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Biochemical and Biophysical Research Communications 335 (2005) 1132–1139
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Expression of myristoyltransferase and its interacting proteins in epilepsy

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Received 20 June 2005
Available online 10 August 2005

Abstract

N-Myristoylation is a co-translational, irreversible addition of a fatty acyl moiety to the amino terminus of many eukaryotic cellular proteins. This modification is catalyzed by *N*-myristoyltransferase (NMT) and is recognized to be a widespread and functionally important modification of proteins. The myristoylated Src family kinases are involved in various signaling cascades, including the *N*-methyl-D-aspartate receptor functions. We examined the expression of NMT and its interacting proteins to gain further insight into the mechanisms in epileptic fowl. Higher expression of NMT1 and NMT2 was observed in carrier and epileptic fowl whereas expression of heat shock cognate protein 70, an inhibitor of NMT, was lower. Furthermore, protein–protein interaction of NMT with *m*-calpain, caspase-3, and p53 was established. The interaction of NMT2 with caspase-3 and p53 was weak in epileptic fowl compared with normal chicks while the interaction of NMT1 with *m*-calpain was weak in epileptics. Understanding the regulation of NMT by specific inhibitors may help us to control the action of this enzyme on its specific substrates and may lead to improvements in the management of various neurological disorders like Alzheimer's disease, ischemia, and epilepsy.

Keywords: *N*-Methyl-D-aspartate receptors; Src family kinases; *N*-Myristoyltransferase; Heat shock cognate protein 70; NMT inhibitor protein 71; Methionine aminopeptidase 2

Recently, the role of lipid modification of proteins and protein function has received attention. N-Myristoylation is a co-translational, irreversible addition of a fatty acyl moiety to the amino terminus of many eukaryotic cellular proteins. This modification is catalyzed by *N*-myristoyltransferase (NMT) and is recognized to be a widespread and functionally important modification of proteins [1–5]. King and Sharma [6] provided the first evidence for the existence of multiple forms of bovine brain NMT. In addition, McIlhinney et al. [7] identified two forms of NMT in bovine brain cortex. Subsequently, Glover and Felsted [8] showed that bovine brain exists as a heterogeneous mixture of NMT subunits. In humans, two forms of NMT (NMT1 and NMT2) have been identified and cloned [9].

Myristoylation is necessary for the proper functioning of some Src family protein kinases [10]. Members of these family protein kinases are expressed in the central nervous system and have been implicated in the regulation of glutamate *N*-methyl-D-aspartate (NMDA) receptors. In turn, neural excitability and plasticity are affected [11]. Electrophysiological studies have shown that tyrosine kinases can modulate the activities of post-synaptic ion channels [11–13], and tyrosine kinase signaling is required for synaptic plasticity [14]. Sanna

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.190

et al. [15] reported the role of Src family kinases in the pathophysiology of epilepsy and the possibility of considering Src family as a potential therapeutic target for antiepileptic therapy. Various reports including those from our laboratory have demonstrated that NMT plays a major role in several signaling cascades of cardiovascular, cancer, and neurodegenerative diseases. The role of NMT in epileptic condition has yet to be explored.

Originally, we reported the elevation of NMT activity in rat [16], human colorectal [17], and gallbladder [18] carcinomas. NMT and $pp60^{src}$ mRNA levels were generally elevated in a subset of human colon cancer cell lines [19]. We also reported that $pp60^{c\text{-}src}$ was regulated by various endogenous proteins such as methionine aminopeptidase 2 (MetAP2), NMT, and NMT inhibitor protein 71 (NIP71) in human colon cell line HT29 [20]. In addition, we demonstrated that NIP71 is homologous to heat shock cognate protein 70 (Hsc70) [21]. Recently, we reported a high expression of NMT in human brain tumors [22].

Protein myristoylation is a co-translational process that occurs after the removal of methionine. MetAP is the enzyme responsible for the removal of methionine from the NH_2-terminus of newly synthesized proteins [23]. Eukaryotic organisms express two different isoforms of this enzyme, MetAP1 and MetAP2 [24]. Previously, we had demonstrated the higher expression of MetAP2 in human colorectal adenocarcinoma [25] and colon cancer cell lines [20]. Furthermore, $pp60^{c\text{-}src}$ expression was correlated with the expression of MetAP2 and NMT in HT29 cells [20]. To our knowledge, no reports are available related to MetAP2 and any-type of neurological disorders.

