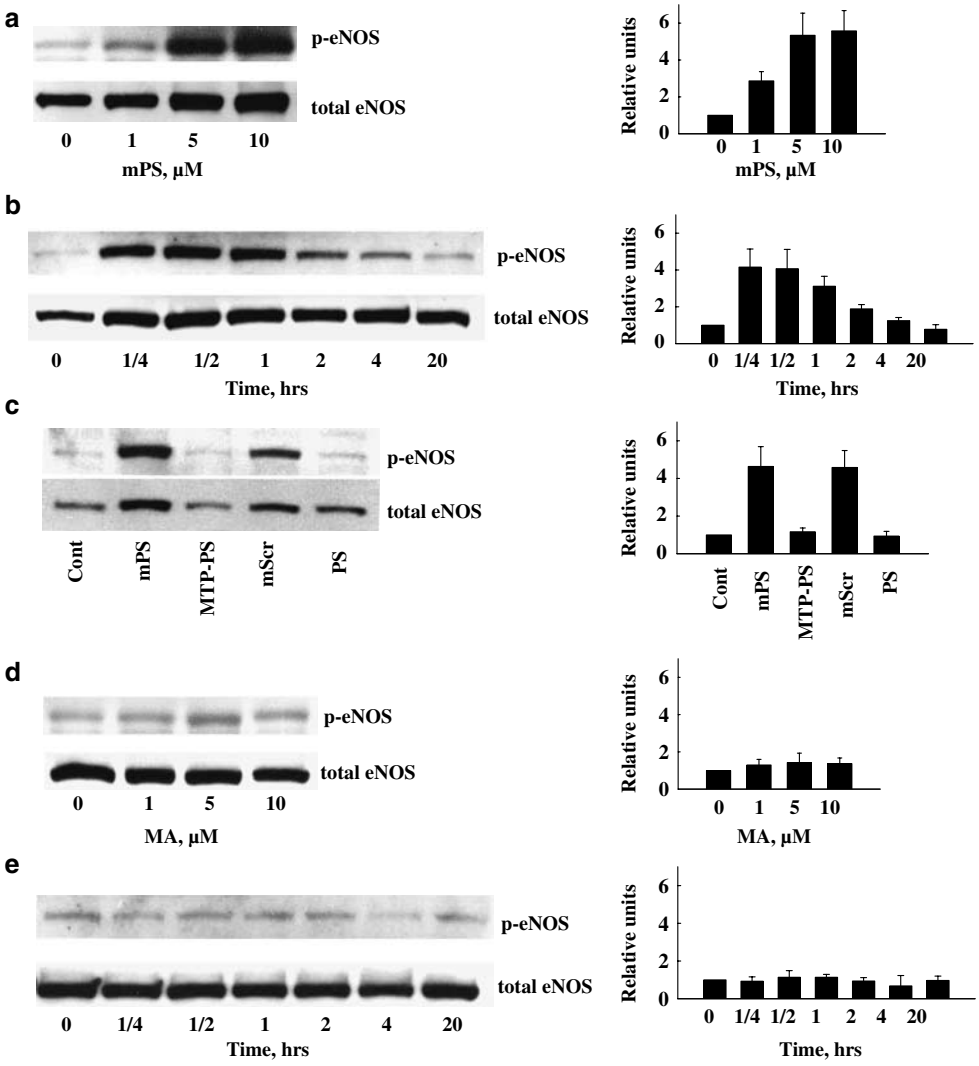

Figure 1 eNOS is phosphorylated at Ser^{1177} by mPS treatment in a dose- and time-dependent manner. In panel a, concentration dependence for mPS stimulation is demonstrated. PAEC were treated with the indicated concentrations of mPS for 15 min in serum-free DMEM, and phosphorylation of eNOS (Ser^{1177}) was analyzed by Western blotting. In panel b, the time course of eNOS phosphorylation in PAEC treated with mPS is shown. PAEC were treated with 10 μM mPS in serum-free DMEM for the indicated times, and phosphorylation of eNOS was analyzed by Western blotting. In panel c, eNOS is phosphorylated by mPS and mScr, but not by pseudosubstrate (PS) or MTP-PS. PAEC were treated with different peptides: mPS, mScr, MTP-PS or PS at a concentration of 10 μM for 15 min in serum-free DMEM, and phosphorylation of eNOS (Ser^{1177}) was analyzed by Western blotting. Panels d and e demonstrate the absence of eNOS phosphorylation (Ser^{1177}) in PAEC incubated with myristic acid (MA) for different times and with different concentrations. Three independent experiments have been performed with equivalent results. Western blots images of one of the three experiments as well as densitometric analyses of the data from all experiments (means $\pm$ s.e.) are shown. The phosphorylation levels of eNOS at Ser^{1177} are normalized to total eNOS levels and presented in relative units where control is 1.

was comparable to relaxation induced by ACh (Figure 4). Relaxation induced by mScr was a little weaker than that induced by mPS.

