FEBS Letters 405 (1997) 356–362 FEBS 18367

The role of palmitoyl–protein thioesterase in the palmitoylation of endothelial nitric oxide synthase

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Received 27 January 1997

Abstract Palmitoylation of eNOS is required for targeting to plasmalemmal caveolae and agonist-promoted depalmitoylation leads to eNOS translocation, modifying the agonist response. To date, one palmitoyl–protein thioesterase (PPT) has been purified and cloned. To explore the role of PPT in eNOS palmitoylation, we first established that PPT mRNA and protein are expressed in endothelial cells. Coexpression of PPT and eNOS in heterologous systems (COS and Sf-9 cells) resulted in a marked reduction in [^{3}H]palmitate labeling of eNOS. We found, however, that co-expression did not alter subcellular targeting of eNOS, but that [^{3}H]palmitate incorporation into cellular lipids, in particular palmitoyl-CoA, was significantly reduced. These results suggest that while PPT expression can significantly alter cellular lipid metabolism, it has no effect on eNOS palmitoylation.

Key words: Nitric oxide synthase; Palmitoyl–protein thioesterase; Palmitoylation; Caveolae; Signal transduction; Endothelial cell

1. Introduction

The endothelial isoform of nitric oxide synthase (eNOS) is an important determinant of blood pressure homeostasis and platelet aggregation [1,2], and is targeted to plasmalemmal caveolae consequent to its dual acylation by the fatty acids myristate and palmitate [3–5]. In addition to eNOS, many other proteins modulating cellular signal transduction, including G-protein-coupled receptors, G-proteins and tyrosine kinases are similarly modified by the covalent attachment of fatty acids [6–8]. Acylation of these proteins, by N-myristoylation and/or thiopalmitoylation, appears to play an important role in targeting of signaling proteins to cell membranes, and may modulate the cellular response to agonist drugs and hormones.

N-myristoylation is an essentially irreversible protein modification catalyzed by well-characterized *N*-myristoyltransferases, and occurs co-translationally (following removal of the N-terminal methionine residue) via an amide linkage of the 14-carbon-saturated fatty acid myristate to the amino-terminal glycine (Gly_2) within a specific N-terminal consensus sequence [9]. By contrast, protein thiopalmitoylation is an easily reversible post-translational modification and involves the formation of a fatty acyl thioester between the 16-carbon-saturated fatty acid palmitate and specific cysteine residues in a variety of membrane-targeted signaling proteins [6–8]. Protein N-myristoylation alone appears to impart insufficient hydrophobicity to stabilize membrane association of the modified protein: many membrane-associated myristoylated proteins are further stablized by electrostatic interactions with membrane phospholipids, or by additional post-translational modifications, including palmitoylation. Palmitoylated proteins appear to be exclusively membrane-associated, and the lability of the palmitoyl–protein thioester bond appears to facilitate the dynamic regulation of protein palmitoylation, and, consequently membrane targeting. For example, agonist-induced depalmitoylation of eNOS likely promotes enzyme translocation from plasmalemmal caveolae, and thereby may importantly influence the agonist response [10].

How are these key regulatory pathways of protein palmitoylation and depalmitoylation controlled? No general consensus sequences for protein palmitoylation have been identified, and the enzymatic machinery involved in protein palmitoylation remains incompletely understood. No protein palmitoylthiotransferase has been purified to homogeneity, and molecular clones encoding this key activity have not yet been isolated [11,12]. Recently, however, a single protein palmitoylthioesterase (termed PPT) was purified, and its molecular clone has been isolated and characterized [13,14]. PPT was recently discovered to localize in cellular vesicles, presumably lysosomes [15], but the biological functions PPT remain somewhat enigmatic, and its potential role in the regulation of eNOS entirely unexplored. The purified PPT thioesterase can catalyze removal of palmitate from palmitoylated proteins such as H-*ras*, and in addition, PPT has been shown to possesses acyl-CoA hydrolase activity that can hydrolyze palmitoyl-CoA in vitro. Despite these advances in understanding the biochemical properties of PPT, the roles of PPT in the regulation of palmitoylated proteins within cells are not yet completely defined. An essential role for PPT in neuronal function was established by the recent discovery that PPT deficiency is the cause of the rare and fatal neurodegenerative disease, infantile neuronal ceroid lipofuchsinosis [16]. Given the rapidly emerging biological role of PPT, it seemed to us imperative that the role of this enzyme in the regulation of eNOS palmitoylation be defined. In these studies, we first establish that PPT is expressed in endothelial cells that express eNOS, and and then explore the role of PPT in eNOS palmitoylation and subcellular targeting using heterologous expression systems.

