Influence of acylation of the peptide corresponding to the amino-terminal region of endothelial Nitric Oxide Synthase on the interaction with model membranes[†]

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[1]ABBREVIATIONS

DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMSO, dymethylsulfoxide; DPH, 1,6-dyphenyl-1,3,5-hexatriene; eNOS, endothelial nitric oxide synthase; NMT, N-myristoyltransferase; •NO, nitric oxide; PC, egg phosphatidylcholine, PG, egg phosphatidylglycerol; TFE, trifluoroethanol; TMA-DPH, 1-(4-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

ABSTRACT

Endothelial Nitric Oxide Synthase (eNOS) is unique among the NOS isoforms in its being dually acylated by the fatty acids myristate and palmitate. Due to its N-terminus consensus sequence, eNOS becomes cotranslationally myristoylated through an amide bond between the first Gly residue and myristic acid. Interestingly, this protein can also become transiently palmitoylated through the formation of two thioester bonds at cysteine residues 15 and 26. Protein palmitoylation of previously myristoylated protein results in the membrane association of eNOS in caveolin-enriched domains. In this work we have synthesized the first 28 amino acids of eNOS *i)* non-acylated, *ii)* singly myristoylated, *iii)* doubly palmitoylated and *iv)* dually myristoylated and palmitoylated. The effect of acylation on the conformation of the peptides has been studied by means of circular dichroism and fluorescence properties of a Trp residue which has been included at position 29. Acylation with either myristic or palmitic acid confers the peptide strech the ability to adopt extended conformations. Subsequently, we have studied the interaction of these four peptides with liposomes of defined composition by means of circular dichroism and fluorescence spectroscopy and their degree of insertion within lipid vesicles measuring the polarization of diphenyl-hexatriene (DPH) and tetramethyl ammonium diphenyl-hexatriene (TMA-DPH)-labeled liposomes. The acylated peptides were able to insert deeply into the hydrophobic core of both neutral and acidic phospholipids. Finally, our data suggest that palmitoylation of previously myristoylated sequences could be responsible of the partition into lipids rafts observed for this type of acylated proteins.

INTRODUCTION

Protein acylation by myristic and palmitic acid is a wide occurring process which is involved in membrane binding as well as in cellular signaling (*1*). Myristoylation is a cotranslational and irreversible process that adds a myristic acid through an amide bond on the N-terminal glycine residue of the protein within a specific consensus sequence. The enzyme responsible for myristoylation is the N-myristoyl transferase (NMT)[1] that catalyzes transfer of myristate from myristoyl-CoA to suitable peptide and protein substrates. To date, nearly twenty NMTs from fungal and mammalian sources have been identified (*2-4*). On the other hand, palmitoylation is a reversible and post-translational process. S-acylation occurs on cysteine residues through a thioester linkage in a wide variety of sequence contexts (*5*). This attachment is more labile than the amide linkage and facilitates the interaction of the acylated protein with membranes (*6*). Recently, it has been discovered the molecular identity of protein acyltransferases that modify palmitoylated proteins (*5*). Two yeast palmitoyl-acyl transferases for intracellular proteins have been identified, Erf2/Erf4 and Akr1 that palmitoylate Ras2 and yeast casein kinase 2 respectively (*7, 8*). Erf2 and Akr1 share a common domain, the Asp-His-His-Cys (DHHC) within a cysteine riche domain. The finding that these unrelated proteins share a common protein domain that is essential for protein acyltransferases activity has lead to the identification of a functional ortholog of the yeast Ras palmitoylransferase, the complex DHHC9/GCP16 (*9*). Nevertheless, non-enzymatic palmitoylation in vitro has also been demonstrated as the palmitoylation of a myristoylated peptide with the N-terminal sequence of the $p62^{Yes}$

protein tyrosine kinase (*10*), the myristoylated Giα protein (*11*), the 25-kDa synaptosomal protein (SNAP-25) (*12*) and small peptides of the β_2-adrenergic receptor (*13*). By using artificial sequences we have obtained data which support the non-enzymatic protein palmitoylation within mammalian cells (*14*).

The dually acylated eNOS enzyme produces the endothelium derived nitric oxide (•NO) which is an important signaling molecule involved in many biological processes such as the regulation of blood pressure, platelet aggregation and the maintenance of the vascular tone both in veins and arteries. Diminished •NO production has been implicated in the pathogenesis of a variety of vascular disorders including atherosclerosis and pulmonary hypertension (*15-17*). The synthesis of endothelium derived •NO is a calcium and calmodulin-dependent process. This enzyme is dually acylated by myristic acid on the N-terminal glycine and by palmitic acid on two cysteine residues: Cys15 and Cys26 (*18-20*). Myristoylation of eNOS is required for the subcellular location (*20, 21*), the efficient production of •NO (*22*) and subsequent palmitoylation (*19*), but it does not seem that myristoylation of eNOS alone could be responsible for the association of eNOS with membranes (*23*). Both myristoylation and palmitoylation are required to target eNOS to specialized membrane microdomains named caveolae where it is associated with caveolin at low cellular calcium concentrations (*23, 24*). The association of eNOS to caveolin leads to an inhibition of eNOS activity (*23*). The cellular stimulation with agonists that increase the intracellular concentration of Ca^{2+} promotes the binding of calmodulin to eNOS and relieves the inhibition of eNOS enzyme by caveolin (*18*).