Phosphorylation of eNOS in response to mPS and mScr requires intact cells

We have shown above that mPS and mScr induce activation of eNOS in endothelial cells and, as a result, relaxation of vessels. To assess whether myristoylated peptides stimulate eNOS by acting directly on the enzyme or by induction of cellular signaling cascades regulating enzyme activity, isolated membranes from PAEC were treated with 10 μM mPS or mScr and eNOS activity was measured. Neither mPS nor mScr increased L-citrulline formation in isolated membrane preparations (Figure 5) demonstrating that the stimulation of eNOS activity by mPS and mScr requires intact cells and likely involves intracellular signaling mechanisms.

mPS and mScr-induced activation of eNOS are mediated through the PI3K/Akt pathway and require intracellular Ca^{2+}

We next tried to identify the molecular events involved in the activation of eNOS in PAEC in response to myristoylated peptide treatment. We have demonstrated that mPS and mScr induce phosphorylation of different kinases in PAEC: Akt, ERK1/2, p38 MAPK. In a number of studies it has been

shown that eNOS is an important Akt substrate and that eNOS is activated by phosphorylated Akt (Dimmeler *et al*., 1999; Fulton *et al*., 1999). Although the data on the role of ERK1/2 and p38 MAPK in the phosphorylation of eNOS are controversial (Bernier *et al*., 2000; Wang *et al*., 2001; Schmidt *et al*., 2002; Anter *et al*., 2004), coactivation of eNOS, ERK1/2, and p38 MAPK have been reported to occur during exposure of endothelium to VEGF (Rousseau *et al*., 1997; Kevil *et al*., 1998; Breslin *et al*., 2003). To determine which of these kinases are involved in activation of eNOS, we used a pharmacological approach (Figure 6). Pretreatment of PAEC with wortmannin, a specific inhibitor of PI3 K, which is an upstream activator of Akt, completely abolished phosphorylation of Akt and significantly reduced phosphorylation of eNOS by mPS and mScr (Figure 6) indicating that the PI3K/Akt pathway is involved in mPS-mediated phosphorylation of eNOS.

Because an increase in intracellular free Ca^{2+} ($[Ca_i^{2+}]$) is one of the major mechanisms activating eNOS, we also assessed the role of intracellular and extracellular Ca^{2+} in activation of eNOS in response to mPS or mScr (Figure 6). Incubation of PAEC in Ca^{2+}-free medium did not affect mPS-induced phosphorylation of eNOS and Akt and partially attenuated mScr-induced phosphorylation of eNOS and Akt. Pretreatment of PAEC with a cell-permeable $[Ca_i^{2+}]$ chelator (BAPTA/AM) completely abrogated the phosphorylation of eNOS and Akt. These data demonstrate that changes in $[Ca_i^{2+}]$ are important for activation of eNOS in response to mPS or mScr treatment.

PD98059 – an inhibitor of the MEK1/2 – ERK1/2 pathways – did not affect the phosphorylation of eNOS and Akt. An inhibitor of p38 MAPK – SB203580 – partially blocked the Akt phosphorylation but had no effect on eNOS. From these data, we have concluded that ERK1/2 and p38 MAPK are not involved in mPS and mScr-induced phosphorylation of eNOS.

