Palmitoylation of Inducible Nitric-oxide Synthase at Cys-3 Is Required for Proper Intracellular Traffic and Nitric Oxide Synthesis*

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A number of cell types express inducible nitric-oxide synthase (NOS2) in response to exogenous insults such as bacterial lipopolysaccharide or proinflammatory cytokines. Although it has been known for some time that the N-terminal end of NOS2 suffers a post-translational modification, its exact identification has remained elusive. Using radioactive fatty acids, we show herein that NOS2 becomes thioacetylated at Cys-3 with palmitic acid. Site-directed mutagenesis of this single residue results in the absence of the radiolabel incorporation. Acylation of NOS2 is completely indispensable for intracellular sorting and NO synthesis. In fact, a C3S mutant of NOS2 is completely inactive and accumulates in intracellular membranes that almost totally co-localize with the Golgi marker β-cop. Likewise, low concentrations of the palmitoylation blocking agents 2-Br-palmitate or 8-Br-palmitate severely affected the NO synthesis of both NOS2 induced in muscular myotubes and transfected NOS2. However, unlike endothelial NOS, palmitoylation of inducible NOS is not involved in its targeting to caveolae. We have created 16 NOS2-GFP chimeras to inspect the effect of the neighboring residues of Cys-3 on the degree of palmitoylation. In this regard, the hydrophobic residue Pro-4 and the basic residue Lys-6 seem to be indispensable for palmitoylation. In addition, agents that block the endoplasmic reticulum to Golgi transit such as brefeldin A and monensin drastically reduced NOS2 activity leading to its accumulation in perinuclear areas. In summary, palmitoylation of NOS2 at Cys-3 is required for both its activity and proper intracellular localization.

The gaseous radical nitric oxide (NO) modulates biological function in a wide range of tissue types, acting either as a signaling molecule or as a toxin. Three human NOS isoforms have been cloned and characterized. Among them, NOS2 (sometimes referred to as inducible NOS or iNOS) is mostly involved in the synthesis of the large amounts of NO that appear in inflammatory and immunologic processes (1, 2).

Both crystallographic and enzymatic studies performed with recombinant proteins expressed in Escherichia coli have shown that the N terminus end of the three mammalian NOSs is not involved in NO synthesis but rather in subcellular targeting of the mature polypeptide chain (2, 3). For instance, the PDZ domain of NOS1 (residues 1–90) interacts with dystrophin and becomes localized to the sarcolemma of fast twitch fibers (4). In fact, deletion of the first 226 amino acids of NOS1 results in a catalytic protein that synthesizes NO at a similar rate than the full-length protein (5). Likewise, the N-terminal end of NOS3 is covalently and irreversibly myristoylated at Gly-2 and reversibly palmitoylated at Cys-15 and Cys-26 in a well described process responsible for its targeting to caveolae (6, 7). In addition, deletion of the first 52 amino acids of NOS3 does not affect catalytic activity, reflecting that this sequence stretch is not part of the enzymatic machinery but is involved in intracellular traffic (8).

As in the case of NOS1 and NOS3, the N-terminal end of NOS2 is very likely involved in subcellular targeting. In fact, deletion of the first 65 amino acids of a recombinant NOS2 expressed in E. coli results in a mutant protein that behaves like the wild-type counterpart (9). However, much less is known about the post-translational modifications of NOS2 in vivo within eukaryotic cells, such as acylation, phosphorylation, or its binding to other cellular proteins. Interestingly, when a peptide corresponding to the first 17 amino acids of NOS2 is used to elicit a rabbit antiserum the resulting antibodies recognize a soluble form of the enzyme, but not its membrane-bound counterpart (10). In contrast, both the particulate and the soluble NOS2 were recognized by a serum elicited against the C terminus end of the protein (10). Likewise, the presence of a post-translational modification in NOS2 resulting in membrane attachment with an increased molecular mass has also been reported (11, 12). Although this membrane association of NOS2 has been well characterized in a number of tissues (11–14), the mechanisms underlying the translocation of NOS2 to the particulate fraction and its subsequent dissociation is poorly characterized.