In this work we have synthesized four peptides corresponding to the first 28 amino acids of eNOS with different degree of acylation: non-acylated, singly myristoylated on amino-terminal Gly, doubly palmitoylated on the two cysteine residues and dually myristoylated and palmitoylated peptides. At the carboxyl-end, we have added a tryptophan residue in order to monitor the fluorescence properties of each peptide. Subsequently, we have studied the structural properties of these four peptides and their interaction with liposomes of defined composition.

EXPERIMENTAL PROCEDURES

Materials. Egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol and sphingomyelin were provided by Avanti Polar Lipids. 1,6-dyphenyl-1,3,5-hexatriene (DPH) and 1-(4-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes. All other reagents were from Merck.

Synthesis of peptides. Synthesis was carried out on an automated multiple peptide synthesizer (AMS 422, Abimed) using a solid-phase procedure and a standard Fmoc chemistry in a base of 25 μmol. It was used a N-α-Fmoc-DMP resin [4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Novabiochem) with Fmoc-protected amino acids activated in situ with PyBop (benzotriazole-1-yl-oxi-tris-pyrrolidinophosphonium hexafluorophosphate) in the presence of N-methylmorpholine and 20% piperidine/dimethylformamide for deprotection. The protecting side-chain groups

were as follows: Asn and Gln (Trt), Glu (OtBu), Ser (tBu), Lys and Trp (Boc) and Cys (Acm). Peptides were cleaved from the resin with 82.5 % triflluoroacetic containing 5% phenol, 5 % water, 5 % thioanisole, 2.5 % ethane dithiol as scavengers (*25*), precipitated and washed with cold mehyl tert-buyl ether, water-extracted, lyophilized and purified by reverse-phase HPLC using a SuperPac® Pep-S C2/C18 column (Pharmacia). Two different systems were used to purify the peptides. The non-acylated and myristoylated peptides were dissolved in 5% Acetonitrile in 0.1% trifluoroacetic acid (TFA) and a gradient of acetonitrile/0.1% TFA from 5 to 60% in 35 min and from 60 to 100% in 5 min was employed to elute them at a flow rate of 1 ml/min. In the case of palmitoylated and dually acylated peptides, ethanol (60%, v/v) in 0.1% TFA served as initial mobile phase, and peptides were eluted with a 5-100% linear gradient of propan-2-ol/0.1%TFA, in 35 min at 0.7 ml/min. The absorbance at 214 nm was monitored continuously and relevant peaks were collected manually, pooled and lyophilized. The purified peptides were characterized by amino acid analysis, performed on a Beckman 6300 amino acid analyzer, PAGE electrophoresis, HPLC and fast atom bombardment mass spectrometry using a Bruker mod. Reflex III mass spectrometer. Due to the low solubility of peptides in aqueous buffers, a 5-10 mg/ml stock solution of the peptide in trifluoroethanol (TFE) or dimethylsulfoxide (DMSO) was used in the various assays described in this work and kept a -20 ^{o}C between uses. The final organic solvent concentration was always kept under 2% (v/v).

Acylation of peptides. Myristoylation and palmitoylation of peptides were performed on the synthesis resin. The myristic acid was activated with diciclohexylcarbodimide that renders the asymmetric anhydride and added in excess to the peptide attached to the resin.

This causes the formation of the amide bond. Once the peptide was myristoylated, it was cleaved from the resin. The palmitoylation was carried out according to the protocol of Beeckman et al. (*26*). After the synthesis, the protected peptide-resin was washed with dimethylformamide (DMF) and the side chains of cysteine residues were liberated with 0.1 M mercury acetate in DMF. After completion of the reaction the resin was washed copiously with DMF. Then, the sulfhidryl groups were reduced with β-mercaptoethanol/DMF (9:1) for three hours. Free SH groups were palmitoylated on the resin by coupling using 8 eq. of PyBop, 8 eq. of palmitic acid, 16 eq. of diisopropylethylamide (DIEA) in DMF, for 16 hours at 37 ^{o}C. Then, the peptide was cleaved from the resin.

Vesicle preparation. In all cases a lipid film was obtained by drying a chloroform solution of the lipid under a current of nitrogen; this film was kept under vacuum for 5 hours. The phospholipid was resuspended at a concentration of 1 mg/ml in medium buffer (5 mM Tris, pH 7.0, 100 mM NaCl, 5 mM MES, 5 mM sodium citrate, 1 mM EDTA) or 20 mM sodium phosphate buffer, pH 7.0 for 1 hour at 37 ^{o}C and eventually vortexed. This suspension was sonicated in a bath sonicator (Branson 1200), and was subsequently subjected to at least 15 cycles of extrusion in a Liposofast^{tm}-Basic apparatus (Avestin) with 100 nm polycarbonate filters (Avestin). A 0.14 mM final phospholipid concentration was used in all the experiments.