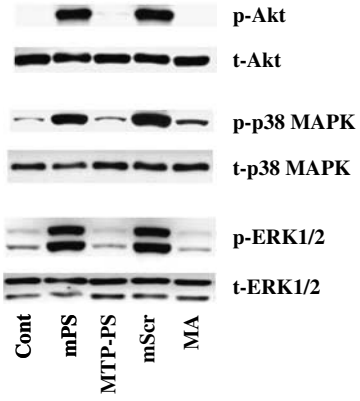

Figure 2 mPS and mScr induce phosphorylation of Akt, ERK1/2 and p38 MAPK. PAEC were incubated with 5 μM mPS, mScr, MTP-PS or MA for 15 min. After the incubations, cells were washed and scraped in RIPA buffer. Cell lysates were resolved by SDS–PAGE, and immunoblots were probed with antibodies against Ser^{473}-phospho-Akt (pAkt), phospho-ERK1/2 (p-ERK1/2), or phospho-p38 MAPK (p-p38MAPK). Antibodies against nonphosphorylated kinases were used to detect total kinase (t-kinase) expression in PAEC. Three independent experiments have been performed with equivalent results. Western blot images of one of the experiments are shown.

Cell specificity of the effects of mPS and mScr on eNOS, Akt ERK1/2 and p38 MAPK

Considering that the intracellular response to extracellular stimuli can be species and cell-type specific, we studied mPS and mScr effects on the following cell types: AoEC, PSMC, HEK 293, 3T3-L1. eNOS, present only in endothelial cells, was phosphorylated in response to mPS or mScr treatments in both AoEC and PAEC (Figure 7). Akt and ERK1/2 were activated in endothelial cell lines (PAEC, AoEC) but not in PSMC, HEK 293 or 3T3-L1. p38 MAPK was activated in endothelial cell lines, PSMC and 3T3-L1 but not in HEK293. Thus, myristoylated peptides did not activate any of the enzymes we studied in HEK 293 cells in culture and activated only p38 MAPK in PSMC and 3T3-L1, suggesting that the effects of mPS and mScr described here appear to be somewhat specific for endothelial cells.

Discussion

The initial goal of our work was to investigate the effects of mPS, which is regarded as a selective inhibitor of PKCζ (Eichholtz *et al*., 1993; Thiam *et al*., 1999), on eNOS activity in endothelial cells. Myristoylation is a modification of peptides that promotes cell permeability and is assumed to have no effect on the functional properties of the peptide within the cell. Working with mPS and its negative control mScr we discovered that myristoylation confers new properties to

Table 1 Effect of myristoylated peptides on eNOS phosphorylation (Ser^{1177})

$mPS_{PKC\xi}$	myr-N-SIY*RR*GA*RR*W*RK*L-OH	+ + + +
MScr	myr-N-*R*LY*RKR*IW*R*SAG*R*-OH	+ + + +
$mPS_{PKC\eta}$	myr-N-T*RKR*Q*R*AM*RRR*VHQING-OH	+ + + +
m1	myr-N-*R*LY*RKR*IW-OH	+ + +
mPS_{PKA}	myr-N-G*R*TG*RR*NAI-NH2	+ + +
$mPS_{PKCEGFR}$	myr-N-*RKR*TL*RR*L-OH	+ +
$mPS_{PKC19-27}$	myr-N-FA*RK*GAL*R*Q-OH	+
m2	myr-N-*R*SAG*R*–OH	+
m3	myr-N-*R*VHL*K*D*RR*AI-NH2	–
Marip	myr-N-*KK*AL*RR*QGAVDAL-OH	–
m4	myr-N-SNYL*K*DHQAI-NH2	–
m5	myr-N-VLF*K*DHW*R*I-OH	–

Peptides are ordered from most (top) to least (bottom) effective. Cationic AA are marked in italic letters and anionic AA are underlined. The effect on eNOS was assessed by densitometric analysis of Western blotting. The activity of $mPS_{PKC\xi}$ was expressed as a '+ + + +' and the effects of other peptides on eNOS were estimated relative to mPS $_{PKC\zeta}$.

