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Endothelial nitric oxide synthase is myristylated

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The enzyme responsible for the synthesis of endothelium-derived relaxing factor and/or nitric oxide in the endothelium has been described as a particulate enzyme, whereas other isoforms of nitric oxide synthase are soluble enzymes. Here we are reporting that endothelial cells metabolically incorporate myristate (C14), but not palmitate (C16), into nitric oxide synthase. We are postulating that the endothelial-derived nitric oxide synthase is a particulate enzyme because of the fatty acid acylation of the protein which 'anchors' the enzyme into the membrane either directly or via another membrane-bound protein.

Myristic acid; Palmitic acid; EDRF; L-Arginine; Bovine aortic endothelial cell

1. INTRODUCTION

Nitric oxide synthase enzymatically converts L-Arginine and oxygen to L-citrulline and nitric oxide (NO) [1–4]. NO may be involved in signal transduction in the brain [5,6], is a cytostatic and tumoricidal agent of cytokine or endotoxin-induced macrophages [7] (reviewed in [8]), is responsible for endothelium-dependent vasodilatation of vascular smooth muscle [9–11] and inhibition of platelet aggregation [12]. Different isoforms of NO synthase have been purified and characterized from the brain [13–16], cytokine/endotoxin-induced RAW macrophages [17,18], and endothelial cells [19] (reviewed in [20]). Type I NO synthase isolated from rat, porcine, and human brain and Type III NO synthase isolated from bovine aortic endothelial cells are regulated by Ca^{2+}/calmodulin [13–16,19,21]. The Type I NO synthase is located in the soluble fraction of brain homogenates and the purified enzyme migrates at a molecular mass of 150–160 kDa under denaturing conditions [13–16]. Type II NO synthase is a soluble, cytokine-inducible enzyme that does not seem to be regulated by Ca^{2+}/calmodulin. Its denatured molecular mass was determined to be about 130 kDa [17,18]. In bovine aortic endothelial cells, Type III NO synthase is located predominantly (cultured cells [22] or exclusively (native cells [23]) in the particulate fraction with the purified enzyme from both sources migrating at a molecular mass of 135 kDa under denaturing conditions. [19]

Therefore, we sought to investigate the possible mechanisms involved in membrane association of the endothelial NO synthase. Some eukaryotic proteins are co- or post-translationally modified by the addition of a saturated fatty acid, such as myristate (C14) or palmitate (C16), or a poly-isoprene group, such as farnesol (C15) or geranylgeranol (C20). These modifications can have profound effects on the subcellular localization and function of the modified protein (reviewed in [24]). Incubation of bovine aortic endothelial cells with [^{3}H]myristate or [^{3}H]palmitate demonstrated that only the myristate was incorporated into Type III NO synthase.

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2. MATERIALS AND METHODS

Bovine aortic endothelial cells (BAE cells, passage 9) [25] were grown to approximately 80% confluency in 175 cm^2 plates in Dulbecco's Modified Essential Medium containing 10% fetal bovine serum (Gibco). The medium was then removed and replaced with 10 ml of serum-free medium. Approximately 350 μCi of [^{3}H]myristic acid (NEN, 30.7 Ci/mmol) or [^{3}H]palmitic acid (NEN, 30.7 Ci/mmol) was added per tissue culture plate. In some plates, additional non-radiolabeled myristic acid or palmitic acid was added at a 100× molar ratio. The cells were then incubated at 37°C for 4, 16, or 24 h. The cells did not reach confluency after 24 h. [^{3}H]Myristic acid incorporation was found to be significantly less in confluent cells. The medium was then removed and the cells washed with phosphate-buffered saline (PBS) twice. The cells were scraped from the plates in 5 ml of PBS and each plate washed with 5 ml of PBS to insure complete removal of all the cells. The cells were spun down at 4°C for 12 min at 2,200 rpm. They were resuspended in 1 ml of cold buffer 1 (50 mM Tris-HCl pH, 7.4, 0.1 mM EDTA, 0.1 mM EGTA, and 12 mM 2-mercaptoethanol) and homogenized in a glass/teflon homogenizer in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 2 μM leupeptin, and 1 μM aprotinin). The crude homogenates were centrifuged at 100,000 × g for 60 min. The cytosolic fractions were removed and frozen at −70°C. The particulate fractions were resuspended in 0.5 ml of buffer 2 (buffer 1 containing 10% glycerol), rehomogenized in the presence of protease inhibitors, solubilized with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma; 20 mM) for 20 min at 4°C with gentle rotation of the sample, and then recentrifuged at 100,000 × g for 30 min. The CHAPS extracts were removed and frozen at −70°C. The cytosolic