Circular dichroism measurements. Circular dichroism spectra were obtained on a Jasco J-715 spectropolarimeter using a 0.1 cm pathlength cuvette at 25 °C. The peptide

concentration employed was 80 μM. The buffer used was 20 mM sodium phosphate, pH 7.0 or TFE. A minimum of four spectra was accumulated for each sample and the contribution of the buffer was always subtracted. The resultant spectra were smoothed using J-715 Noise Reduction software. The values of mean residue molar ellipticity $[\Theta]_{MRW}$ were calculated on the basis of 100 as the average molecular mass per residue and they are reported in terms of degree x cm^2 x $dmol^{-1}$. The spectra in the presence of lipid vesicles were recorded after incubating for 1 h at 37 ^oC. To minimize the effect of light scattering on the shape and magnitude of the spectrum, lipid-peptide mixtures were sonicated before the spectra were recorded.

Fluorescence measurements. Fluorescence studies were carried out on a SLM Aminco 8000C spectrofluorimeter, fitted with a 450 W xenon arc and equipped with Glan Thompson polarizers. A 0.2x1 cm quartz cuvette was used. The buffer used was medium buffer, pH 7.0. The peptide concentration was 3 μM and the lipid concentration was 0.14 mM. The temperature in the cuvette was maintained by a Polystat Hubber circulating water bath. Excitation was performed at 275 nm, and the emission spectra were recorded over the range 285-450 nm. The contribution of the buffer was always subtracted.

Fluorescence polarization. Two different probes were employed in the depolarization experiments: 1,6-dyphenyl-1,3,5-hexatriene (DPH) and 1-(4-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). The former was dissolved in tetrahydrofuran and added to the lipid chloroform solution at a 1:500 molar ratio, and the latter was dissolved in methanol and added to the lipid chloroform solution at a 1:100 molar ratio. The lipid was

hydrated in medium buffer at a concentration of 1 mg/ml. The sample was divided into 0.5 ml aliquots, each of them at 0.14 mM lipid concentration. DMSO was added to one of these aliquots (control) and peptide from a concentrated DMSO stock solution was added to the others (0.4, 1.4 and 4 μM final concentration). The samples were measured in 0.2x1 cm fluorescence cuvettes. The temperature in the cuvette was maintained by a Polystat Hubber circulating water bath. Excitation was performed at 365 nm and the emission wavelength was 425 nm.

RESULTS

Solid-phase synthesis and acylation of peptides. Following the methods described under Experimental Procedures, four peptides of 29 amino acids corresponding to the amino-terminal region of eNOS have been obtained: non-acylated peptide (eNOS), myristoylated peptide (myr-eNOS), doubly palmitoylated peptide (eNOS-pal) and dually myristoylated and palmitoylated peptide (myr-eNOS-pal) (Table 1). All peptides were purified by reverse-phase HPLC. eNOS and myr-eNOS were dissolved in 0.1% TFA/5% acetonitrile and separated with a gradient of acetonitrile. The increased hidrophobicity of eNOS-pal and myr-eNOS-pal made necessary to dissolve them in 0.1% TFA/60% ethanol and use a gradient of 5-100 % propan-2-ol to separate them. Figure 1 shows representative HPLC profiles of purified peptides. All the peptides eluted as single peaks. Incorporation of the myristic acid to the eNOS sequence considerably increased the elution time (from 21.7 to 25.2 min). The presence of two palmitic acids rendered a peptide which eluted at almost

85% propan-2-ol while the additional myristic acid present in myr-eNOS-pal made the peptide to elute at 100% propan-2-ol. The identity of the peptides was confirmed by amino acid analysis and mass spectrometry. Table 2 shows the molecular mass obtained by mass spectrometry. The experimental data are virtually identical to the values calculated from the amino acid sequence.

CD spectra of peptides. The conformation of the peptides was determined by circular dichroism. Figure 2 shows the far-UV circular dichroism spectra of non-acylated and acylated peptides in aqueous buffer and in the presence of an organic solvent, TFE. In aqueous solutions, eNOS showed a CD spectrum characteristic of a non-ordered structure with a minimum centered near 200 nm and a shoulder at 220 nm. However, the acylated peptides showed a shift of the minimum to higher wavelength, showing spectra with a minimum at 220 nm. In spite of the differences in the magnitude of the spectra of acylated peptides the spectral features are different from the non-acylated and are those of extended structures. The increased population of ordered conformations upon introduction of myristoyl and palmitoyl groups should be the result of the hydrophobic environment provided by the acyl chains. TFE has been used to identify the propensity of a polypeptide sequence to adopt ordered structures. When all four peptides are dissolved in 100 % TFE their spectra showed a maximum at 190 nm and two minima at 205 and 220 nm, with molar ellipticity values ranging from -13700 for myr-eNOS-pal to -15500 deg cm^2 $dmol^{-1}$ for eNOS (Figure 2). These are characteristics of peptides adopting a α-helical conformation.

Interaction of the peptides with phospholipid vesicles. To explore the ability of the peptides to interact with model membranes of different composition two different approaches have been used: fluorescence emission spectra of peptides in presence of lipid vesicles and fluorescence depolarization of two probes embedded in the bilayer to monitor changes in the transition properties of the phospholipids.

The peptides have been designed to contain a tryptophan residue at position 29 which would serve as a fluorescent probe to monitor the interaction with lipid vesicles. One end of the molecule was chosen because of the minimal induced conformational perturbation and the C-terminal one because the N-terminal Gly had to be myristoylated. The fluorescence emission spectra of eNOS and acylated peptide alone or in presence of PC and PG vesicles are depicted in Figure 3. The position of the maximum of the emission spectra of eNOS peptide (354 nm) in medium buffer was indicative of a highly exposed environment of the tryptophan residue (*27*). Acylation with myristic acid did not modify the position of the maximum but the palmitate induced a significant decrease (347.5-349 nm). Moreover, the acyl chains caused a decrease in the quantum yield of tryptophan in such a way that independently of the fatty acid content the fluorescence intensity was reduced to 50% of the original value (Figure 3B-D, solid lines).