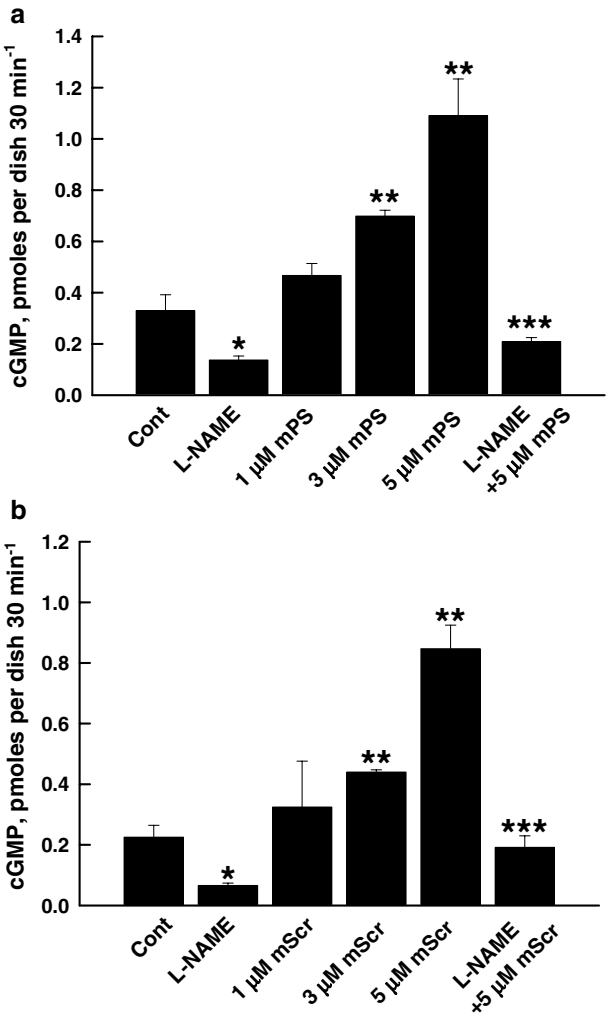

Figure 3 Effects of mPS (a) and mScr (b) on accumulation of intracellular cGMP in PAEC. PAEC grown in 35 mm dishes were incubated with different concentrations of myristoylated peptides for 30 min, and cGMP accumulation in cells was determined using a cGMP detection kit (Amersham) according to the manufacturer's instructions. In some experiments, PAEC were pretreated with L-NAME (100 μM) for 1 h. *$P<0.05$ vs control; **$P<0.01$ vs control; ***$P<0.001$ vs 5 μM mPS (a) and vs 5 μM mScr (b).

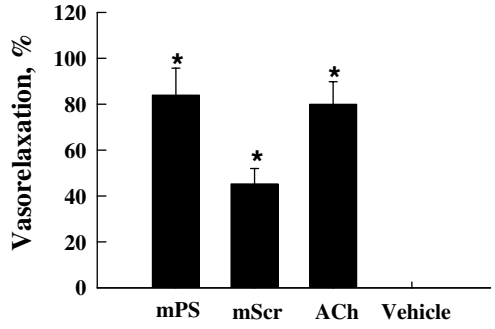

Figure 4 mPS- and mScr-induced vasorelaxation of U-46619-contracted PAS from mice. PAS were precontracted with a thromboxane mimetic U-46619 (0.5 μM) as described in Methods. After stable contraction, PAS were incubated with mPS or mScr (both 5 μM) for 30 min after which vascular tone was assessed. In parallel experiments, the vasorelaxing effects of myristoylated peptides were compared with the effects of ACh (5 μM, 30 min). Data from 4 independent experiments are presented. *$P<0.05$ vs control (vehicle).

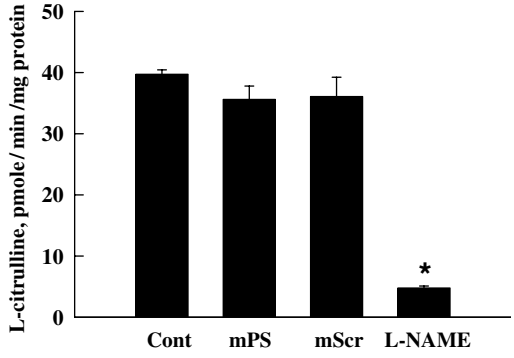

Figure 5 Assay of eNOS activity in total membrane fractions isolated from PAEC. The total membrane samples (400 μg of protein) were incubated in assay buffer with or without (control) the presence of 10 μM mPS, 10 μM mScr or 100 μM L-NAME for 30 min at 37°C. After these treatments, eNOS activity was measured as described in Methods. Data are means ± s.e.; $n=3$ for each treatment group. *$P<0.05$ vs control.

peptides making them able to activate several kinases and eNOS in endothelial cells.