2. Materials and methods

2.1. Biological reagents

Plasmid constructs encoding wild-type eNOS, myristoylation-deficient eNOS (alanine substituted for Gly^2, termed myr^-, and palmitoylation-deficient eNOS (serines substituted for Cys^{15} and Cys^{26}, termed $palm^-$) in the expression vector pBK-CMV (Stratagene) have been described previously [3,4]. The plasmid pCMV-PPT con-

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PII S0014-5793(97)00222-6

taining the full-length 940 bp bovine PPT cDNA was a kind gift from S. Hofmann [14]. A rabbit anti-peptide polyclonal anti-eNOS antiserum we have extensively characterized was used for immunoprecipitation of eNOS from cell lysates [3]; a mouse monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY) was employed for immunoblot analyses. Purified polyclonal rabbit anti-PPT antibody was a kind gift of S. Hofmann [14]. Recombinant baculovirus constructs encoding eNOS (BV-ecNOS) and PPT (BV-PPT, gift of S. Hofmann) have been described previously [14,17]. Purified PPT isolated from baculovirus-infected insect Sf9 cells was a generous gift of S. Hofmann.

2.2. Cell culture and transfection

COS-7 cells were maintained in culture and transiently transfected with eNOS or PPT cDNAs using the DEAE-dextran method; bovine aortic endothelial cells were cultured as previously described [18,19] and used between passages 6 and 11. Insect Sf-9 cells were maintained in spinner culture and infected with recombinant baculovirus as described previously [17]. Transfected COS-7 cells or recombinant baculovirus-infected insect Sf9 cells were biosynthetically labeled with [^{3}H]palmitic acid, and eNOS was immunoprecipitated and analyzed by SDS-PAGE and fluorography exactly as described previously [3,4]. NADPH-diaphorase assays of transfected COS-7 and Sf-9 cells were performed as previously described [3,17]. Immunoblots using the eNOS antibody as probe were analyzed by chemiluminescence according to the manufacturer's instructions (Renaissance, DuPont NEN, Wilmington, MA). Immunoblot analyses of PPT in endothelial cell lysates used a 1:2500 dilution of purified rabbit anti-PPT antibody as a primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (Pierce) was used for chemiluminescencent detection of PPT.

2.3. Northern blot analysis

Total RNA was harvested from confluent BAECs using TRIZOL reagent (BRL Life Sciences) following the manufacturer's protocols. Agarose electrophoresis and northern blot analyses were performed using standard protocols, using as probe a ^{32}P-labeled PPT cDNA (obtained from a Pst-1 digest of plasmid pCMV-PPT); filters were exposed to Kodak XAR film using intensifying screens at −70°C for 24 h; molecular weight markers (RNA MW II markers) were from Boehringer Mannheim Biochemicals.

2.4. RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) of endothelial cell transcripts were performed as previously described [20]. Briefly, total RNA was isolated from confluent BAECs, and cDNA was synthesized using Superscript II reverse transcriptase (BRL Life Sciences). PCR (30 cycles) was performed using an annealing temperature of 55°C. Forward (5′-TGCTTGTGGCTCTTGGCTCTCG-3′) and reverse (5′-TGCGGTATATGTCCT-CCCTGAT-3′) primers specific for bovine PPT [14] were predicted to amplify a 574 bp DNA fragment. The plasmid encoding PPT was used as a positive control; a sample of BAEC RNA identically processed, but otherwise lacking reverse transcriptase, provided a negative control.

2.5. Thin-layer chromatography

Sf-9 cells from suspension spinner culture were transferred to P-60 plates and infected with recombinant baculovirus expressing PPT (BV-PPT). Seventy-two hours after infection, cells were to biosynthetically labeled with [^{3}H]palmitate. In parallel, uninfected Sf-9 cells were maintained and biosynthetically labeled under identical conditions. Extraction of cellular lipids and TLC were performed following previously described protocols [21]. Liquid scintillation counting confirmed that equal ^{3}H-counts were present in lipid extracts from both infected and uninfected cells. The lipid extracts were then subjected to aqueous extraction to enrich for palmitoyl-CoA, using established protocols [21]. Following reverse-phase TLC on KC18 Silica Gel 60 TLC plates (Whatman) developed in (5:3:2) butanol/water/acetic acid, plates were air-dried, sprayed with fluor (En^{3}Hance, DuPont), and exposed to Kodak XAR film using intensifying screens at −70°C for 12-48 h.