We demonstrate in this article that the palmitoylation of NOS2 at Cys-3 is a process necessary for its intracellular transit toward subcellular domains where NO synthesis is required. In fact, the site-directed mutant where this Cys-3 has been converted into a Ser residue did not incorporate the radioactive fatty acid and irreversibly aggregated in the Golgi compartment, becoming completely devoid of activity.

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Palmitoylation of NOS2

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Glutamine, antibiotics, cell culture media (Dulbecco’s modiﬁed Eagle’s medium, sulﬁnic acid (4-aminozenesulfonic acid), N-(1-naphthyl)ethylenediamine dihydrochloride, LPS (from Salmonella enteritidis), brefeldin A, monensin, 2-Br-palmitate, and nickel-nitrotriacetic acid resin, transfect agents Escort-III and Escort-IV, and Hoechst were purchased from Sigma. Trypsin-EDTA and fetal bovine serum were from BioWhittaker Europe. The source of the various antibodies used in this work is as follows: anti-caveolin-1 polyclonal (C13630) and anti-NOS2 polyclonal (N32030) were from Transduction Laboratories. Anti-β-tubulin I (T7516) monoclonal antibody and anti-β-esp (PA1–061) were purchased from Santa Cruz Biotechnology. Anti-NOS2 monoclonal antibody (N9657) was purchased from Sigma. The rabbit polyclonal anti-GFP serum was raised in our laboratory as previously described (12, 15). Goat anti-GFP was from Eusera.com. Recombinant human IFN-γ was from PepTech. Protein A-Sepharose, 2′,5′-ADP-Sepharose, Dowex resin, ECL reagents, Cy3- and Cy3-labeled secondary antibodies, and radioisotopes were from Amersham Biosciences.

The C2C12 myotubes were grown in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a 5% CO2 atmosphere at 37 °C. Differentiation of ~70% confluent myoblasts into myotubes was performed in Dulbecco’s modiﬁed Eagle’s medium supplemented with antibiotics and glutamine plus 50 μM insulin in the absence of serum for 3 days (12). Stimulation of myotubes, transfection, immunoprecipitation, immunofluorescence, and determination of the NO release were performed essentially as previously reported (12, 15).

Molecular Cloning and Construction of the 16 NOS2 Chimeras—We have previously described the cloning and expression of the full-length wild-type NOS2 (12), which possessed the initial sequence MACPKW-FLFKVSKYQSD . . . We have created the following mutant NOS2-GFP constructs: wild-type, the single mutants A2C, G3S, K4E, K6E, and the double mutants K6E/K10E, P4E/K6E, and P4E/K8E. Every construct was digested with XbaI for 2 h and EcoRI was then added for 5 min to induce a partial digestion (there are internal EcoRI sites of a pUC-linker-GFP construct (12, 15). In this construct we had a 4.2 kb in the case of the large chimeras and 1 kb in the case of smaller constructs.) The larger band obtained from this digestion containing the isoelectric point of the isoform was ligated into the pGEM-T vector (Invitrogen) and subcloned in the expression vector pCWori was used to transform competent BL21 cells (Novagen). When we coexpressed the vector for calmodulin was already inserted (19). Four liters of 2× YT media were used for protein expression at 22 °C. The protein was puriﬁed using two aﬃnity columns as previously described (20).

Incubation of Live Cells with BODIPY-Texas Red-Ceramide—The live C07 cells transfected with the various GFP chimeras were incubated with BODIPY-Texas Red ceramide and subcellular fractionation was then performed. The ceramide 3′-phosphate conjugated to BODIPY (PA1–061) was purchased from Molecular Probes (Eugene, OR) and diluted as previously reported (12, 15). For subcellular fractionation, cells were incubated with 1 μM Tris, pH 7.0, for 24 h, whereas the entire gel was incubated with a buﬀered solution of 1 μM hydroxylamine.

