Expression, Localization, and Correlation of N-Myristoyltransferase and Its Inhibitor in Bovine Eye

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PURPOSE. N-myristoyltransferase (NMT) is a ubiquitously distributed eukaryotic enzyme that catalyzes myristoylation of proteins. Very little is known about the process of myristoylation, particularly in the eye. In this study, the distribution, expression, correlation of NMT and its inhibitor (NMT inhibitor protein, NIP) were investigated.

METHODS. Whole bovine eyes were either homogenized or regionally dissected to determine the activity and localization of NMT and NIP. Dissected tissues were homogenized, and Western blot analysis was performed using polyclonal anti-NMT and anti-NIP antibodies. The NMT activity was assayed using cAMP-dependent protein kinase or pp60src derived peptide as a substrate. Fresh samples were then prepared for immunohistochemical analysis and probed with polyclonal anti-NMT and anti-NIP antibodies.

RESULTS. The total bovine eye cytosolic fraction displayed both NMT and NIP expression. NMT was present in all regions of the eye at various levels of expression. The highest expression of NMT was in the cornea, whereas NIP was present in the retina, optic nerve, sclera, and choroid only. NIP expression was highest in the optic nerve, sclera, and retina. NMT activity was observed in the cornea, iris, and retina after DEAE-Sephrose CL-6B column chromatography. The inhibitory activity of crude homogenate on recombinant human NMT activity was measured. NIP isoforms and expression were observed in various regions of the eye and will lead to further understanding of the relationship of NMT with NIP.

CONCLUSIONS. The varied expression of NMT in different regions of the eye reveals a regulatory relationship of NMT with NIP. These findings indicate that NMT and NIP are present in various regions of the eye and will lead to further understanding of visual signaling in ocular cells. (Invest Ophthalmol Vis Sci. 2004;45:1674–1679) DOI:10.1167/iovs.03-1202

Myristoylation is a cotranslational modification of proteins with myristic acid. This modification regulates both protein function and localization.1,2 Myristoylated proteins are acylated through an amide linkage to their N-terminal glycine residue, a reaction catalyzed by N-myristoyltransferase (NMT).3–6 Myristoylated proteins in the cell have diverse biological functions such as signal transduction, cellular transformation, and oncopogenesis.3–6 Myristoylated proteins include the catalytic subunit of cAMP-dependent protein kinase,7 the β-subunit of calcineurin,8 the α-subunit of several G-proteins,9 cellular and transforming forms of pp60src10 and several tyrosine kinases and proteins essential for the assembly, maturation, and infectivity of mature virus particles, such as the murine leukemia virus Pr65src precursor11 and poliovirus VPO polypeptide precursor.12 NMT knockout Drosophila resulted in various embryonic cytoskeletal and cell morphologic defects and apoptotic cell death, suggesting the significance of myristoylation.13 During tumorigenesis, the importance of protein myristoylation was first suggested by the fact that myristoylation of the viral oncogene pp60src is required for membrane association and cell transformation.14,15 Elevated expression of NMT in colorectal and gallbladder cancer has been reported from our laboratory,16–17 suggesting NMT could be a prognostic marker for cancer.

N-myristoylation of protein is crucial for HIV-1 Gag,18–20 which renders proper functions and survival. NMT inhibitors were found to prevent the binding of Gag to membrane and virus assembly.21 Thus, NMT is considered to be one of the key proteins for both HIV-1 and its host cell. It has been shown that, with decreased expression levels of NMT, HIV structural proteins were expressed gradually in HIV-1 infection of the human T-cell line CEM. This is attributed to the virus’s strategy for persistent replication. Also, there is decrease in the mRNA levels of human NMT isoforms and the NMT activities in the course of infection.22–25