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and CHAPS extract fractions from each variable condition were then loaded onto 200 μl columns of preswollen 2',5'-ADP-Sepharose (Pharmacia) and each fraction was recirculated twice. Each column was washed with 1 ml of buffer 2 containing 0.5 M NaCl and then with 1 ml of buffer 2. The nitric oxide synthase fraction was eluted with 450 μl of buffer 2 containing 10 mM NADPH (Boehringer Mannheim) into microconcentrator units (10,000 mwco; Millipore). The buffers for the CHAPS extract fractions all contained 10 mM CHAPS, whereas the buffers for the soluble fractions contained no detergent. The NADPH eluates were then concentrated to approximately 30 μl volumes. These samples were run on 7.5% denaturing SDS-PAGE [26] and stained with Coomassie blue so that the amount of nitric oxide synthase in each lane could be assessed. No difference in protein content of each variable condition was ever noted (data not shown). The gels were then destained, exposed to the fluorographic reagent, EN^{3}HANCE (DuPont-NEN), dried, and exposed to Hyperfilm-MP (Amersham) at −70°C for 2 weeks. The films were processed with a Kodak-XO-mat processor. Densitometry was performed with a Leica Quantimet Q570 image analyzer. Optical density readings were taken using the quantitative autoradiographic software of the image analyzer.

3. RESULTS AND DISCUSSION

The detergent (CHAPS) extract from BAE cells homogenates purified on 2',5'-ADP-Sepharose produced approximately 5 bands on a Coomassie blue-stained SDS-PAGE including the 135 kDa band that represents Type III NO synthase [19]. This band was positively identified as endothelial NO synthase using a monoclonal antibody generated against the purified particulate enzyme from BAE cells [27]. The corresponding autoradiography of a SDS-PAGE of 2',5'-ADP-Sepharose purified NO synthase prepared from BAE cells exposed to [^{3}H]myristate alone during log growth demonstrated incorporation only in the 135 kDa band (Fig. 1). In order to verify the specificity of this reaction, additional BAE cells were incubated in the presence of 100× molar ratio of unlabeled myristic acid. This markedly reduced the incorporation of [^{3}H]myristate in the 135 kDa band (Fig. 1). The ADP-purified soluble fraction from BAE cells showed no measurable incorporation of radiolabelled myristic acid, however the total amount of soluble nitric oxide synthase is very low [22] and none could be detected by Coomassie blue staining (data not shown). When the same experiments were repeated with [^{3}H]palmitate, none of the particulate (Fig. 1) or soluble (data not shown) proteins eluted from 2',5'-ADP-Sepharose were labeled.

Fatty acids may be oxidized and the resulting acetyl-CoA fragments may be metabolically incorporated into amino acids for cellular protein synthesis. To ensure that the incorporation of radioactive myristic acid into NO synthase was specific, we incubated BAE cells with [^{3}H]myristic acid for 4, 16 and 24 h. The same specific incorporation into Type III NO synthase was seen at each incubation time with an increase in intensity of the radiolabeled NO synthase with increasing incubation time (Table I). This suggests a specific modification of the endothelial NO synthase to be myristylated. Three known isoforms of NO synthase have now been cloned