3. Results

PPT is expressed by cultured endothelial cells. Experiments

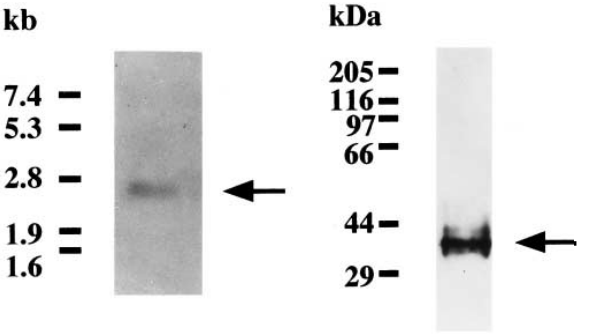

Fig. 1. PPT expression in endothelial cells. Left panel: Northern blot analysis of RNA extracted from BAEC probed with ^{32}P-labeled PPT cDNA, as described in the text. An arrow marks the location of the 2.5 kb PPT transcript. The right panel shows an immunoblot of BAEC extracts resolved by SDS-PAGE, electroblotted and probed with PPT antibody. The arrow indicates the position of PPT protein. This experiment was performed twice in duplicate, yielding identical results.

seeking to establish a role for PPT in eNOS palmitoylation explored first whether PPT is expressed in endothelial cells that express eNOS. Bovine aortic endothelial cells (BAEC) have been extensively studied in culture, and are known to express eNOS. Northern blots of total RNA obtained from confluent BAEC were probed with the full-length 940 bp bovine PPT cDNA and demonstrate a single band at approximately 2.5 kb (Fig. 1, left panel). This is similar in size to the major PPT transcript identified in bovine brain [14]. Expression of PPT mRNA in BAEC was further confirmed by RT-PCR experiments: PCR primers for bovine PPT amplified the predicted 574 bp product in cDNA prepared from BAEC, with appropriate positive and negative controls confirming PPT expression in these cells (data not shown). Immunoblot analysis of endothelial cells reveals a broad band at ~38 kDa (Fig. 1, right panel), as previously described in bovine brain tissues; this broad band appears to reflect glycosylation of PPT [14].

3.1. Co-transfection of eNOS and PPT cDNAs into COS-7 cells

We next explored the effects of PPT expression on eNOS palmitoylation. COS-7 cells, which express neither eNOS nor PPT, were utilized to establish a heterologous system to study eNOS and PPT co-expression using appropriate expression vectors. Our previous work has demonstrated that recombinant wild-type eNOS transfected into COS-7 cells undergoes acylation and subcellular targeting to plasmalemmal caveolae in a manner essentially identical to that observed in endothelial cells [3,4,10]. COS cells have also been used to study PPT processing and activity [14]. We transfected COS-7 cells with wild-type eNOS alone, or in combination with wild-type PPT, and 48–72 h later performed metabolic labeling with [^{3}H]palmitate, followed by immunoprecipitation. Control cells were also transfected with either the eNOS palmitoylation-deficient mutant (palm$^-$), or with the eNOS myristoylation-deficient mutant (myr$^-$), and processed in parallel by immunoprecipitation, SDS-PAGE and fluorography. As shown in Fig. 2 (left panel), there is ^{3}H-labeling of wild-type eNOS transfected alone, but only trace labeling of eNOS co-transfected with PPT; similar amounts of total eNOS protein in each lane were documented by immunoblot analysis, as shown