RESULTS

Palmitoylation of Both Cytokine-induced and Transfected NOS2—When C2C12 muscular myotubes are challenged with a mixture of LPS and IFN-γ a clear resemblance with the phenotype shown by the wild-type NOS2 construct were metabolically labeled with [3H]tritiated palmitic acid into the proteins was visualized by phosphorimaging and autoradiography of the polyvinylidene diﬂuoride membrane. A control labeling experiment was equally performed using a chimeric Fyn kinase GFP and its mutant chimera Fyn(G22A) fused to GFP that included the first 15 amino acids of the N-terminal end of the sequence (17).

Assay of NOS2 Activity—Because NOS2-derived nitric oxide decomposes in nitrites and nitrates, we performed the Griess assay according to our published protocol (12). A volume of 0.5 ml of sample was incubated with 50 μl of a 100 mM sulfanilic acid solution and 50 μl of a 10 mM N-1-naphthyl ethylenediamine dihydrochloride solution. The mixture was allowed to react for 15 min, and the absorbance value at 540 nm was determined. Every sample was analyzed in triplicate. Fresh solutions of sodium nitrite were regularly prepared as standards. In addition, in COS7 cells transfected with the various NOS2 constructs we also performed the L-[14C]arginine to L-[14C]citrulline assay as previously described (18).

Recombinant Expression of NOS in E. coli and Puriﬁcation—NOS2 cloned in the expression vector pCWori was used to transform competent BL21 cells (Novagen) where the coexpression vector for calmodulin was already inserted (19). Four liters of 2× YT media were used for protein expression at 22 °C. The protein was puriﬁed using two aﬃnity columns as previously described (20).

Neutral Hydroxylamine Treatment—The wild-type NOS2-GFP chimera was immunoprecipitated using anti-GFP antibodies and divided into separate gels. One of these gels was treated with SDS-PAGE gel was treated with 1 μM Tris, pH 7.0, for 24 h, whereas the other gel was incubated with a buﬀered solution of 1 μM hydroxylamine.

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Palmitoylation of NOS2

In a series of experiments, COS7 cells transfected with the wild-type-GFP and C3S-GFP constructs were incubated with the 125I-labeled fatty acid analogue of palmitic acid as previously described (16). As depicted in Fig. 1A, addition of the palmitoylation inhibitors 2-Br- and 8-Br-palmitate decreased NOS2 activity when measured as NO release in a dose-dependent manner. Inhibition of palmitoylation was performed both in muscular C2C12 myotubes challenged with LPS/IFN-γ (left plot) and in COS7 cells transfected with a NOS2-GFP vector (right plot). Comparable levels of NOS2 were observed in each case, as determined by immunoblotting (depicted under both plots).

The changes observed in the activity of NOS2 transfected in COS7 cells were also determined using the L-[14C]arginine to L-[14C]citrulline assay (Fig. 1B). As expected, incubation of wild-type NOS2 with 20 μM 2- and 8-Br-palmitate lead to decreased levels of citrulline, as previously observed when the levels of nitrites were determined using the Griess assay. Likewise, the C3S mutant of NOS2 displayed less than 10% of the activity of its wild-type counterpart.

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The changes observed in the activity of NOS2 transfected in COS7 cells were also determined using the L-[14C]arginine to L-[14C]citrulline assay (Fig. 2B). As expected, incubation of wild-type NOS2 with 20 μM 2- and 8-Br-palmitate lead to decreased levels of citrulline, as previously observed when the levels of nitrites were determined using the Griess assay. Likewise, the C3S mutant of NOS2 displayed less than 10% of the activity of its wild-type counterpart.

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Inhibitors of protein palmitoylation do not affect the activity of recombinant NOS2 activity per se. Panel A shows a Coomassie Blue-stained gel of recombinant NOS2 expressed in E. coli (~2 μg of purified protein were loaded). The effect of increasing doses of 8-Br-palmitate on the activity of recombinant NOS2 protein is shown on the right. COS7 cells were also transfected with the full-length wild-type NOS2 and its activity was determined using the 14C-labeled L-Arg conversion assay (C). Immunoprecipitated NOS2 obtained from [3H]palmitate-labeled COS7 cells was incubated with either 1 M Tris, pH 7.0, or 1 M hydroxylamine, pH 7.0, for 24 h. The gels were dried and residual radiolabel was detected by autoradiography. The position of the NOS2-GFP chimera is indicated by the arrow.