When PC vesicles were added to eNOS peptide a slight decrease of the fluorescence intensity was observed without modifying the position of the maximum while the presence of PG vesicles also reduced slightly the fluorescence intensity but shifted the position of the maximum of the spectrum of eNOS to a lower wavelength (349 nm) (Figure 3A). The fluorescence properties of acylated peptides were modified upon interaction with neutral

and acidic phospholipids vesicles. The Trp residue occupied a more hydrophobic environment as a consequence of the interaction as evidenced by the blue shift of the fluorescence maximum (from 355-349 to 348-341 nm). On the other hand, the fluorescence intensity increased upon interaction being the increase much higher in the presence of PG than of PC. In fact, the fluorescence intensity at the maximum of the spectrum of myr-eNOS-pal in the presence of PG is almost double of that of the parent peptide, eNOS. Thus, the fluorescence quenching which appears as a consequence of acylation completely disappears upon interaction with negatively charged phospholipids. This would be indicative of a conformational change which moves further away the Trp from the side chains responsible of the quenching.

The effect of eNOS and acylated peptides on the thermotropic behavior of synthetic phospholipids was assessed by fluorescence depolarization of DPH and its polar derivative TMA-DPH incorporated into the bilayer. The peptides were allowed to interact with DMPC and DMPG vesicles for 1 hour at 37 ^{o}C. The two fluorescent probes were employed in the assays so that the fluidity of both the inner and the outer part of the bilayer could be monitored. DPH is assumed to be aligned with the phospholipid acyl chains giving information of the hydrophobic core of the bilayer (*28, 29*), whereas TMA-DPH has a shallower location due to the anchoring of its non-fluorescent polar moiety to the lipid/water interface and interacts with both the phospholipid polar head groups and the fatty acyl chain region, probably as far down as C8-C10 (*30, 31*).

The addition of acylated peptides to PG and PC vesicles modified the phase transition curves mainly decreasing the fluidity of the bilayers above the transition

temperature. Thus, the amplitude of the phase transition was considerably reduced without significantly affecting the gel-to-liquid crystalline transition temperature. In order to compare the effect of the peptides on the thermotropic behavior of DMPC and DMPG phospholipids, the changes in the amplitude of the thermal transition (ΔP) at increasing peptide concentrations for DPH- and TMA-DPH-labeled phospholipids were plotted (Figure 4). It can be observed that eNOS peptide did not produce any effect on the transition of neutral phospholipids independently of the probe used or the peptide concentration employed (Figure 4A,C) and only a small decrease of the ΔP value was observed when a 4 μM concentration was added to DPH-labeled DMPG vesicles, affecting the phospholipids acyl chain in its fluid state. However, the acylated peptides decreased the amplitude of the thermal transition in a concentration dependent manner. At 4 μM peptide the transition of DPH-labeled DMPC vesicles is almost completely abolished when myristoylated, palmitoylated and dually acylated peptides were present (Figure 4A). When acidic vesicles were used, only myr-eNOS-pal cancelled the transition at the maximum concentration employed although the other two peptides also affect the microviscosity above the transition temperature, being higher the effect of eNOS-pal than that of myr-eNOS. When TMA-DPH was used to monitor less deep domains similar results were obtained except that only the dually acylated peptide was able to cancel the transition and that the myr-eNOS induced more significant changes than eNOS-pal in TMA-DPH-labeled DMPC vesicles (Figure 4C). Control experiments carried out in the presence of equivalent concentrations of the free fatty acid (myristic or palmitic acid) indicated that all the effects should be due to the amino acid moiety of the acylated peptide since the acyl chain did not

modify either the order parameter nor the transition temperature (data not shown). Then, the presence of any fatty acids allows the peptide to interact with hydrophobic domains of the bilayer.

The effect of the peptides on the thermal transition of more complex vesicles was also studied. Figure 5 shows the fluorescence depolarization profiles of DPH-labeled DMPC vesicles containing 10 % of both cholesterol and sphingomyelin, two lipids present in the lipid composition of caveolae, alone and in the presence of 0.14 μM and 1.4 μM peptide concentrations. The thermal transition of the vesicles in the absence of peptide is less steep than that of pure DMPC because of the presence of cholesterol. The addition of cholesterol to a pure phospholipid bilayer abolishes the normal sharp thermal transition between gel and liquid crystalline (l_c) phases, giving the membrane properties intermediate between two phases (*32*). Below the T_m, cholesterol weakens the packing of the headgroup increasing the fluidity of the ordered gel phase while above the T_m the reduction in freedom of acyl chains causes the membrane to condense with a concomitant decrease in fluidity (*33*). At 0.4 μM peptide concentration only myr-eNOS-pal diminished the transition amplitude affecting the acyl chain mobility both below and above the transition temperature. While myr-eNOS and eNOS-pal reduce the transition amplitude by 30% affecting only the acyl chains in the fluid state the dually acylated peptide almost completely cancelled the transition at 1.4 μM.