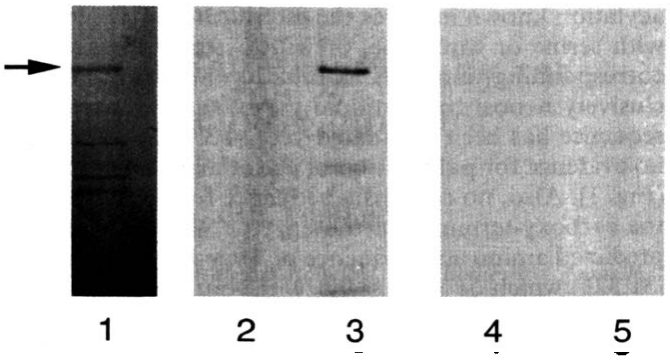

Fig. 1. Autoradiographic results of incorporation of [^{3}H]myristic acid and [^{3}H]palmitic acid into endothelial NO synthase. All proteins shown were 2',5'-ADP-Sepharose purified from BAE cells and ran on a 7.5% denaturing SDS-PAGE as outlined in section 2. Lanes: (1) Coomassie blue stained for total protein (arrow denotes the position of Type III NO synthase, 135 kDa); (2) incubation of BAE cells with [^{3}H]myristate in the presence of 100 × unlabeled myristate for 16 h; (3) incubation of BAE cells with [^{3}H]myristate for 16 h; (4) incubation of BAE cells with [^{3}H]palmitate in the presence of 100× unlabeled palmitate for 16 h; (5) incubation of BAE cells with [^{3}H]palmitate for 16 h.

[28–32]. None of the enzymes appear to have a hydrophobic signal sequence that would identify it as a membrane-bound protein. However, the endothelial NO synthase [31,32] does possess a consensus sequence at the amino terminus for *N*-myristyl transferase [33]. Amino-terminal myristylation involves an amide linkage to an amino-terminal glycyl residue catalyzed by the enzyme *N*-myristyl transferase following the removal of the initiation methionine residue by the enzyme methionine aminopeptidase. The amino-terminal glycyl residue is critical to myristylation and at the fifth and eighth residues from the amino-terminal end certain amino acids are also preferred. It has been shown that myristylation is a very early co-translational event that occurs before the first 100 amino acids of a nascent polypeptide chain are polymerized [33–35]. Myristylation of a protein may confer membrane association within a cell, although some proteins have been shown to be myristylated, but remain soluble proteins [24]. We postulate for Type III NO synthase that myristylation determines its predominant localization in the particulate fraction of BAE cells. The second form of fatty acid

Table I

Absolute optical density of autoradiographs from SDS-PAGE of the Type III NO synthase protein band

Time	Optical density [^{3}H]myristate only	Optical density [^{3}H]myristate plus 100 × unlabeled myristate
4 h	0.0035	0.0002
16 h	0.0183	0.0015
24 h	0.0171	0.0003

acylation known involves the esterification of palmitate with serine or threonine, or with cysteine to form the corresponding thioester. Palmitation appears to be exclusively a post-translational event and no consensus sequence has been elucidated yet [24,33–35]. We found no evidence for palmitation of endothelial NO synthase (Fig. 1). Also, no consensus sequence for prenylation at the carboxy-terminus of the enzyme was found in the predicted amino acid sequence of Type III NO synthase [31,32], which is consistent with our finding of no change in the subcellular localization of endothelial NO synthase activity after inhibition of HMG-CoA-reductase with lovastatin (unpublished results).

The Type I NO synthase from rat brain and Type II isoform from induced macrophages are both soluble proteins [13–18] and the cloned amino acid sequences do not contain an *N*-myristyl transferase consensus sequence [28–30]. In addition to the mainly soluble activity, smaller quantities of particulate NO synthase activity has been found in cytokine-induced macrophages [36–38] and a recent report suggests the presence of a 150 kDa insoluble NO synthase in the rat cerebellum [39]. The nature of these particulate NO synthases is unclear at this time.

In conclusion, we postulate that endothelial Type III NO synthase incorporates a saturated fatty acid, myristate, but not palmitate, which determines its membrane association. In vitro mutagenesis studies that substitute the N-terminal glycine of endothelial NO synthase will have to be performed to corroborate this finding. Myristylation may facilitate Type III NO synthase binding to a cellular membrane directly or via a myristyl protein receptor similar to the myristylated $pp60^{v\text{-}src}$ protein [40].

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