We initially assessed phosphorylation of eNOS at Ser^{1117} (which is known to lead to activation of eNOS) in response to mPS treatment. We found that mPS induced profound phosphorylation of eNOS in the first 15 min of treatment, but the phosphorylation lasted only a few hours (4 h). This time course of mPS action as a PKC inhibitor in PAEC was not in agreement with the literature. Thus, Johnson *et al.* (1996) reported that after administration of pseudosubstrate into cardiac myocytes using a saponin-induced permeabilization protocol, the effects of pseudosubstrate lasted for 3 days. Moreover, if the effect of mPS on eNOS activity in PAEC was due to inhibition of PKC, other cell-permeable versions of this pseudosubstrate should have the same effect on eNOS. However, we found that MTP-PS did not induce phosphorylation of eNOS whereas a scrambled version of mPS (mScr) had the same effect on eNOS activation as mPS. In addition, mPS and mScr activated Akt, ERK 1/2, and p38 MAPK in PAEC, whereas MTP-PS did not induce changes in phosphorylation of all of these enzymes. Based on our observations that only the myristoylated peptides mPS and mScr induced changes in PAEC, we have concluded that modification of peptides with myristoylation changed their properties making them able to activate a variety of enzymes in PAEC. Myristic acid alone and nonmyristoylated versions of the pseudosubstrate (PS and MTP-PS) did not induce eNOS phosphorylation in PAEC. The termination of action in a few hours is consistent with the time necessary for de-esterification of peptides by intracellular esterases.

The ability of myristoylation to change the properties of peptides was first noticed by Harris *et al.* (1999). These authors, using insulin-secreting β-cells, demonstrated that myristoylation altered the selectivity of pseudosubstrate peptides such that all myristoylated peptides tested, even those lacking pseudosubstrate domains, acted as PKC inhibitors. We confirmed their results using different myristoylated and

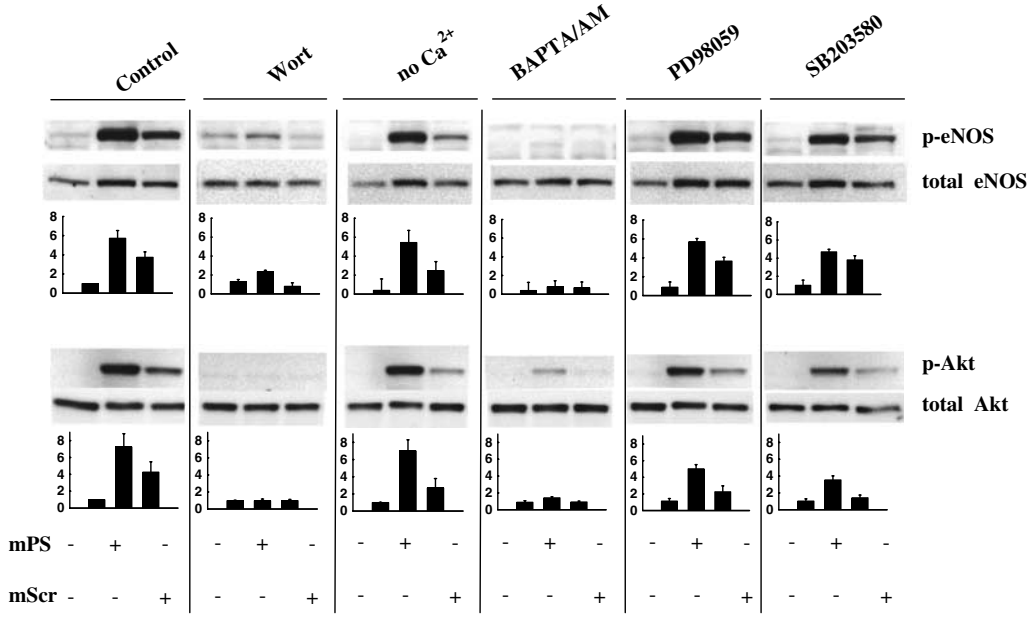

Figure 6 Effects of pathway inhibitors on phosphorylation of eNOS and Akt in response to mPS or mScr treatment. PAEC were serum-starved overnight and then treated with either 5 μM mPS or 5 μM mScr for 15 min. Different groups of cells were pretreated for 1 h with 100 nM wortmanin, 30 μM PD98059, or 1 μM SB 203580. In experiments without extracellular Ca^{2+}, PAEC were washed and treated in HBSS without Ca^{2+} and supplemented with 2 mM EGTA. To remove intracellular Ca^{2+} cells were pretreated with a cell-permeable chelator of Ca^{2+} – BAPTA/AM (10 μM) for 10 min in Ca^{2+}-free HBSS. The immunoblots shown are representative of 3 independent experiments. The quantification of phosphorylation levels was performed as specified in Figure 1.