CD of mixtures peptide-phospholipid. The CD spectra of the peptides in the presence of lipid vesicles of both acidic and neutral phospholipids are shown in Figure 6. The spectra

shown are those obtained at a protein:lipid ratio of 1:10 although it was observed a gradual change of the spectrum from the one in the absence of added phospholipids vesicles to the one at that protein:lipid ratio. Increasing the lipid concentration did not modify the spectrum any further. When challenged by either DMPG or DMPC vesicles the peptides underwent conformational changes as indicated by the CD spectra. The largest change corresponded to eNOS peptide. From an existing non ordered conformation the peptide adopted an extended conformation, featured by a minimum at 213 nm, in the presence of DMPG. However, the presence of DMPC did not modify the shape of eNOS peptide spectrum and only a decrease of the molar ellipticity values was observed (from -5070 at 199 nm to -12200 deg cm^2 $dmol^{-1}$ at 201 nm). This fact could be due to the presence of less aggregated structures on the surface of the bilayer (*34*).

The extended conformation induced by acylation, characterized by a spectrum with a minimum at 216-218 nm is modified somehow as a consequence of the interaction with acidic phospholipids since a small shift of the position of the minimum and a decrease in the ellipticity value were observed. The spectra of acylated peptides in presence of DMPG vesicles were similar with a minimum at 214-219 nm and ellipticity values ranging -10700 for myr-eNOS, -11200 for eNOS-pal and -12800 deg cm^2 $dmol^{-1}$ for myr-eNOS-pal (Figure 6). The interaction of acylated peptides with neutral phospholipids modified the CD spectrum to a smaller extent. In the presence of DMPC vesicles, the CD spectra of myristoylated, palmitoylated or dually acylated peptides exhibited a minimum centered at 218-222 nm and molar ellipticity values ranging from -5800 to -6900 deg cm^2 $dmol^{-1}$ (Figure 6).

DISCUSSION

Covalent attachment of fatty acids to proteins is a common form of protein modification which has been shown to influence both structure and interaction with membranes. To shed some light on the membrane-perturbing properties of acylated proteins we have synthesized a 28 amino acids peptide corresponding to the N-terminal region of endothelial nitric oxide synthase, eNOS. Two targeting signals seem to be contained within the eNOS sequence. To begin with, the N-terminus end of the protein, which is irreversibly myristoylated, can become reversibly palmitoylated at positions 15 and 26, in a process known to be involved in caveolar translocation. On the other hand, mutagenesis data seem to indicate that residues 350-358 of eNOS directly interact with caveolin-1 within endothelial cells hence modulating its enzymatic activity (*35*). *In vivo*, the subcellular localization of the 1203 amino acid eNOS is dictated by its N-terminus end. In fact, constructs of the first 35 (*20*) or 55 amino acids (*14*) of eNOS are sufficient to determinate a subcellular distribution similar to the wild-type protein. We might, therefore, infer that all the information necessary for the subcellular targeting of eNOS might be contained within these first 30 residues. With that in mind, we have analysed the structural and functional properties of the isolated N-terminus end of eNOS in order to inspect if its translocation to sphingomyelin-cholesterol enriched caveolar subdomains can be attributed to the acylation state. Consequently, in our system, the effect of the caveolin-1-eNOS direct interaction does not contribute to the peptide-lipid interactions that we observed, allowing us to study each effect in isolation.

From the original sequence we have obtained three additional peptides with different degree of acylation: myristoylated, doubly palmitoylated and dually myristoylated and palmitoylated. The four peptides are highly pure as demonstrated by HPLC, amino acid analysis and mass spectrometry.

From a structural point of view it has been previously shown both that palmitoyl groups increase (*36*) and decrease (*37*) the helical content of pulmonary surfactant protein-C. CD spectra show that the peptide corresponding to the N-terminal sequence of eNOS owns a great conformational flexibility being able to adopt different secondary structures when challenged by different types of environment. Thus, eNOS peptide has a non-ordered conformation in phosphate buffer. When the peptide is dissolved in TFE, a solvent which can induce stable conformations in peptides which are unstructured in solution (*38*), it adopts mainly a helical conformation. However, acylation itself, either myristic and/or palmitic, confers the peptide the ability to adopt extended conformations, indicated by the fact that the CD spectrum of all acylated peptides has a minimum at 220 nm characteristic of β-sheet. On the other hand, the fact that eNOS and acylated peptides show different fluorescence emission maxima could suggest that acylation causes peptide aggregation or micelle formation (*39*) although the conformational changes observed by CD spectra could also be responsible for the more hydrophobic environment of the Trp residue. Although N-terminally truncated eNOS have been crystallized and their atomic structure obtained, none of them contain the sequence stretch described in this work. We have demonstrated herein that the myristoylated N-terminal amino-acid peptide of eNOS adopts a β-strand conformation in solution. This observation strongly suggests, although it does not confirm,

that this extended conformation might adopt a β-strand that could associate with the N-terminal β-sheet lariat observed in the truncated three-dimensional structure obtained from crystallographic data (*40*).

With respect to the role of acylation on the interaction with membranes, there are controversial examples in the literature. Thus, it has been shown that the N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with phospholipids bilayers in the absence of acylation (*41*). However, palmitoylation of peptides derived from the cysteine-rich domain of SNAP-23 is essential for membrane association (*42*). In several other cases a lipid moiety represents an additional binding energy necessary to achieve stable interactions (*for a review see 43*).