Certain endosomes (12, 15, 17, 21). On the other hand, coatomer proteins are involved in regulating the transport between the endoplasmic reticulum and the Golgi complex as well as in intra-Golgi transport. Hence, antibodies against β-cop are frequently used as ER to cis-Golgi markers (22, 23). When the subcellular distribution of the full-length WT and C3S mutant of NOS2 along the sorting pathways were compared, consistent differences could be observed (Fig. 3). Whereas, the non-palmitoylated mutant clearly accumulated in perinuclear areas that colocalized with both BODIPY-Texas Red-ceramide and β-cop, the wild-type phenotype showed a more clear distribution throughout the cytosol. When we quantified the colocalization percentages for the BODIPY-Texas Red-ceramide and β-cop we obtained the data shown in Fig. 3. Although the wild-type NOS2 partially colocalized with the Golgi markers in certain perinuclear locations, the C3S mutant displayed an almost complete overlap with β-cop and BODIPY-Texas Red-ceramide, indicative that the correct sorting of the protein has been interrupted in the Golgi apparatus. Only 17% of the total WT GFP fluorescence colocalizes with BODIPY labeling, whereas 95% of the C3S GFP fluorescence colocalizes with BODIPY staining, reflecting that the wild-type chimera has partially passed through the Golgi in transit to areas closed to the plasma membrane. On the other hand, 12% of WT GFP fluorescence colocalizes with β-cop labeling, in contrast with the C3S mutant, where 63% of its GFP fluorescence overlaps with β-cop staining. When the red-green colocalization is considered, WT GFP fluorescence displays an almost complete colocalization with both the BODIPY and β-cop fluorochromes (100 and 99%), whereas the GFP fluorescence of the C3S chimera only “covers” 24% of the total BODIPY fluorescence and 81% of the total β-cop fluorescence. These results not only indicate that the full-length NOS2-GFP chimera moves along the traffic machinery of the COS7 cells in transit through the ER-Golgi-TGN compartments, but also provide direct evidence that the palmitoylation of the cysteine residue located at position 3 of its sequence is required for this process.