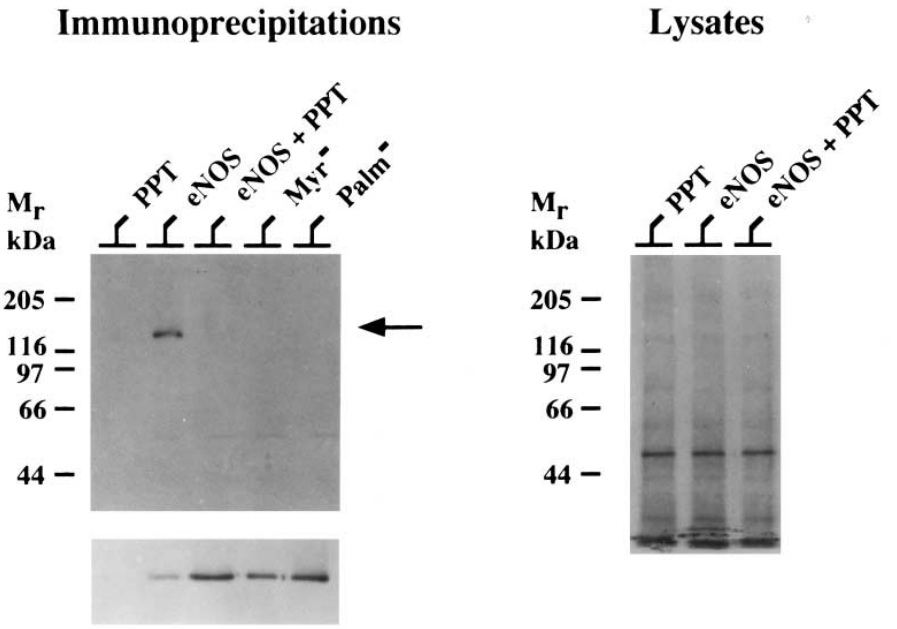

Fig. 2. Effects of PPT expression on [^{3}H]palmitate biosynthetic labeling of eNOS in transfected COS-7 cells. COS-7 cells were transfected with cDNAs encoding PPT and/or eNOS, myristoylation deficient eNOS (myr−) or palmitoylation deficient eNOS (palm−). 48–72 h following transfection, cells were labeled for 2 h with [^{3}H]palmitate and harvested. In these experiments, all ^{3}H-labeling of eNOS and of cellular lysates was abrogated by treatment with 1 M hydroxylamine (pH 7.0), indicating that the labeling resulted from thioester linkages to [^{3}H]palmitate (data not shown). Left panel: Cellular homogenates immunoprecipitated with anti-eNOS serum were analyzed by SDS-PAGE on 7% gels followed by fluorography. The film shown was exposed to X-ray film for 1 week at −70°C using intensifying screens. Shown under this panel is are immunoblot analyses of these same samples, analyzed in parallel and probed using a monoclonal eNOS antibody. The righthand panel shows the parallel analysis of total cellular lysates prepared from the transfected COS-7 cells (10 μg total protein) and subjected to SDS-PAGE and fluorography. The data shown are representative of four similar experiments.

below the fluorogram. As expected, no [^{3}H]palmitate labeling of eNOS is seen in cells transfected with the palmitoylation- or myristoylation-deficient mutants of eNOS; eNOS protein expression is entirely absent in cells transfected with PPT alone. In order to explore whether PPT co-transfection generally affected protein ^{3}H-palmitoylation, total cellular lysates from transfected cells were also examined by SDS-PAGE and fluorography (Fig. 2, right panel). There was no significant difference in overall ^{3}H-labeling of cellular proteins following transfection with PPT. This observation argues against a post-hoc action of PPT on palmitoylated proteins in the cellular lysates. To exclude the possibility that a specific PPT-mediated depalmitoylation of eNOS occurs in cellular lysates, we mixed lysates of eNOS transfected COS cells with lysates from cells transfected with either PPT or vector alone. We observed no difference in ^{3}H-labeling of eNOS following 24 h incubations (4°C) with either lysate (data not shown). In previous studies, we have seen no evidence of post-lysis artifacts following co-transfection under similar reaction conditions [22].

Interpretation of this negative result is confounded by the fact that only about 10% of the COS-7 cells in the dish successfully undergo transfection using these protocols, and a marked effect on protein labeling in the minority of cells actually expressing PPT might be obscured by the large majority of cells that remain untransfected. In order to overcome this limitation, we infected insect Sf-9 cells with recombinant baculovirus expressing eNOS and PPT cDNAs, under conditions we have previously shown to result in expression of recombinant enzyme in the majority of treated cells [17].