In the cellular cytoplasm, only two of the four peptide species that we describe in this work are present, that is myr-eNOS and myr-eNOS-palm. According to the circular dichroism data, both of them are able to display very similar secondary structures both in solution and in association with vesicles composed of DMPC or DMPG. However, both of them display distinct degree of perturbation of the lipidic moiety according to our fluorescence spectra and fluorescence depolarization measurements. Fluorescence polarization studies show that the polypeptide sequence corresponding to the N-terminal region of eNOS is able to interact with model membranes composed of acidic phospholipids although the reduction of the transition amplitude is much smaller than that induced by any of the acylated peptides and only is observed at the highest concentration assayed. Probably the effect is promoted by the net charge that this amino acid sequence possesses, +2. As a consequence of this ionic interaction the peptide adopts a β-sheet like

structure. However, the behavior observed for the acylated peptides is that of an integral membrane protein which strongly restricts the mobility of the phospholipids acyl chains, suggesting the insertion of the peptide into the membrane. At a peptide concentration of 4 μM, myr-eNOS-pal completely abrogated the thermal transition which indicates that the peptide interacts with the hydrophobic portion of the bilayer, preventing part of the phospholipids molecules from undergoing the transition characteristics of pure phospholipids species (*44*). Since equivalent amounts of the corresponding free fatty acid had no effect on the thermotropic behavior of the phospholipids it seems reasonable to conclude that the effect of acylated peptides are due to the insertion of the peptide into the hydrophobic core of bilayer probably mediated by the interactions between the fatty acid moiety of the peptide and the acyl chains of the phospholipids. The fact that acylated peptides are able to insert into bilayers of both neutral and acidic phospholipids point out that hydrophobic interactions are more important than ionic ones in eliciting the effects observed for these peptides.

The interaction of the peptides with phospholipids vesicles brings about conformational changes as indicated by fluorescence spectroscopy and circular dichroism. The largest change takes place when eNOS interacts with acidic phospholipids since from a non-ordered structure the peptide adopts spectroscopic features of extended conformations. The changes are higher upon interaction with acidic than with neutral phospholipids although in both cases the spectral features of an extended conformation are maintained. Then, it seems clear that there exists a high tendency of the N-terminus of eNOS to associate with acidic phospholipids. Remarkably, negatively charged phospholipids such as

phosphatidylserine and phosphatidylglycerol are known to inhibit eNOS activity through its binding to the calmodulin-binding amphipathic helix that interconnects both domains (*45*). Hence, the N-terminus end of eNOS might target the protein to membrane areas enriched in negatively charged phospholipids, where the reported inhibition might exist. When the intracellular calcium levels are raised, and calmodulin binding to eNOS activated the electron transfer and •NO synthesis, this inhibitory association with phospholipids might be lost (*45*).

Finally, another role of lipid modification is to promote membrane targeting. It is a general trend that a single lipophilic modification is not sufficient to target a protein to a specific membrane and two lipid moieties are necessary (*43*). Specifically, myristate and palmitate are necessary and sufficient to promote targeting to rafts (*46, 47*). In the case of eNOS it has been shown that palmitoylation only takes place in protein molecules which have been previously myristoylated (*14*). Since this protein is regulated through a palmitoylation-depalmitoylation cycle, it would be transiently associated with cholesterol-sphingolipid-enriched subdomains present in the cellular membrane (*48, 49*).

It is remarkable that in pure PC liposomes, the myr-eNOS peptide and the myr-eNOS-pal peptide display similar membrane-perturbing properties at a 1.4 μM peptide concentration (Fig. 4A). Interestingly, when we introduce cholesterol/sphingomyelin at low levels (Fig. 5A or B) the perturbation exerted by the tri-acylated peptide becomes much more profound. This observation clearly states that palmitoylation *per se* of previously myristoylated sequences introduces a strong tendency to partition into l_0 lipid domains, characteristic of lipid rafts. Moreover, since the amino acid sequence of all the peptides is

the same and only myr-eNOS-pal seems to specifically interact with rafts, the hydrophobic interactions of both myristic and palmitic acids should be responsible of the targeting specificity. However, it has been recently shown that short sequences such as Gly-Cys bearing only palmitoyl group both at the N-terminal and at the side chain of Cys can associate with raft domains (*50*). This would mean that the amino acid sequence also play a role in determining their association with a lipid bilayer. Since no proteins are incorporated into our vesicles, it can be inferred that partitioning of eNOS protein in particular and dually acylated proteins in general into rafts is due to the association of their acylated sequences with lipid rafts without requiring protein-protein interactions.

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TABLES

Table 1. Synthesized peptides corresponding to the N-terminal sequence of eNOS[a].

Peptide	Sequence
eNOS	GNLKSVGQEPGPPCGLGLGLGLGLCGKQ**W**
myr-eNOS	myr-GNLKSVGQEPGPPCGLGLGLGLGLCGKQ**W**
eNOS-pal	GNLKSVGQEPGPPC(pal)GLGLGLGLGLC(pal)GKQ**W**
myr-eNOS-pal	myr-GNLKSVGQEPGPPC(pal)GLGLGLGLGLC(pal)GKQ**W**

[a] myr, myristic acyl chain; pal, palmitic acyl chain. The tryptophan residue added is showed in bold.