Subcellular Distribution and Radioactive Labeling of NOS2-GFP Chimeras Mutated in the N-Terminal End—A detailed analysis revealed that the N-terminal end of NOS2 includes hydrophobic amino acids (i.e., Pro, Trp, Phe, Leu, and Val) together with three basic Lys residues at positions 6, 10, and 12 (Fig. 4A). To reveal the requirements for NOS2 palmitoylation, we designed mutations in amino acids located at the N-termi-
The N-terminal end of full-length NOS2-GFP (boxed in Fig. 4A) included an additional Cys residue at position 2 as well as mutants P4K, P4K/W5K, K6E, K6E/K10E, and P4E/K6E. Immunofluorescence data indicated that all the full-length chimeras were excluded from the cell nucleus and associated with perinuclear areas to a different extent (Fig. 4B). Interestingly, introduction of an additional palmitoylatable Cys residue rendered a mutant protein that partially associated with the plasma membrane (Fig. 4B). Although the substitution of hydrophobic residues for basic residues only resulted in limited changes when compared with the wild-type phenotype, the introduction of acidic residues at the N-terminal end of NOS2 yielded a slight increase in perinuclear labeling, especially in the K6E NOS2 chimera (Fig. 4B). Because NOS2 activity has been shown to be inhibited by synthetic caveolin peptides (24) and by caveolin-1 within muscular cells (12), we considered that the full-length NOS2 might possess various targeting motifs. To elucidate the role of the N-terminal end in the absence of interference from the caveolin binding motif (FPGCPFNGW) that is found spanning residues 325–333, we created deletional chimeras of NOS2-GFP containing only the first 94 amino acids of NOS2 fused to GFP. These constructs would presumably localize to certain subcellular locations dictated by the N-terminal acylation, hence bypassing a possible targeting to caveolae. Short chimeras were constructed of wild-type NOS2-GFP and also of mutants C3S, P4K, P4K/W5K, K6E, K6E/K10E, P4E/K6E, and A2C. Arrows indicate the plasma membrane labeling observed both in the wild-type NOS and A2C GFP short chimeras (Fig. 4C). Remarkably, unlike the full-length chimeras, most of their short counterparts displayed a partial nucleus staining. When we compared the subcellular distribution together with the radioactive fatty acid incorporation (Fig. 4D), we could conclude that palmitoylation can be correlated with a certain amount of the recombinant chimera in the completion of the subcellular transit leading to the plasma membrane. On the other hand, the short mutants that failed to become palmitoylated, such as C3S, K6E, K6E/K10E, P4E/K6E, and P4K never reached the plasma membrane, and in many cases showed a clear perinuclear localization (Fig. 4, C and D). Consequently,
the presence of both the basic residues Lys-6 and Lys-10 together with the hydrophobic residue Pro-4 seem to be determinant in governing the proper palmitoylation of the NOS2-(1–94) chimeras as well as their subcellular traffic. In addition, at least in the case of WT and A2C chimeras (which become palmitoylated), the targeting information conferred by these first 94 amino acids of NOS2 allows a full transit past the trans-Golgi network and clear localization in the plasma membrane (shown by arrows in Fig. 4C). Nevertheless, as previously shown in our double immunofluorescence studies, 83% of the WT full-length GFP chimera has already exited the TGN and is in transit to the plasma membrane in a catalytically active state. On the other hand, to explain the divergence in subcellular distribution displayed by the short and long chimeras we propose that one of the multiple protein-protein interactions reported for NOS2 might be responsible for this differential targeting (caveolin binding, interaction with Nap110 or kalirin, etc).

**Palmitoylation of NOS2 Is Not Involved in Caveolae Targeting**—Because in the case of endothelial NOS, palmitoylation of Cys-15 and Cys-26 is necessary for its translocation to caveolae (6, 7), we isolated Triton X-100-insoluble domains enriched in caveolin-1 where acylated proteins are frequently found. Interestingly, in transfected COS7 cells neither the wild-type NOS2 nor the C3S mutant associated with Triton X-100-insoluble rafts (Fig. 5). A positive caveolin-1 immunodetection in the more buoyant fractions was indicative of caveolae, whereas β-cop appeared enriched in fractions with large non-caveolar membranes. We tested the putative caveolar targeting of our long and short wild-type NOS2-GFP chimeras. The majority of the wild-type NOS2-GFP appeared in the high density fractions (at the bottom of the tube) and a very limited amount of protein co-fractionates with caveolae (Fig. 5A, fractions 5–8), where a single degradation band was clearly observed. Full-length mutant C3S was unable to associate with caveolae. The short GFP chimeras (amino acids 1–94) of both wild-type and C3S NOS2 were partially degraded, and only a very small fraction of the total wild-type NOS2 (but not the C3S mutant) could be observed in caveolae (Fig. 5B). This piece of data contrasts with the clear plasma membrane distribution of a significant population of the short wild-type NOS2-GFP according to immunofluorescence data (see above) from which one might expect a significant caveolae enrichment. When live COS7 cells transfected with the full-length NOS2 construct were kept at 4 °C and 1% cold Triton X-100 was added, the GFP fluorescence progressively vanished, reflecting that NOS2 localizes to Triton X-100-soluble regions. Hence, although NOS2 significantly associated with total intracellular membranes (Fig. 1C), the majority of these membranes are apparently not in rafts/caveolae enriched in cholesterol and sphingomyelin, which might be Triton X-100-insoluble (Fig. 5C), in agreement with the data obtained in the sucrose gradients (Fig. 5A). In fact, according to our immunofluorescence data, full-length and short NOS2-GFP chimeras colocalize with caveolin only partially in the plasma membrane and in intracellular areas of the Golgi apparatus (yellow staining in Fig. 5D).