3.2. *Infection of Sf-9 cells with baculovirus containing eNOS and PPT cDNAs*

We have previously established that recombinant eNOS expressed in insect Sf-9 cells undergoes myristoylation and palmitylation, and is appropriately targeted to the particulate subcellular fraction in infected cells [17]. Recombinant PPT expression in insect Sf9 cells has, in similar fashion, been characterized by Camp et al. [14], who found that the recombinant enzyme is enzymatically active. In our experiments, we found the recombinant baculovirus constructs infected the majority of treated Sf9 cells: ~90% of Sf-9 cells treated with BV-PPT could be found to express the protein (by immunohistochemical analysis), and ~50% of the Sf9 cells treated with BV-eNOS expressed the enzyme (using the NADPH-diaphorase assay, as previously described [17]. Sf9 cells infected with BV-PPT or BV-eNOS, alone or in combination, were biosynthetically labeled with [^{3}H]palmitate and analyzed by immunoprecipitation and by subcellular fractionation, as discussed below. There was strong [^{3}H]palmitate labeling of eNOS immunoprecipitated from cells infected with the BV-eNOS virus alone, but, co-infection of BV-eNOS with BV-PPT led to a marked attenuation in eNOS ^{3}H-labeling, with no change in the total quantity of eNOS enzyme expressed (Fig. 3). In order to explore the effects of PPT expression on [^{3}H]palmitate labeling of total cellular proteins, we analyzed lysates prepared from biosynthetically labeled cells by SDS-PAGE and fluorography (Fig. 3, upper panel), and found that ^{3}H-labeling of several proteins, in addition to eNOS, is attenuated by PPT. This indicates that the

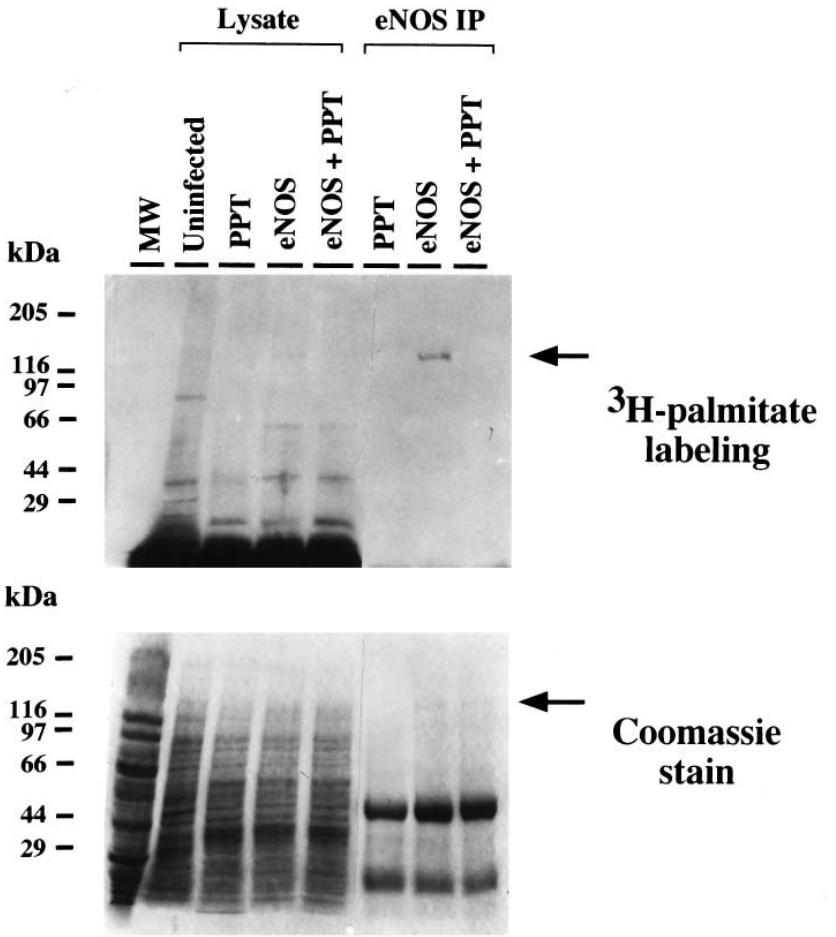

Fig. 3. Effects of PPT expression on [^{3}H]palmitate biosynthetic labeling of eNOS in insect Sf-9 cells. Three days after infection with recombinant baculovirus expressing eNOS, PPT or co-infected with both viruses, Sf-9 cells were labeled for 2 h with [^{3}H]palmitate and harvested. The upper panel shows the fluorogram of SDS-PAGE analysis both of ^{3}H-labeled total cellular lysates as well as the pattern seen following immunoprecipitation of eNOS. The lower panel shows the results of Coomassie Blue staining of cellular lysates of infected cells following SDS-PAGE, documenting equivalent total protein in cellular lysates as well as similar eNOS content in Sf-9 cells infected with eNOS or with eNOS plus PPT. An arrow indicates the position of eNOS. The experiment shown was repeated 3 times with equivalent results.

effect of PPT on [^{3}H]palmitate labeling is not specific to eNOS.