Table 2. Molecular mass of synthesized peptides of the N-terminal sequence of eNOS.

Peptide	Calculated mass [a] (Da)	m/z (Da)
eNOS	2836.3	2834.2
myr-eNOS	3046.5	3046.2
eNOS-pal	3312.7	3310.6
myr-eNOS-pal	3522.9	3520.7

[a] The mass of peptides was calculated from the amino acid sequence of peptides and taking into account molecular mass of myristic acid (228.21) and palmitic acid (256.24).

LEGENDS

FIGURE 1. HPLC elution profile of reverse phase purified peptides. (A) eNOS; (B) myr-eNOS; (C) eNOS-pal; (D) myr-eNOS-pal. Peptides were purified by a 5-100% acetonitrile gradient (A, B) or a 5-100% Propan-2-ol linear gradient (C, D). The elution times are indicate.

FIGURE 2. Far-UV circular dichroism spectra of eNOS and its acylated peptides alone or in the presence of TFE. (A) eNOS; (B) myr-eNOS; (C) eNOS-pal; (D) myr-eNOS-pal. (●) 20 mM sodium phosphate, pH 7.0; (○) 100% TFE. The peptide concentration was 80 μM. These spectra are representative of those obtained for three different preparations.

FIGURE 3. Fluorescence emission spectra of eNOS and its acylated peptides in the presence of lipid vesicles. (A) eNOS; (B) myr-eNOS; (C) eNOS-pal; (D) myr-eNOS-pal. Medium buffer (solid line); PC vesicles (short-dashed line); PG vesicles (long-dashed line). Emission spectra were obtained upon excitation at 275 nm. The protein concentration was 3 μM and lipid concentration was 0.14 mM. The buffer employed was medium buffer, pH 7.0.

FIGURE 4. Changes in the amplitude of the thermal transition (ΔP) of DPH (A, B) and TMA-DPH (C, D)-labeled DMPC (A, C) and DMPG (B, D) vesicles with increasing peptide

concentration. (□) eNOS; (Δ) myr-eNOS; (◇) eNOS-pal; (○) myr-eNOS-pal. ΔP value is defined as the difference between the fluorescence polarization values measured at 10 and 45 ^{o}C. The phospholipid concentration was 0.14 mM.

FIGURE 5. Fluorescence polarization of DPH-labeled DMPC-10% Cho-10% SM vesicles. (●) vesicles alone; (○) eNOS; (▼) myr-eNOS; (▽) eNOS-pal; (■) myr-eNOS-pal. The phospholipid concentration was 0.14 mM and the peptide concentrations were 0.4 μM (A) and 1.4 μM (B).

FIGURE 6. Far-UV circular dichroism spectra of eNOS and its acylated peptides in the presence of DMPC or DMPG vesicles. (A) eNOS; (B) myr-eNOS; (C) eNOS-pal; (D) myr-eNOS-pal. (●) peptide alone; (▼) DMPG vesicles; (○) DMPC vesicles. The peptide concentrations was 80 μM. The peptide:lipid ratio was 1:10. The buffer employed was 20 mM sodium phosphate, pH 7.0. These spectra are representative of these obtained for three different preparations.