We initiated our present study to examine the role of NMT in epileptic fowl and its involvement with different apoptotic factors in the hereditary form of primary generalized tonic–clonic seizures. The homozygous recessive fowl (epileptics) reproducibly undergo tonic–clonic seizures when subjected to intermittent photic stimulation (IPS) [26,27]. Heterozygous fowl (carriers), which do not develop any seizure activity and remain refractory to IPS, were used as age-matched controls. In this report, we examined the expression of two different isoforms of NMT and its inhibitor protein, Hsc70 in normal, carrier, and epileptic fowl. We observed a high expression of NMTs in the epileptic birds compared to normal while Hsc70 expression was decreased. In addition, we studied the protein–protein interaction of NMT and signaling molecules, p53, *m*-calpain, and caspase 3, in normal, carrier, and epileptic fowl, and observed that these signaling molecules interact with NMT. Furthermore, we studied the expression of MetAP2, which is involved in the translational process, and found increased expression of this enzyme in epileptics.

Materials and methods

Materials. Polyclonal antibody against human NMT was produced and purified as described [19]. Recombinant human NMT was purified as described by Raju et al. [28]. Anti-Hsc70 was obtained from Affinity BioReagents (Golden, CO). Anti-p53 was obtained from Santa Cruz Biotechnology, USA. Anti-*m*-calpain was obtained from Chemicon International, Temeculla, CA, USA. Radioisotope [^{3}H]myristic acid (39.3 Ci/mmol) was obtained from NEN Life Science Products. *Pseudomonas* acyl CoA synthetase and coenzyme A were obtained from Sigma–Aldrich, Canada. The peptide based on the NH_2-terminal sequence of the type II catalytic subunit of cAMP-dependent protein kinase (GNAAAAKKRR) was obtained from Research Genetics, Huntsville, USA. General laboratory chemicals were of analytical grade.

Subjects. All bird care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals described and approved by the University of Saskatchewan, Saskatchewan, Canada. Epileptic and carrier fowls were developed as described elsewhere [26,27]. Briefly, male adult fowl (~1 year old) and male chicks (1 day old) were bred from a mutant flock of domestic fowl. The fowl were maintained in individual cages on a 12 h light/dark cycle and fed ad libitum with standard chicken feed and water. The fowl were sacrificed by decapitation during seizure-free (or interictal) state and at 30 s after IPS-induced tonic–clonic seizure activity.

Preparation of brain tissue extracts. All procedures were carried out at 4 °C, unless otherwise stated. Forebrains (left and right hemispheres), cerebella, and optic lobes isolated from adult fowl were homogenized in 100 mM Tris–HCl, pH 7.4, containing 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml leupeptin. The crude homogenate was centrifuged for 30 min at 10,000*g* and the supernatant was filtered through glass wool. The clear supernatant was used for NMT activity, immunoprecipitation, and Western blot analysis.

NMT assay. The NMT activity was measured as previously described [29,30]. For the standard enzyme assays, the reaction mixture contained 0.4 μM [^{3}H]myristoyl-CoA, 40 mM Tris–HCl, pH 7.8, 0.5 mM EGTA, 0.1% Triton X-100, and 500 μM peptide substrate, and the clear supernatant was used as the NMT source in a total volume of 25 μl. The reaction was initiated by the addition of radiolabeled [^{3}H]myristoyl-CoA and incubated at 30 °C for 10–30 min. The reaction was terminated by spotting aliquots of incubation mixture onto P81 phosphocellulose paper disks and drying them under a stream of warm air. The P81 phosphocellulose paper disks were washed in three changes of 40 mM Tris–HCl, pH 7.3, for 90 min. The radioactivity was quantified in 7.5 ml of Beckman Ready Safe Liquid Scintillation mixture using a Beckman Liquid Scintillation Counter. One unit of NMT activity was expressed as 1 pmol myristoyl peptide formed per minute per milligram protein.

Immunohistochemical method. Immunohistology of the normal, carrier, and epileptic brain tissues was performed as described previously [31]. Tissues fixed in 10% formaldehyde and dehydrated in ascending solutions of ethanol and xylene were embedded in paraffin. Five sections of 5-μm thickness were prepared from tissue blocks and placed on slides coated with silane. The slides were kept at 55 °C for 45 min in an oven to improve adherence of sections. The sections were deparaffinized and rehydrated in xylene and descending concentrations of ethanol. Endogenous peroxidase was neutralized by a 30-min incubation in 0.5% hydrogen peroxide in methanol. The antigens were unmasked by treating the sections with 2 mg pepsin/ml 0.01 N HCl for 45 min. This incubation time was determined with a series of trials and was found to yield maximum staining.

The sections were then blocked with 1% bovine serum albumin in PBS for 30 min, followed by incubation with anti-NMT1 polyclonal antibody at a concentration of 0.58 mg/ml for 90 min and with HRP-