3.3. *Effect of PPT co-expression on eNOS subcellular distribution*

The attenuation of [^{3}H]palmitate labeling of cellular proteins induced by PPT might reflect a variety of different mechanisms. One possbility is that PPT is directly or indirectly promoting the depalmitoylation of diverse palmitoyl–protein substrates. An alternative explanation is that PPT expression might lead to a decrease in the specific activity of the [^{3}H]palmitate in the cellular pool of palmitic acid in transfected cells (plausibly through its previously characterized activity as an acyl-CoA hydrolase). These two hypothesis will be addressed in turn. We have previously established that the palmitoylation-deficient eNOS mutant (palm$^-$) is partially redistributed to the soluble subcellular fraction, relative the the wild-type enzyme. If the marked decrease in [^{3}H]palmitate labeling of eNOS caused by PPT co-expression truly reflects an attenuation in the overall level of eNOS palmitoylation state, a concordant change in subcellular localization of eNOS should be detected. We therefore performed subcellular fractionation of lysates prepared from COS-7 cells transfected with cDNAs encoding eNOS or PPT, individually or in combination; the myristoylation- and palmitoylation-deficient mutants of eNOS served as key controls. (COS-7 cells are used for these experiments because we have not yet developed recombinant baculovirus expressing the eNOS acylation mutants). Lysates prepared from transfected cells were resolved by ultracentrifugation into soluble and particulate fractions, and then analyzed by immunoprecipitation with eNOS antiserum followed by SDS-PAGE electrophoresis and immunoblot analysis (Fig. 4). These experiments showed no difference in subcellular localization between eNOS alone or eNOS co-transfected with PPT. In both cases the majority ($>95\%$) of eNOS is found in the particulate fraction and less than 5% in the soluble fraction (Fig. 4, upper panel). The subcellular distribution of the palmitoylation deficient mutant, on the other hand, is similar to that reported previously (55% particulate and 45% soluble), and the myristoylation-deficient mutant was found almost entirely in the soluble fraction. PPT expression was not systematically altered by co-expression with eNOS, nor was the ratio of particulate to soluble PPT changed substantively. The absence of any change in the subcellular distribution of eNOS in the presence of PPT expression suggests that the decrease in [^{3}H]palmitate labeling of

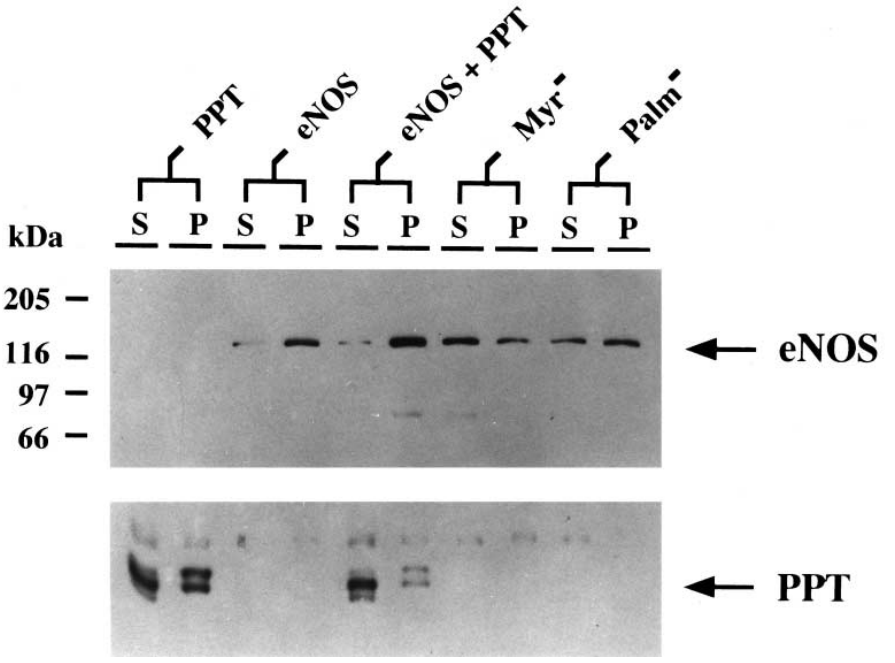

Fig. 4. Effects of PPT co-expression on eNOS subcellular distribution in transfected COS-7 cells. COS -7 cells were transfected with cDNAs encoding PPT or eNOS, alone or in combination; the myristoylation deficient eNOS (myr−); or the palmitoylation deficient eNOS (palm−). Seventy-two hours after transfection, the COS-7 cells were harvested and sonicated and subjected to cellular fractionation by ultracentrifugation followed by immunoprecipitation with anti-eNOS or anti-PPT antibodies, as described in the text. The top panel shows the immunoblot analysis of a eNOS-immunoprecipitated samples resolved on a 9% polyacrylamide gel and probed with the eNOS monoclonal antibody. The bottom panel shows the immunoblot analysis of PPT immunoprecipitated samples resolved on a 12% polyacrylamide gel and probed with PPT antiserum. The data shown are representative of three independent and identically designed experiments.

eNOS seen with PPT coexpression does *not* result from the removal of palmitate from eNOS, but more likely represents a change in the specific activity of the [^{3}H]palmitoyl-CoA substrate due to PPT expression in the biosynthetically labeled cells.