**Compounds That Alter the ER to Golgi Transit Interface with the Activity of NOS2 and with Its Subcellular Localization**—The possibility therefore exists that the proper sorting of NOS2 might be indispensable to reach complete activity. Hence, we tested next the effect of both brefeldin A and monensin on the NOS2 activity and subcellular distribution both in C212 mouse myotubes challenged with LPS/IFN-γ and in COS7 cells transfected with a full-length NOS2-GFP chimera. Brefeldin A treatment, which inactivates Arf1, is known to lead to the dissociation of COPI and other peripheral proteins from Golgi membranes, resulting in Golgi enzymes redistributing to the ER as the Golgi structure disassembles (25). On the other hand, the ionophore monensin is commonly used to partially disrupt the integrity of the Golgi apparatus and to inhibit vesicular transport in eukaryotic cells (26). Induction of NOS2 expression in muscular myotubes followed by the addition of both brefeldin A and monensin drastically diminished the NO synthesis to 29 and 41% of the control levels, respectively (Fig. 6A). These alterations in the secretion pathways resulted in a dispersive phenotype of the Golgi apparatus accompanied by a clear vesicularization in the cytosol (Fig. 6A). Similarly, NOS2-GFP transfected in COS7 cells was also severely affected by treatment with brefeldin A and monensin (Fig. 6B), which lowered the amount of NO detected to 32 and 38%, respectively, of the control levels treated with the vehicle only. Likewise, these treatments affected the intracellular distribution of NOS2-GFP (Fig. 6B), with brefeldin A resulting in large perinuclear aggregates and monensin inducing the formation of dispersive “patches” of NOS2 throughout the cytosol.

The “Gain of Function” Mutant A2C/C3S That Restores a Potential Palmitoylation Site Partially Rescues the Wild-type Phenotype—Next, we created both a full-length and a NOS2-GFP transfected in COS7 cells and caveolae were purified in a discontinuous sucrose gradient in the presence of 1% Triton X-100 at 4 °C. After centrifugation, the tubes were equally divided into 12 fractions and each was immunoblotted against NOS2, caveolin-1, and β-cop (used here as a Triton X-100 soluble marker). B, the NOS2-(1–94)-GFP wild-type and its C3S mutant chimeras were also transfected in COS7 cells and caveolae were purified in a discontinuous sucrose gradient in the presence of 1% Triton X-100 at 4 °C. C, live COS7 cells grown on coated glass coverslips transfected with the wild-type NOS2-GFP construct were submerged in medium at 4 °C and then pre-chilled 1% Triton X-100 in phosphate-buffered saline was added. Photographs of the same field were taken every 10 s in a 10-min period. D, the subcellular distribution of both the full-length and NOS2-(1–94)-GFP constructs were analyzed by laser confocal microscopy in a triple staining with anti-caveolin-1 (red) and cell nuclei (blue).
Agents that disturb the ER to Golgi traffic affect NOS2 activity and subcellular distribution. A, muscle C2C12 myotubes were challenged with LPS/IFN-γ and 12 h later 10 μM brefeldin A (lower left panel) or 10 μM monensin (lower left panel) were added to the cell culture for 16 h. After treatment, the NOS2 activity was determined with the Griess assay and the cells were fixed and analyzed by laser confocal microscopy. NOS2 activity as shown in the upper left panel. A control experiment with the vehicle (methanol) was performed in parallel (upper right panel). B, COS7 cells were transfected with the wild-type NOS2-GFP chimera and 12 h later 10 μM brefeldin A (lower left panel) or 10 μM monensin (lower left panel) were added to the cell culture for 16 h. After treatment, the NOS2 activity was determined with the Griess assay and the cells were fixed and analyzed by laser confocal microscopy. NOS2 activity is shown in the lower left panel. A control experiment with the vehicle (methanol) was performed in parallel (upper right panel) (mean ± S.E., n = 4 experiments in triplicate, * p < 0.05).