Rhodopsin, a visual pigment in vertebrate rod photoreceptors, is activated by light, which leads to hydrolysis of GTP and closure of the GTP-gated channel to induce membrane hyperpolarization.24,25 Recovarin is a calcium-binding protein that inhibits rhodopsin phosphorylation by rhodopsin kinase until rhodopsin is activated by light.26 The posttranslational modification of N-terminal glycine of recovarin is essential for the association of recovarin with rod segment membranes at high Ca2+ concentrations.27,28 Recovarin is membrane bound in the dark adapted state and a decrease in bound Ca2+ induced by light causes conformational changes in recovarin. This result in binding of Ca2+ to recovarin and extrusion of the myristoyl group as “myristoyl switch,” which is unique to recovarin.29

In our continuing interest in protein myristoylation, we initiated an investigation to study the localization, expression, and activity of NMT in the eye. In view of the fact that the eye is a heterogeneous organ containing many different regions, very little is known about the mechanism of NMT action; we report here for the first time the distribution of NMT in the different regions of eye and its regulatory relationship with NMT inhibitor protein (NIP).

METHODS

Materials

9,10-3H myristic acid (39.3 Ci/mmoll) was purchased from Perkin Elmer Life Sciences (Boston, MA). Pseudomonas acyl CoA synthetase, phe-
nymethylsulfonyl fluoride, soybean trypsin inhibitor, pepsin benza-
idine, and monoclonal anti-β-actin antibody were from Sigma-Aldrich
(Toronto, Ontario, Canada). Peptide substrate based on the N-terminal
were applied to a DEAE-Sepharose CL-6B column (1.4
were of analytical grade and obtained from Sigma-Aldrich.
were purchased from Dako Corp. (Carpinteria, CA). General laboratory
ondary and anti-von Willebrand antibodies for immunohistochemistry
Elmer Life Sciences. Horseradish peroxidase (HRP)
and HRP-conjugated secondary antibody were from BioRad Laborato-
Fig. 1. NMT activity in different
regions of the eye. Crude homoge-
nates of different regions of the eye
were applied to a DEAE-Sepharose
CL-6B column and 1 M NaCl elute
were assayed for NMT activity using
pp60{"SO} (●) or cAMP-dependent pro-
tein kinase A (◻)-derived peptide.
Only cornea, iris, and retina dis-
played NMT activity. Data are ex-
pressed as the mean ± SD of results
in three independent experiments.

Preparation of Homogenates
All methods conformed to the ARVO Statement for the Use of Animals
in Ophthalmic and Visual Research. Fresh bovine eyes were obtained
from an abattoir, transported on ice, and used immediately. Various
bovine eye tissues (cornea, lens, iris, retina, vitreous body, optic nerve,
sclera, and choroid) were dissected and homogenized (Polytron;
Brinkman Instruments, Westbury, NY), five times for 30 seconds each
in 2 mL ice-cold phosphate-buffered saline (PBS) containing 0.1 mM
EGTA and 10 mM 2-mercaptoethanol. This was further centrifuged at
10,000 g for 30 minutes to obtain a clear homogenate. Total eye
cytosol was prepared from the whole bovine eye with a meat grinder
followed by homogenization in a blender for 1 minute in PBS contain-
ing 2 mM EDTA, 10 mM 2-mercaptoethanol, 100 mg/L phenylmethyl-
sulfonyl fluoride, 100 mg/L soybean trypsin inhibitor, and 200 mg/L
benzamidine. Centrifugation at 100,000g at 4°C for 1 hour yielded the
cytosolic fraction. The crude homogenates of different regions of eye
were applied to a DEAE-Sepharose CL-6B column (1.4 × 1 cm) pre-
equilibrated with PBS containing 0.1 mM EGTA and 10 mM 2-mercap-
toethanol. The column was subsequently washed with two bed vol-
umes of the above-described buffer, and proteins were eluted with
buffer containing 1 M NaCl.

