Ablation of Arg-tRNA-protein transferases results in defective neural tube development

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INTRODUCTION

The arginylation branch of the N-end rule pathway is a ubiquitin-mediated proteolytic system in which post-translational conjugation of Arg by ATE1-encoded Arg-tRNA-protein transferase to N-terminal Asp, Glu, or oxidized Cys residues generates essential degradation signals. Here, we characterized the ATE1−/− mice and identified the essential role of N-terminal arginylation in neural tube development. ATE1-null mice showed severe intracerebral hemorrhages and cystic space near the neural tubes. Expression of ATE1 was prominent in the developing brain and spinal cord, and this pattern overlapped with the migration path of neural stem cells. The ATE1−/− brain showed defective G-protein signaling. Finally, we observed reduced mitosis in ATE1−/− neuroepithelium and a significantly higher nitric oxide concentration in the ATE1−/− brain. Our results strongly suggest that the crucial role of ATE1 in neural tube development is directly related to proper turn-over of the RGS4 protein, which participate in the oxygen-sensing mechanism in the cells. [BMB Reports 2016; 49(8): 443-448]

Keywords: Arginylation, ATE1, G protein signaling, N-end rule, Neural tube

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studies, however, we frequently observed various gross defects of neurons as well, such as malformation of the brain and cerebral hemorrhages, but their origin and identity were elusive.

In the present study, we demonstrated that in ATE1-null murine embryos, neural-tube genesis is severely defective, and this problem may be the primary cause of embryonic mortality of the mutant mice. ATE1 expression was more prominent in the embryonic brain and spinal cord than in the heart: this pattern overlapped with the migratory path of neural crest cells. Moreover, the ATE1-null embryonic brain showed stabilized RGS proteins, defective G protein signaling, and a higher concentration of NO. Consistent with these biochemical observations, proliferation of ATE1+/− neuroepithelial cells in the developing primary neural tube was significantly impaired. Taken together, our results suggest that the crucial role of ATE1 in neural tube development may be related to the proper turnover of the RGS4 protein instead of secondary events of blood vessel malformation; the Arg/N-end rule pathway may function as an oxygen sensor and free-radical scavenger.

RESULTS

Midgestation death and neural abnormality of ATE1−/− mice

Genotyping of litter embryos at E14.5 retrieved from the ATE+/− intercross revealed no live homozygous mutants (Fig. 1A), indicating that the deletion of the ATE1 gene was lethal at midgestation. To examine the timing and pathological nature of the embryonic death of ATE1−/− mice, we dissected embryos at E14.5 from a heterozygous mutant intercross revealed no live homozygous mutants (Fig. 1A), indicating that the deletion of the ATE1 gene was lethal at midgestation. To examine the timing and pathological nature of the embryonic death of ATE1−/− mice, we dissected embryos from E11.5 to E14.5. Arrows indicate internal hemorrhages. (A) Gross morphological features of wild-type and ATE1−/− mouse embryos at E14.5 from a heterozygous mutant intercross. (B) Gross features of surviving ATE1−/− embryos from E11.5 to E14.5. Arrows indicate internal hemorrhages. (C) Close-up morphological analysis of wild-type (a, c) and ATE1−/− embryonic brains in transverse and sagittal views. (D) H&E staining of cross-sections of wild-type and ATE1−/− spinal cords (near the fourth ventricle) at E12.5 (a, b) and E13.5 (c, d). The ventral part is at the bottom. SC, spinal cord; CP, cartilage primordium; DRG, dorsal root ganglion. Arrowheads indicate cystic space. Kidney slices at E13.5 if, g) showed no noticeable difference between wildtype and ATE1−/− murine embryos.

Expression of LacZ from the targeted ATE1 allele in the central nervous system (CNS)

Expression of a LacZ reporter which is integrated in the ATE1 allele was prominent in the brain and neural tube judging by β-galactosidase activity (Fig. 2A), which was consistent with ATE1 mRNA expression from in situ hybridization analysis (16). We also examined expression of ATE1 in histological slices using the anti-RGS4 antibody (Fig. 2B). The RGS4 protein is extremely short-lived in the wild-type background but is stabilized in ATE1−/− animals. Therefore, ATE1−/− mice can serve as a rare experimental model for monitoring the RGS4 expression. At E11.5, when the neural folds still had not closed, the RGS4 protein was expressed at the boundary between the non-neural ectoderm and neural plates (Fig. 2B). As the embryo grew, the RGS4-positive cells migrated ventrally and cranially into the DRG, notochord, and sympathetic ganglia, which are also the destinations of migrating neural crest cells. This finding suggests that degradation of the RGS4 protein by ATE1 is closely associated with the migration or differentiation of neural crest cells during embryogenesis. In
Fig. 2. ATE1 is expressed in the CNS and migrates during neural tube development. (A) LacZ staining of whole embryos of wild-type (a, e), ATE1+/− (b, f), and ATE1−/− (c, g) mice at E13.5. Enlarged views of the brain (h) and heart (i) of ATE1−/− embryos. (B) Immunostaining for RGS4 on cross-sections of ATE1−/− embryonic tissues indicates that RGS4 expression was highly colocalized with the migration path of neural crest cells, which includes ventral migration to DRG and other migratory ends.