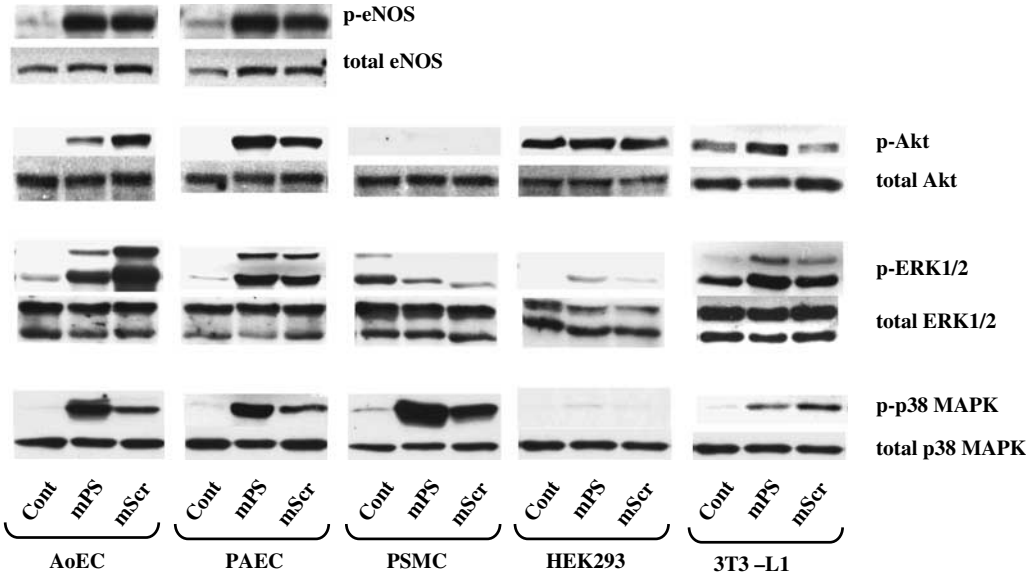

Figure 7 Phosphorylation of eNOS, Akt, ERK1/2, and p38 MAPK in response to mPS or mScr treatments in AoEC, PAEC, PSMC, HEK 293, and 3T3-L1. Cells were cultured under the same conditions and were incubated with 5 μM mPS or 5 μM Scr for 15 min. After the incubation, cells were washed and scraped in RIPA buffer. Cell lysates were resolved by SDS–PAGE, and immunoblots were probed with antibodies against Ser^{1177}- phospho-eNOS, Ser^{473}- phospho-Akt, phospho-ERK1/2, or phospho-p38. The immunoblots shown are representative of three independent experiments.

nonmyristoylated peptides using an *in vitro* PKC assay (data not shown). However, the effects of myristoylated peptides in PAEC could not be explained merely by inhibition of PKC. MTP-PS, which is also an inhibitor of PKC, did not induce any remarkable changes in phosphorylation of all tested enzymes.

To assess whether any peptide modified by myristoylation would induce eNOS phosphorylation in PAEC, we tested the ability of a variety of myristoylated peptides to phosphorylate eNOS. The results showed that not all peptides induced phosphorylation of eNOS. We cannot yet draw the exact rule for predicting peptide activity, but for the effects described in

this study the length of peptide and the presence of positively charged amino acids (AA) are more important than the AA sequence. We found that all active peptides contained several positively charged AA and were at least six AA in lengths. The combination of basic amino acids with myristoylation is well known in the literature as a 'myristate plus basic' motif and serves to promote membrane binding of many naturally-occurring proteins (Resh, 1999). Myristate inserts hydrophobically into the lipid bilayer whereas basic amino acids form electrostatic interactions with the headgroups of acidic membrane phospholipids including PI $(4,5)P_2$. There is evidence that in resting cells PI $(4,5)P_2$ is sequestered in cholesterol-rich domains by interaction with the hydrophobic/basic motif of myristoylated proteins such as MARCKS, NAP-22, CAP-23, GAP-43 (Laux *et al*., 2000; Epand *et al*., 2005; McLaughlin & Murray, 2005). The modulation of availability of free PI $(4,5)P_2$ by these proteins regulates a number of cell signaling cascades in cells. We hypothesize that myristoylated peptides with the effects described here also could act through interaction and modulation of plasma membranes lipids.