N-myristoyltransferase Assay
N-myristoyltransferase activity was assayed as described previ-
ously. The reaction mixture contained 40 mM Tris-HCl (pH 7.4), 0.1
mM EGTA, 10 mM MgCl2, 5 mM adenosine triphosphate (ATP), 1 mM
LiCoA, 1 μM [3H]myristic acid (7.5 μCi), and 0.3 U/mL Pseudomonas
acyl-CoA synthetase in a total volume of 200 μL. The reaction was
performed for 30 minutes at 30°C. The conversion to [3H]myristoyl-
CoA was generally greater than 95%. The assay mixture contained
freshly generated [3H]myristoyl-CoA, 225 mM Tris-HCl (pH 7.4), 0.5
mM EGTA, 0.45 mM 2-mercaptoethanol, 1% Triton X-100, 500 μM of
pp60{"SO} -derived peptide or cAMP-dependent protein kinase- derived
peptide and crude homogenate of various regions of the eye as a
source of NMT in a total volume of 25 μL. The transferase reaction
was initiated by the addition of radiolabeled myristoyl-CoA and was
incubated at 30°C for 30 minutes. One unit of NMT activity was expressed
as 1 picomole of myristoyl peptide formed per minute.

N-myristoyltransferase Inhibition Assay
NIP was assayed by its inhibitory activity against standard human NMT.
Crude homogenates of various regions of the eye were assayed for NIP
activity against purified human NMT (0.2 μg/assay) in the presence of
cAMP-dependent protein kinase-derived peptide. A control exper-
iment was performed in the absence of eye homogenates, and human
NMT activity was considered to be 100%. All other conditions were as
just described, except the assay buffer contained 40 mM Tris-HCl (pH
7.4).

Sodium Dodecyl Sulfate–Polyacrylamide Gel
Electrophoresis and Western Blot Analysis
SDS-PAGE (10%) was performed according to the method of Laemmli. Western blot analyses were performed essentially as de-
scribed by Towbin et al. Briefly, equal amounts of proteins were
subjected to 10% SDS-PAGE and transferred to a nitrocellulose mem-
brane. The membrane was blocked with 5% powdered milk in PBS-
TWEEN 20 (PBST) and polyclonal anti-human NMT or anti-NIP antibody
or monoclonal anti-β-actin was added and incubated overnight at 4°C.
After washing with PBST, the membrane was incubated with HRP-
conjugated secondary goat anti-rabbit or anti-mouse antibody. Immu-
noreactive bands were visualized on x-ray film by using chemilumines-
cence reagents.
Immunohistochemistry

Immunohistochemistry was performed as described previously. Bovine eyes were fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Briefly, the sections were deparaffinized, rehydrated, incubated with hydrogen peroxide (0.5% in methanol), treated with pepsin (2 mg/mL 0.01 N HCl) for 45 minutes, and blocked with 1% BSA in PBS for 30 minutes. Sections were incubated with anti-human NMT antibody (1:100) for 90 minutes and HRP-conjugated secondary antibodies (1:100) for 45 minutes followed by color development. Controls included omission of primary antibody or both primary and secondary antibodies or staining with anti-von Willebrand Factor antibody (dilution, 1:100).

Other Methods

Protein concentrations were determined by the method of Bradford using BSA as a standard.

RESULTS

N-Myristoyltransferase Activity in the Eye

To analyze the role of myristoylation in the bovine eye, we examined NMT activity in the crude homogenates of cornea, lens, iris, retina, vitreous body, optic nerve, sclera, and choroid, using pp60src or cAMP-dependent protein kinase–derived peptide as a substrate. NMT activity was not observed in any region of the eye. However, when the crude homogenate was applied to a DEAE-Sepharose CL-6B column, the high-salt (1 M NaCl) eluent of the cornea, iris, and retina showed NMT activity (Fig. 1). NMT activity was high in the presence of pp60src-derived peptide compared with that in the presence of cAMP-dependent protein kinase–derived peptide (Fig. 1). The observed NMT activity after DEAE-Sepharose chromatography suggests that an unidentified inhibitor(s) of NMT may have been removed. To test this possibility, the inhibition of standard human NMT was performed by homogenates of various regions of the eye. Retina, optic nerve, sclera, and choroid showed inhibitory effects on human NMT activity (Fig. 2). The whole cytosolic fraction of eye was devoid of NMT activity, whereas it had an inhibitory effect on human NMT in vitro (data not shown).