In addition, because neural crest cells migrate into the heart and vessels, it is possible that impaired migration of neural crest cells may lead to the cardiovascular defects in ATE1−/− embryos.

Stabilized RGS proteins in ATE1-null mice and reduced activities of downstream effectors

Previously, we identified a set of RGS proteins (RGS4, RGS5, and RGS16) as the primary in vivo substrates of murine ATE1 (12). Nonetheless, our understandings were mainly obtained by means of in vitro generated RGS proteins and transiently transfected RGS proteins in ATE1-null MEFs because of a lack of good antibodies. Using recently produced monoclonal antibodies (17, 18), we found that the levels of endogenous RGS4 dramatically increased in ATE1−/− whole embryos and embryonic brains (Fig. 3A and 3B). The RGS4 protein was virtually undetectable in wild-type and ATE1+/− littermate controls. The effect of ATE1 inactivation on RGS16 levels was not as striking as that on RGS4, but the RGS16 protein was also stabilized in ATE1−/− mice. We also found that the amount of RGS4 was greater in the brain than in the heart (data not shown). Quantitative RT-PCR analysis of total RNA showed no significant difference between the mutants and wild-type embryonic brains at E13.5; this finding indicates that downregulation of RGS proteins takes place at the posttranscriptional stage (Fig. 3C). These results point to a strong spatiotemporal correlation between protein arginylation and its substrate, RGS4, in the developing murine brain.

Considering the role of RGS proteins as negative regulators in G protein-mediated signaling as well as the prominent expression of ATE1 in the brain and spinal cord, we tested whether the G protein-coupled receptor (GPCR) signaling pathway in the ATE1-mutant brain is defective. To this end, we first examined the downstream kinase activity of the GPCR signaling pathway by an immunoblot assay using embryonic brains (Fig. 3D). Previously, we reported that the activity of ERK1 and ERK2—downstream effectors of Gq and Gi signaling pathways—is impaired in the whole embryo (19). In embryonic brain extracts, phosphorylated forms of ERK1/2 and MEK1 were significantly downregulated, while total kinase levels were comparable to wild-type levels. To further characterize the effects of ATE1 on MAPK components, we examined responsiveness of kinase activities to serum stimulation in primary culture of wild-type and ATE1−/− MEFs (Fig. 3E). Primary MEFs were harvested at multiple time points after 24 h serum starvation and subsequent 10% serum activation, and kinase activities were measured by immunoblotting using activated kinases-specific antibodies. The activities enhanced by serum stimulation were observed as early as 15 min later. Nevertheless, significantly decreased levels of active forms of RAF1, MEK1, and ERK1/2 were observed.
detected in ATE1−/− MEFs compared to wild-type MEFs (Fig. 3F), suggesting that the ATE1-RGS pathway tightly regulates the MEK1-ERK1/2 pathway. Taken together, these findings are consistent with another report showing that RGS proteins downregulate G protein signaling in primary cardiomyocytes (19) and suggest that Gs signaling may be reduced in ATE1-null cells because of stabilization of negative regulators of this signaling pathway (RGS proteins) in the brain.

Defective neuronal-cell proliferation in the ATE1-null embryonic brain

Based on the finding that MAPK signaling in the ATE1−/− brain was reduced and RGS-regulated Gs and Gq signaling pathways were defective, we hypothesized that the aforementioned neural defects of ATE1−/− embryos originate from decreased proliferation. In addition, although the formation and shaping of the neural plate of ATE1−/− embryos appeared to be normal, the closure of the neural groove to form the neural tube was slower than that of wild-types. For quantitative comparison, we examined the rates of neural-cell proliferation in the ATE1−/− neural tube by immunostaining for H3P, a mitosis marker, from E11.5 to E13.5 (Fig. 4A). In wild-type embryos, H3P-positive cells were usually observed at earlier stages (E11.5 and E12.5), but there were little mitotic cells at E13.5. ATE1−/− embryos showed a markedly reduced number of cells with relative H3P-positive staining in the neural tube (especially in the ventricular layers) in comparison with those in wild-type littermates (Fig. 4B). The mitotic index of wild-type neuroepithelial cells was 13.3%, while in wild-type littermates (Fig. 4B). The mitotic index of especially in the ventricular layers) in comparison with those of cells with relative H3P-positive staining in the neural tube decreased cellular proliferation in the