3.4. PPT expression in insect Sf9 cells attenuates biosynthetic incorporation of [^{3}H]palmitate into [^{3}H]palmitoyl-CoA

We next explored the effects of PPT on [^{3}H]palmitate biosynthetic labeling of cellular lipids. Sf-9 cells, rather than COS cells, were studied for this purpose: the majority of Sf9 cells become infected with BV-PPT and robustly express the protein, and there is thus a minimal background of uninfected cells. Expression of PPT leads to a reduced incorporation of [^{3}H]palmitate labeling of total cellular lipids (Fig. 5), as documented by TLC of organic extracts of infected and uninfected biosynthetically labeled cells. Palmitoyl-CoA was enriched by performing an aqueous extraction from the organic phase using established methodologies [21], and we found a marked reduction in ^{3}H-labeled palmitoyl CoA is seen in Sf-9 cells expressing PPT compared to wild-type cells. It therefore seems most plausible that the marked decrease in ^{3}H-biosynthetic labeling of palmitoylated proteins consequent to PPT expression reflects an attenuation in the specific activity of the cellular [^{3}H]palmitoyl-CoA pool.

4. Discussion

The present studies have shown that PPT and eNOS are both expressed in endothelial cells, suggesting a possible link between these two key proteins. Indeed, PPT co-expression with eNOS leads to a marked decrease in [^{3}H]palmitate biosynthetic labeling of eNOS. However, this decrease in [^{3}H]palmitate labeling of eNOS leads to no change in its subcellular distribution, as is characteristic of the palmitoylation-deficient mutant eNOS [4]. Instead, the dramatic decrease in eNOS [^{3}H]palmitate biosynthetic labeling induced by PPT appears to be explainable on the basis of its effects on cellular lipid metabolism. The widespread but highly variable tissue distribution of PPT might thus importantly influence the study of protein palmitoylation in different cell types.

PPT, the only mammalian acyl thioesterase identified to date, posseses the important enzymatic activity of removing palmitate from cysteine residues of mature proteins [13,14]. However, the other enzymatic activities characteristic of PPT, including its activity as an acyl-CoA hydrolase, might be anticipated profoundly to affect cellular lipid and proteolipid metabolism, with important consequences for the flux of labeled lipids (e.g. [^{3}H]palmitate) into cellular pools in biosynthetic labeling experiments. In these studies, we found that PPT expression markedly attenuates the [^{3}H]palmitate biosynthetic labeling of eNOS, and of other palmitoylated proteins. However, this decrease in short-term biosynthetic protein [^{3}H]palmitate *labeling* of eNOS does not appear to be associated with an overall decrease in steady-state enzyme *palmitoylation* (using the surrogate marker of subcellular distribution), and the effect can perhaps be best explained by the effects of PPT on cellular lipid metabolism. We propose that PPT, perhaps through its palmitoyl-CoA hydrolase activity, leads to a decrease in specific activity of the cellular [^{3}H]palmitoyl CoA pool such that after biosynthetic labeling, less [^{3}H]palmitate is incorporated into eNOS and other palmitoylated proteins. The differential effects of PPT on the ^{3}H-labeling of diverse cellular proteins might possibly reflect differences in their suit-