One of the effects of myristoylated peptides in PAEC was an activation of eNOS demonstrated in the Western blot analysis of phosphorylated eNOS and in measurements of cGMP accumulation. We found that mPS and mScr induced relaxation of PAS, which was considerable and comparable to relaxation induced by the classical vasodilator ACh. An increase in eNOS activity after treatment with myristoylated pseudosubstrate of PKCα/β was also reported in human umbilical vein endothelial cells (Spyridopoulos *et al*., 2002). The authors attributed this result to specific inhibition of PKC. However, based on our data we propose that the induction of eNOS activity they observed was due to a new property of myristoylated peptides. Taken together, the results mentioned above suggest that myristoylated peptides have a variety of biological activities not recognized here-to-fore and can be considered as a new class of activators of eNOS.

We have shown that mPS and mScr-mediated phosphorylation of eNOS appear to require an intact cell, suggesting that this effect is mediated through intracellular signaling mechanisms and not through a direct action of mPS and mScr on eNOS. We demonstrated that the PI3K/Akt pathway and a rise in $[Ca_i^{2+}]$ are involved in phosphorylation of eNOS in response to peptide treatment. ERK1/2 and p38 MAPK were also activated in PAEC after treatment with myristoylated peptides but are not involved in the regulation of eNOS phosphorylation.

The number and type of enzymes activated by mPS and mScr depend on cell type. mPS and mScr induced their most dramatic changes in endothelial cells – AoEC and PAEC. We do not know yet which factors are responsible for the distinctive response of endothelial cells to mPS and mScr treatment. We initially assumed that an elevation of $[Ca_i^{2+}]$ or activation of PI3 K – an upstream activator of Akt – might govern the cell-specific responses to mPS or mScr. However, measurements of $[Ca_i^{2+}]$ with a Ca^{2+} indicator Fura-2/AM showed that the rise of $[Ca_i^{2+}]$ after treatment with myristoylated peptides was common to all cells tested: PAEC, HEK 293, PSMC (data not shown). Thus, an elevation of $[Ca_i^{2+}]$ is necessary for the effects of myristoylated peptides on eNOS activity (removal of $[Ca_i^{2+}]$ abolished mPS- and mScr-induced phosphorylation of eNOS and Akt (Figure 6)) but is not sufficient,that is, the increase in $[Ca_i^{2+}]$ in PSMC and HEK 293 cells after mPS or mScr treatment did not lead to activation of Akt (Figure 7). There is a possibility, which requires experimental confirmation, that the elevation of $[Ca_i^{2+}]$ in different cell types in response to myristoylated peptides involves different cellular pools, and this might be at least partially responsible for the cell-specific signal transduction.

We do not fully understand the physiological significance of myristoylated peptides in cell functioning. Myristoylation of proteins is a common process in cells (Resh, 1999; Farazi *et al*., 2001). It is possible that myristoylated peptides are also formed in cells during protein degradation or maturation and might have physiological significance for self-defense of cells against viral infection. Recently, an antiviral agent from the insect Heliothis virescens was identified (Ourth, 2004). It was shown that this antiviral factor is an *N*-myristoylated peptide containing six amino acids with *in vitro* antiviral activity against HIV-1 and Herpes simplex virus-1 and is likely a metabolite of melanin biosynthesis. The author suggested that myristoylation plus 'basic motif ' of amino acid composition of this antiviral factor facilitated its binding to the inner side of the plasma membrane where it may inactivate HIV-1 assembly and/or budding from infected cells.

From our study, we have drawn two important conclusions: (1) myristoylated pseudosubstrate of PKC in addition to inhibition of PKC possesses the ability to activate a variety of intracellular enzymes in cells including a number involved in eNOS activation and NO production. In light of this finding, studies using mPS as an inhibitor of PKC should be interpreted with caution, especially in endothelial cells. (2) Increase in eNOS activity seems beneficial for many diseases such as ischemic stroke, hypertension, and atherosclerosis (Arnal *et al*., 1999; Vallance & Hingorani, 1999; Huang, 2003; Barbato & Tzeng, 2004; Endres *et al*., 2004). Myristoylated peptides are able to activate eNOS both *in vivo* and *in vitro*, and hence have the potential to be used as pharmacological compounds for acute activation of eNOS.

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