athermal N-end rule pathway. The Arg/N-end rule pathway may have a stabilizing (nonarginylated) residue (21). When we measured the concentration of NO metabolites, there were significantly larger amounts of nitrates and nitrates both in whole embryos and in embryonic brains of ATE1−/− mice. This result is consistent with our previous finding in MEFs that RGS proteins require a series of N-terminal modifications, including oxidation of N-terminally exposed Cys-2 (12). This is a consequence of competition among the post-translational modifications at the Cys-2 including acetylation and palmitoylation, and probably, the major determining step at the crossroads to the arginylation and acetylation branch of the N-end rule pathway. The Arg/N-end rule pathway may function as a licensing step to regulate GPCR signaling depending on the cellular oxygen concentration, which also varies among developmental stages. Although the underlying

Fig. 4. ATE1-null embryos show impaired proliferation of neuroepithelial cells. (A) Immunostaining for H3P on cross-sections of wild-type and ATE1−/− neural tubes at E11.5, E12.5, and E13.5 (counterstaining with DAPI). Red blood cells (RBC) showed nonspecific signals. (B) Merged images of neural tube areas from panels A. (C) Quantification of mitotic cells in the neuroepithelium. (D) Amounts of NO metabolites in wild-type and ATE1−/− whole embryos (top) and embryonic brains (bottom) at E13.5 were measured by the Griess assay. Values represent means ± SD (n = 3). *P < 0.05 (two-tailed t-test).
neural-tube defects in the cerebral ventricular system may account for the embryonic abnormalities. Our findings indicate that neuroepithelial cells and the ATE1-RGS circuit are implicated in regulating neuronal migration. Functioning of ATE1 is necessary for proper activation of GPCR signaling pathways, and the delayed closure of the neural groove of ATE1−/− mice appeared not due to the defected paracrine signal of the nearby neural crest cells. Neuronal cells undergo mitosis when they are located lumenally; this activity is a part of closure of hollow neural tubes. Therefore, the cellular-proliferation defects in ATE1−/− mice may lead to malformation of the ventricular zone accompanied with various CNS alterations. Because the neuronal stem cell migration of ATE1−/− embryos was normal, the delayed closure of the neural groove of ATE1−/− mice appeared not due to the defected paracrine signal of the nearby neural crest cells. Functioning of ATE1 is necessary for degradation of RGS proteins and for proper activation of GPCR signaling pathways probably in a cell-autonomous manner. Our findings indicate that neural tube closes during embryogenesis and this knowledge will elucidate not only the physiological function of ATE1 in mammals but the connection of GPCR signaling with migration of neural crest cells during embryonic development. A recent study on adult ATE1-null mice (created by the Cre-Lox method) showed, however, a strikingly broad range of phenotypes (22), indicating that the ATE1-RGS-G protein circuit may be involved in a greater variety of essential biological pathways than previously thought. A likely explanation would be that the reduced availability of O2 at the developing neural tube areas may be critical for stabilizing the RGS proteins, GPCR signaling, and proper proliferation of neuroepithelium under normal physiological conditions. Therefore, the oxygen-sensing mechanism by the Arg/N-end rule pathway is the additional layers of the regulatory steps of G protein signaling to maintain the proper development of the CNS.