FIG. 6. The gain of function mutant A2C/C3S is palmitoylated, active, and does not accumulate in perinuclear areas. The panel A shows the subcellular localization of the gain of function A2C/C3S-GFP long and short chimeras as visualized by laser confocal microscopy. COS7 were transfected with the constructs, and fluorescence was analyzed 30–36 h after transfection. GFP fluorescence was visualized with an excitation wavelength of 488 nm. NO synthesizing activity of the wt and C3S mutant (Fig. 7A). In fact, the full-length NOS2 A2C/C3S mutant displays 51% activity when compared with the WT polypeptide (Fig. 7B). When we added radiolabeled palmitic acid and immunoprecipitated the A2C/C3S transfected in COS7 cells, a distinctive incorporation of the radioisotope could be observed (Fig. 7C). Hence, engineering a surrogate palmitoylatable Cys residue at the N-terminal end of NOS2 renders a polypeptide that becomes palmitoylated and partially rescues the wild-type phenotype in terms of subcellular distribution and activity.

DISCUSSION

The major finding described in this work is the fact that inducible nitric-oxide synthase is S-acylated with palmitic acid in Cys-3 of its sequence. Palmitoylation occurs both in NOS2 transcriptionally induced in muscular myotubes insulin with proinflammatory stimuli as well as in transfected NOS2 and takes place through a hydroxylamine-sensitive thioester bond. However, when recombinant NOS2 was expressed in a bacterial expression system, the purified enzyme was not inhibited by levels of 8-Br-palmitate that had a profound effect when added to COS7 cells transfected with NOS2.

FIG. 7. The gain of function mutant A2C/C3S is palmitoylated, active, and does not accumulate in perinuclear areas. The panel A shows the subcellular localization of the gain of function A2C/C3S-GFP long and short chimeras as visualized by laser confocal microscopy. COS7 were transfected with the constructs, and fluorescence was analyzed 30–36 h after transfection. GFP fluorescence was visualized with an excitation wavelength of 488 nm. NO synthesizing activity of the wt and C3S mutant compared with wild-type NOS2 determined with the Griess assay (B). Radioactivity labeling of COS7 cells transfected with the NOS2-(1–94) A2C/C3S chimera incubated in the presence of [3H]palmitic acid followed by immunoprecipitation with anti-GFP antibodies. The position of the NOS2-GFP chimera is indicated by the arrow (C).

Unlike NOS3, where palmitoylation is a prerequisite for caveolar targeting, interaction with caveolin, and protein inactivation, NOS2 requires the acylation for its proper sorting along the secretory route after its initial synthesis and localization to the ER. Hence, the proper localization of the highly active NOS2 within the cells might be a tightly regulated process to avoid the toxic effects of NO. Alternatively, NOS2 might be targeted to certain subcellular areas with a large availability of its substrate L-Arg or its cofactor tetrahydrobiopterin.

Remarkably, substitution of Cys-3 for Ser resulted in the loss of fatty acid incorporation, apparent protein misfolding, and aggregation in subcellular membranes of the Golgi apparatus concomitant with a complete loss of NOS activity. Both basic amino acids and the hydrophobic Pro residue at position 4, which are in proximity of the palmitoylated Cys residue, seem to be determinant for the acylation process, because their mutation changed the subcellular distribution of NOS2 and eliminated its S-acylation as well.

Elegant studies performed by Felley-Bosco and co-workers (27, 28) have proven that in Caco cells, only about 1% of the...
total NOS2 protein content was associated with caveolin-1 in caveolae, where it was targeted for proteasomal degradation. In fact, ectopic expression of caveolin-1 promoted NOS2 degradation at a single site on its N terminus (28). Our own studies have shown that, in C2C12 myotubes, NOS2 can also associate with caveolin-1, becoming inhibited, although the same stimuli that up-regulated NOS2 expression seemed to down-regulate caveolin-1 expression via the extracellular signal-regulated kinase pathway (12). The results described herein agree with the pharmacological inhibition of the palmitoyltransferases involved in NOS2 thioacylation might be a useful approach in the treatment of inflammatory diseases associated with increased NOS2 levels.

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