Differential Expression of NMT and NIP Proteins

The crude homogenate showed NMT activity only after DEAE-Sepharose chromatography suggesting partial removal of inhibitor protein(s). Consequently, we investigated the protein ex-
pression profile of NMT in different regions of the eye by Western blot analysis. Crude homogenates of different regions of the eye were probed with polyclonal antibodies raised against NMT. Western blot analysis of eye samples revealed immunoreactive bands for NMT at 58 kDa in all regions of the eye with various levels of protein expression (Fig. 3A). The highest expression of NMT was observed in the cornea, and moderate expression was observed in the retina (Fig. 3A, lane 4 vs. 5) whereas the lens, vitreous body, optic nerve, sclera, and choroid showed low expression of NMT. Though NMT was expressed in all the regions of the eye, no enzymatic activity was observed in the crude homogenates. To explore the possibility of the presence of inhibitor(s) of NMT, we investigated the expression of NIP(s) in crude homogenates of different regions of the eye (Fig. 3B). On probing with anti-NIP antibody, the retina, optic nerve, and sclera showed a major band at 45 kDa. High expression of NIP was observed in the retina, optic nerve, and sclera (Fig. 3B, lanes 5 to 7), whereas the choroid showed low expression. It is interesting to note that the retina and optic nerve showed multiple immunoreactive bands. The explanation for these multiple immunoreactive bands may be the proteolytic degradation of inhibitor protein(s). However, because the retina and optic nerve are heterogeneous tissues containing many different cell types, it is...
possible that the multiple immunoreactive bands originated from different cell types. We did not detect immunoreactive bands in other regions of the eye. The results suggest that protein(s) other than 45-kDa protein may inhibit NMT activity in crude homogenates of cornea, lens, and iris. One possible inhibitor may be serum albumin, which inhibits NMT activity, as we have shown.46 Furthermore, the total eye cytosolic fraction showed expression of both NMT and NIP (Figs. 3A, 3B, lane 9).

Immunohistochemistry

Further regarding our observation of the ubiquitous expression of NMT in all regions of the eye, we investigated the localization of NMT and NIP(s) in various regions of the eye by immunohistochemical methods. Sections from cornea, retina, and optic nerve incubated with only secondary antibody showed no staining, indicating the absence of nonspecific binding (Fig. 4A). Incubation with anti-i-WF antibody, however, outlined the vasculature in those sections (Fig. 4B). Sections prepared from bovine eye and incubated with anti-human NMT antibody showed a distinct reaction in corneal epithelium, whereas the substantia propria was negative (Fig. 4C). Moderate staining was observed for human NMT in the retina (Fig. 4D), whereas the choroid (Fig. 4D) and optic nerve (Fig. 4E) did not show any staining. Furthermore, sections from the eye when incubated with anti-NIP antibody showed a distinct reaction with the retina (Fig. 4F), optic nerve (Fig. 4G), ciliary body, and lens (Fig. 4H), whereas all other sections showed no reaction.

Discussion

All the regions of the eye showed the presence of NMT. The expression of NMT varied within the tissue, with the highest expression in the cornea, iris, and retina. All purified NMT-1 essentially contain a single polypeptide with molecular mass within a range of 49 to 68 kDa.39-40 The molecular mass and cross reactivity with polyclonal antibody raised against NMT-1 suggest that bovine eye NMT belongs to the NMT-1 family. The crude homogenates did not show any NMT activity due to the presence of NIP(s). Therefore, we applied crude homogenate to a DEAE-Sepharose CL-6B column to partially separate NIP(s).

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