DISCUSSION

In the N-end rule-dependent proteolytic system, the N-terminal Arg is post-translationally attached by ATE1 Arg-transferase and functions as a degradation determinant. We previously reported that ATE1−/− mice die because of various cardiovascular defects during embryogenesis (19) although the molecular etiology is still unknown. Our data in this study collectively indicate that ATE1 is also necessary for neurogenesis, and that the CNS phenotype observed in ATE1-null embryos may be due to a mitotic aberration of neuronal cells. Neuronal cells undergo mitosis when they are located lumenally; this activity is a part of closure of hollow neural tubes. Therefore, the cellular-proliferation defects in ATE1−/− mice may lead to malformation of the ventricular zone accompanied with various CNS alterations. Because the neuronal stem cell migration of ATE1−/− embryos was normal, the delayed closure of the neural groove of ATE1−/− mice appeared not due to the defected paracrine signal of the nearby neural crest cells. Functioning of ATE1 is necessary for degradation of RGS proteins and for proper activation of GPCR signaling pathways probably in a cell-autonomous manner. Our findings indicate that neural tube closes during embryogenesis and this knowledge will elucidate not only the physiological function of ATE1 in mammals but the connection of GPCR signaling with migration of neural crest cells during embryonic development. A recent study on adult ATE1-null mice (created by the Cre-Lox method) showed, however, a strikingly broad range of phenotypes (22), indicating that the ATE1-RGS-G protein circuit may be involved in a greater variety of essential biological pathways than previously thought. A likely explanation would be that the reduced availability of O2 at the developing neural tube areas may be critical for stabilizing the RGS proteins, GPCR signaling, and proper proliferation of neuroepithelium under normal physiological conditions. Therefore, the oxygen-sensing mechanism by the Arg/N-end rule pathway is the additional layers of the regulatory steps of G protein signaling to maintain the proper development of the CNS.

MATERIALS AND METHODS

Mice

Exons 1 through 3 of the ATE1 gene were disrupted in CJ7 embryonic stem cells as previously described (16). Embryos at E10.5 to E17.5 were obtained from intercrosses of heterozygous mice in the 129SvEv/C57BL/6 (mixed) genetic background. The presence of a vaginal plug after overnight mating was regarded as E0.5. Genotyping of the yolk sac or tail DNA of each embryo was performed using a standard polymerase chain reaction.

Histological analysis and β-galactosidase staining

For histological analysis, embryos were fixed overnight at 4°C in 4% paraformaldehyde (Fisher Scientific, Hampton, NH) in ice-cold phosphate-buffered saline (PBS). The specimens were incubated with 70% ethanol, dehydrated, embedded in paraffin wax, and sectioned transversely into slices 7 μm thick, followed by staining with hematoxylin and eosin (H&E). To detect the activity of β-galactosidase on the slices, we stained the fixed samples overnight at 37°C in an X-gal solution (1.3 mg/ml potassium ferrocyanide, 1 mg/ml potassium ferricyanide, 0.3% Triton X-100, 1 mM [mmol/l] MgCl2, 150 mM NaCl, and 1 mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside [X-gal, Roche Applied Science, Indianapolis, IN] in PBS [pH 7.4]).

In vivo proliferation assay

The neural-tube slices were incubated with an anti-phospho-histone H3 (H3P) antibody (Upstate, Charlottesville, VA; 1:100 dilution), followed by a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Jackson Immunotech, West Grove, PA). All the antibodies were incubated with the slices for 1 h at room temperature in 1% goat serum in PBS (with 10% Tween 20), and the secondary antibody was diluted 1 to 200 (23). The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) to visualize the nuclei.

Immunotechniques

For immunoblotting analysis, whole embryos or embryonic brains at E13.5 or E14.5 were isolated in lysis buffer (20 mM HEPES [pH 7.5], 150 mM KCl, 10% glycerol, and 0.1 mM EDTA). A soluble fraction was separated by two-step centrifugation (9,000 × g and 100,000 × g), subjected to electrophoresis, blotted to a polyvinylidene fluoride membrane, and probed with an anti-RGS4 or anti-RGS16 antibody (17, 18), followed by reprobing with an anti-actin antibody (Sigma–Aldrich).

Examination of MAPK pathway effectors

To analyze activities of the MAPK pathway, we subjected total extracts from wild-type and ATE1−/− brains and primary mouse embryonic fibroblasts (MEFs) to immunoblotting with antibodies against ERK1/2, MEK1/2, RAF1, their phosphorylated forms (Cell Signaling Technology, Beverly, MA), Gaq (Santa Cruz Biotechnology, Dallas, TX), and actin (Sigma–Aldrich). Quantification was performed using the ImageJ software (version 1.46r, National Institutes of Health, Bethesda, MD).
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Quantification of NO metabolites
For measurement of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) levels in whole embryos and embryonic brains, tissue samples from wild-type and ATE1$^{-/-}$ mice were homogenized in a buffer consisting of 25 mM Tris-HCl (pH 7.5), 0.5 μM EDTA, and 0.5 μM EGTA and were centrifuged at 14,000 $\times$ g for 15 min. Nitrite was assayed using the Griess reaction (24). Nitrate content was determined after conversion of nitrate to nitrite by means of Aspergillus nitrate reductase (Sigma-Aldrich).

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