FIGURE 1

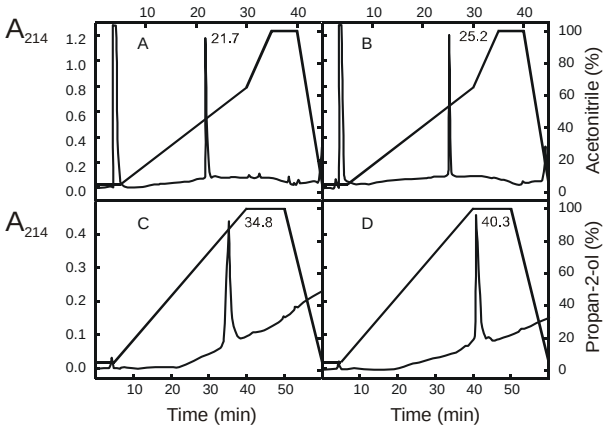


FIGURE 2

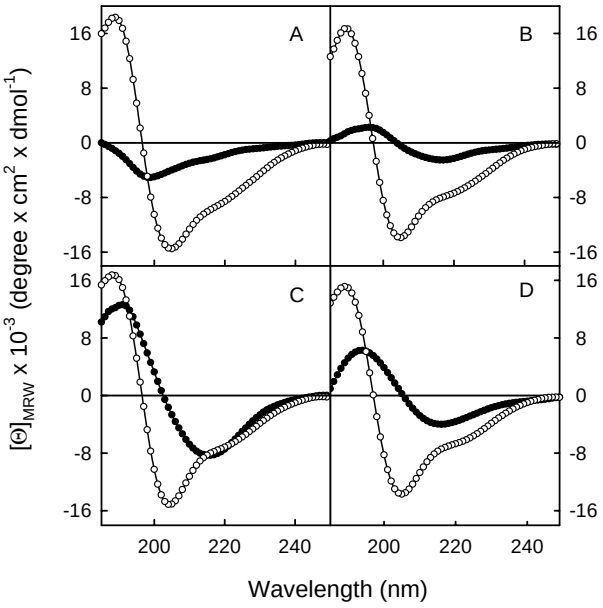


FIGURE 3

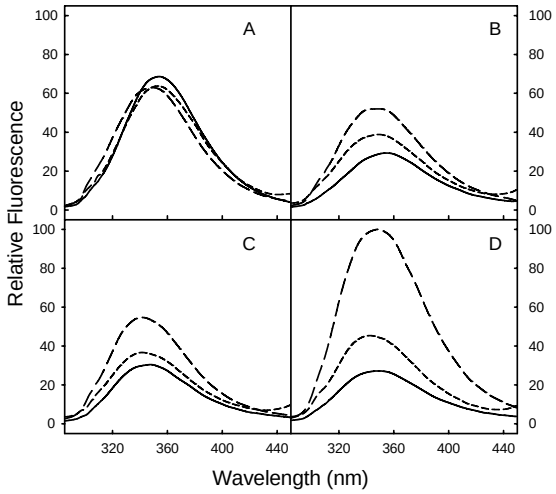


FIGURE 4

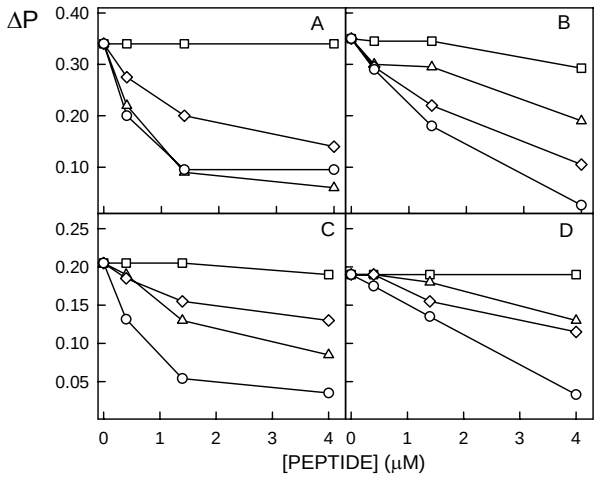


FIGURE 5

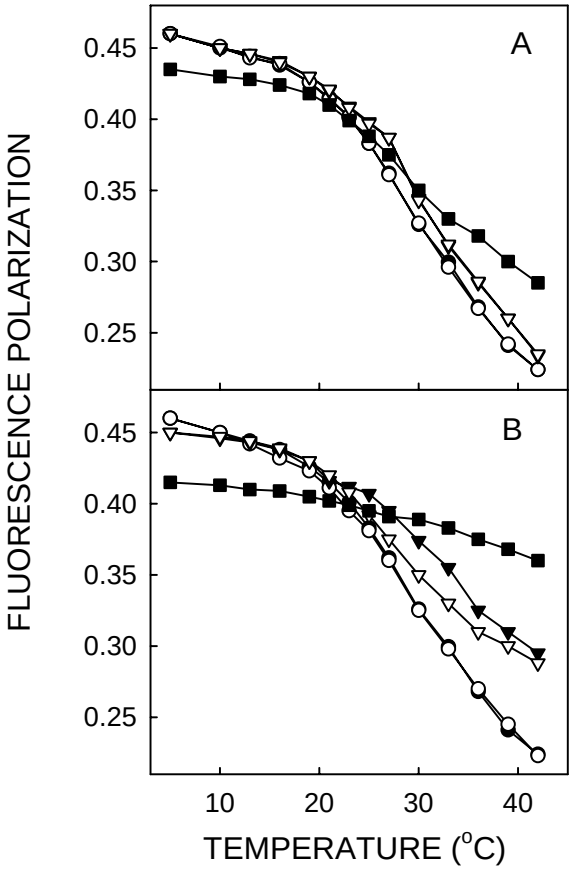
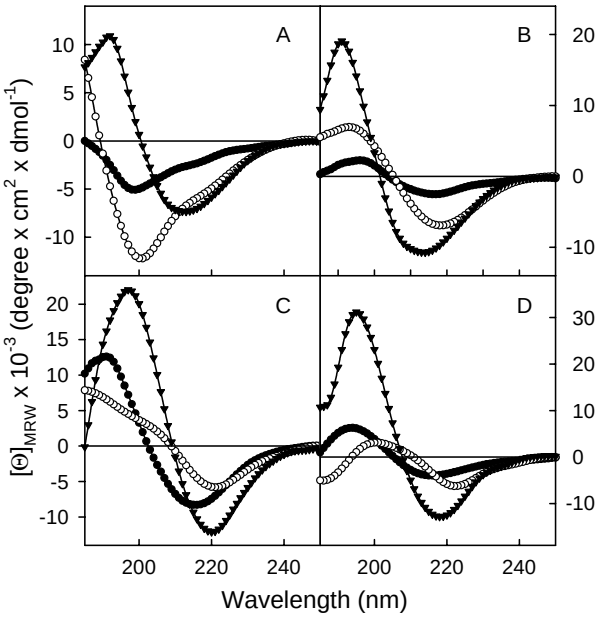


FIGURE 6



FOR TABLE OF CONTENTS ONLY

Influence of acylation of the peptide corresponding to the amino-terminal region of endothelial Nitric Oxide Synthase on the interaction with model membranes

Belén Yélamos, Fernando Roncal, Juan P. Albar, Ignacio Rodríguez-Crespo and Francisco Gavilanes