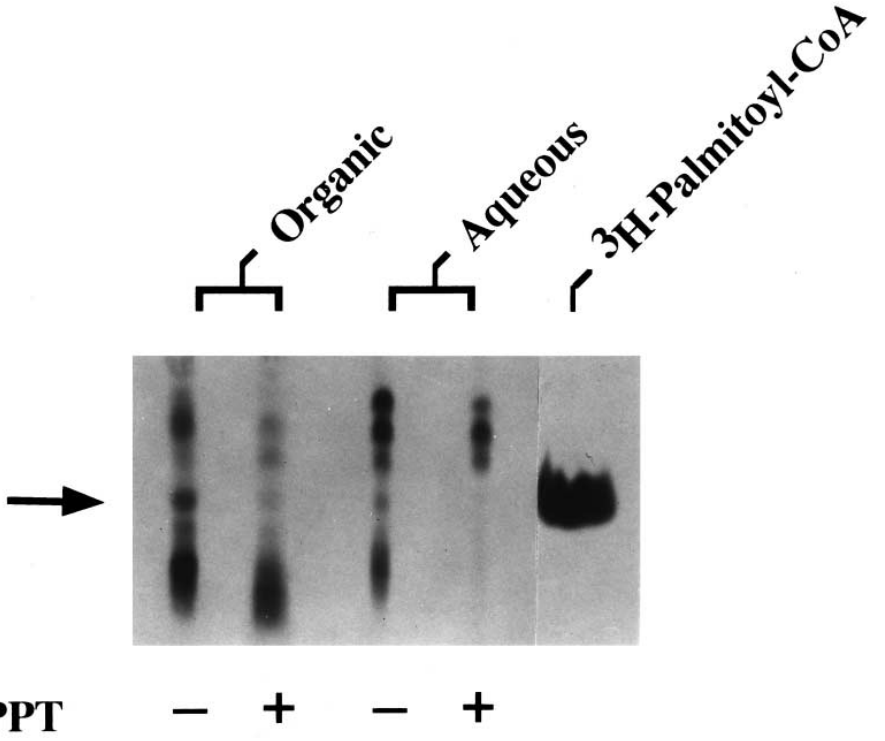

Fig. 5. Effects of PPT expression on [^{3}H]palmitate incorporation into cellular lipids. Cellular lipids extracted from Sf-9 cells, either uninfected or infected with baculovirus expressing PPT, were analyzed by reverse-phase TLC as described in the text. An arrow denotes the position of [^{3}H]palmitoyl-CoA which was run in parallel. Scintillation counting of extracts established that samples prepared from infected and uninfected cells contained equivalent ^{3}H cpm. This experiment was performed 4 times with equivalent results.

ability as substrates for PPT, but, in the context of our other findings, we feel this more likely represents differences in palmitate turnover for these diverse proteins during the period of biosynthetic labeling. Indeed, the predominant membrane assocation of eNOS in the presence of co-expressed PPT argues against an important effect of PPT on steady-state palmitoylation and myristoylation of eNOS (which acylations are the key determinants for eNOS membrane targeting; refs. [3,4]) despite the dramatic effects of PPT on the short-term biosynthetic labeling of the protein. It is problematic, however, to study longer periods of biosynthetic labeling with [^{3}H]palmitate: prolonged incubation of cells with [^{3}H]palmitate is confounded by metabolic conversion of the labeled fatty acid to [^{3}H]myristate and to ^{3}H-labeled amino acids, and interpretation of such labeling experiments are dubious [21].

Biosynthetic labeling with [^{3}H]palmitate has become a standard procedure for analyzing the palmitoylation state of a variety of proteins [21]. The present studies demonstrate that under certain experimental conditions, changes in [^{3}H]palmitate biosynthetic labeling of a given protein may not reflect actual changes in its palmitoylation state; this may be particularly problematic in the characterization of candidate protein palmitoylthioesterases, especially those possessing acyl-CoA hydrolase activity. The mechanism for this effect remains speculative, as detailed enzymological studies of palmitoylthiotransferases are not yet available. Studies with partially purified palmitoylthiotransferases indicate that palmitoyl-CoA can serve as an effective substrate, but the characteristics of purified protein palmitoylthioesterases remain incompletely defined [11,12].

Reversible palmitoylation is a prominent feature of many key signaling proteins in addition to eNOS, including G-protein alpha subunits, cellular oncogenes, and G-protein-coupled receptors. The identity of the protein palmitoylthioesterase(s) responsible for depalmitoylation of eNOS and of other signaling proteins remains obscure, and the identification and characterization of additional candidate protein palmitoylthioesterases remain areas of active investigation.

Acknowledgements: The authors wish to thank Dr. S.L. Hofmann for her generous gifts of bovine PPT cDNA; recombinant baculovirus BV-PPT; samples of purified PPT protein; and anti-PPT antibody, reagents without which none of these studies would have been possible. J.B.M. is a Bugher-AHA Fellow in Cardiovascular Molecular Biology. T.M. is a Wyeth-Ayerst Established Investigator of the American Heart Association and a Burroughs Wellcome Scholar in Experimental Therapeutics. These studies were supported by awards from the National Institute of Health, the American Heart Assocation, and the Burroughs Wellcome Fund.

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