AMP N_1 -oxide potentiates astrogenesis by cultured neural stem/progenitor cells through STAT3 activation

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(Received 26 July 2007; and accepted 23 September 2007)

ABSTRACT

We earlier identified adenosine monophosphate (AMP) N_1 -oxide as a unique compound of royal jelly (RJ) that induces neurite outgrowth from cultured rat pheochromocytoma PC12 cells. In the present study, the effects of AMP N_1 -oxide on the proliferation and/or differentiation of cultured neural stem/progenitor cells (NSCs) were examined. As for cell proliferation, low micromolar concentrations of AMP N_1 -oxide or its parent compound, AMP, similarly enhanced the NSC proliferation-inducing activity of basic fibroblast growth factor (FGF-2), although neither compound tested alone affected cell proliferation. Conversely, high concentrations of AMP N_1 -oxide (over 20 μ M) markedly suppressed cell growth even in the presence of FGF-2. However, this suppression was not observed with AMP. As for cell differentiation, AMP N_1 -oxide, but not AMP, increased the generation of astrocytes in a dose-dependent manner when the cells were cultured in medium lacking FGF-2. The generation of neurons or oligodendrocytes was not influenced by AMP N_1 -oxide. Furthermore, AMP N_1 -oxide increased the phosphorylation of STAT3 (signal transducer and activator of transcription 3), a transcription factor that mediates the expression of astrocyte-specific genes. These results suggest that AMP N_1 -oxide is one of the components that facilitates astrogenesis by NSCs through activation of STAT3.

Royal jelly (RJ) contains a variety of molecules biologically active towards various types of cells (1, 16, 18, 19). However, there are few reports so far demonstrating the effects of RJ on the nervous system. Earlier we reported that an extract of RJ induces neurite outgrowth from cultured PC12 cells, a cell line of rat pheochromocytoma, via adenosine A_{2A} receptors and identified adenosine monophosphate (AMP) N_1 -oxide as the first active component in royal jelly that affects the nervous system (7). AMP N_1 -oxide is a unique compound not found in

natural products other than RJ; and it suppresses the proliferation of PC12 cells and stimulates the expression of neurofilament M, a specific protein of mature neurons in them, thus demonstrating that AMP N_1 -oxide can induce neuronal differentiation of these cells (7). A recent investigation has added the new idea that the AMP N_1 -oxide-induced neurite outgrowth requires integrin signaling (5). These findings prompted us to examine the effects of AMP N_1 -oxide on the central nervous system (CNS), particularly on neural stem cells/progenitor cells (NSCs).

Precise control of proliferation and differentiation of multipotent NSCs is crucial for proper development of the nervous system, because NSCs have self-renewal capacity and multipotent activity to differentiate into neurons, astrocytes, and oligodendrocytes during development (3, 17). Besides being present in the developing embryonic brain, NSCs

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also reside in the adult forebrain (3, 15). These cells give rise to constitutively proliferating progenitor cells (12); and they also can differentiate into neurons (10, 11), suggesting that the injured brain has a capacity for self-repair by activated NSCs. Therefore, NSCs in the mature brain are promising tool for therapy of degenerative neurological disorders including Alzheimer's and/or Parkinson's disease.

In the present study, we examined the influences of AMP N_1 -oxide on the proliferation of NSCs and the generation of neurons or glia from them in comparison with those of AMP.

MATERIALS AND METHODS

AMP and AMP N_1 -oxide. Authentic AMP N_1 -oxide was prepared according to the procedure previously reported (7), and AMP was purchased from Sigma.

Primary cultures. NSCs were obtained from the telencephalon of 15.5-day-old rat embryos, and cultured in a proliferation medium consisting of equal volumes of Dulbecco's modified Eagle's minimum essential medium and F12 medium supplemented with insulin (25 μ g/mL), apo-transferrin (100 μ g/mL), progesterone (20 nM), putrescine (100 μ M), selenium (30 nM), and basic fibroblast growth factor (FGF-2, 10 ng/mL) as described previously (9). Cultures were fed the proliferation medium every day, and passaged 5 days after inoculation. For the proliferation experiments, NSCs were cultured in the proliferation medium, and the additive or synergistic effects of AMP and AMP N_1 -oxide on the total number of cells were analyzed. For the differentiation experiments, NSCs were plated on coverslips coated with poly-L-ornithine, and cultured in the medium lacking FGF-2 (differentiation medium). For evaluation of cell viability, NSCs were treated for 10 min with Hoechst 33342 (100 μ g/mL) that had been mixed with YOPro-I (4 μ M) and propidium iodide (PI; 20 μ g/mL). All 3 dyes were obtained from Molecular Probes.

Immunocytochemical procedures. For fluorescence immunostaining, cells were fixed for 3 min by adding an equal volume of 4% (w/v) paraformaldehyde solution prepared in 0.1 M phosphate buffer (pH 7.4) to the culture medium, and the cells were post-fixed for 10 min with the same fixative solution. After 3 washings with PBS, the cells were treated for 30 min with PBS containing 2% (w/v) skim milk to reduce non-specific antibody binding. They were then reacted with primary antibody at 4°C over-

night. The primary antibodies included mouse anti-neuron-specific class III β -tubulin (Tuj1) antibody (R & D Systems, Minneapolis, MN, USA), rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Copenhagen, Denmark) to detect astrocytes, and mouse anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibody (Sigma, St. Louis, MO, USA) to identify oligodendrocytes. After 3 washings for 5 min with PBS, the cells were incubated with Alexa Fluor 488-conjugated donkey anti-mouse or anti-rabbit IgG antibodies (Chemicon, Temecula, CA, USA) for 2 h at room temperature, washed with PBS, and mounted in Permafluor (Thermo Fisher, Waltham, MA, USA).

Western blotting. NSCs were lysed with a lysis buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 0.1 % sodium dodecyl sulfate (SDS), and 1% Na deoxycholate). The lysates were centrifuged, and the protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Each sample, containing 5 μ g of protein, was electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels. Proteins were transferred to a polyvinylidene fluoride membrane, and nonspecific binding sites were blocked for 2 h at room temperature with 5% skim milk (Morinaga Milk Products, Tokyo, Japan) in 20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 0.05% Tween-20 (TTBS). Next the membranes were incubated with primary antibody against signal transducer and activator of transcription 3 (STAT3) or phospho STAT3 (Sigma), and then with alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA) in TTBS containing 5% skim milk. Finally, the specific protein bands were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indorylphosphate *p*-toluidine salt.

RESULTS AND DISCUSSION

Effects on cell proliferation

NSCs proliferate vigorously as neurospheres in proliferation medium containing FGF-2, but start to differentiate into neurons, astrocytes or oligodendrocytes in FGF-2-free medium (3, 17). The cells were exposed for 7 days to various concentrations of AMP N_1 -oxide or AMP added to the proliferation medium, and the cell number was then counted (Fig. 1). Although AMP at all concentrations tested

tended to elevate the cell number, the increment was significant only at 4.0 μ M. AMP N_1 -oxide significantly increased the cell number at 0.8 μ M, but markedly decreased it at 20 μ M. These results demonstrate that AMP and AMP N_1 -oxide similarly increased the cell number at 0.8–4.0 μ M but that only AMP N_1 -oxide reduced it at 20 μ M. This decrease in cell number could have been caused by reduced proliferation or enhanced cell death. For evaluation of cell viability, NSCs were incubated for 10 min with Hoechst 33342 (100 μ g/mL) that had been mixed with YOPro-I (4 μ M) and propidium iodide (PI; 20 μ g/mL). The intact cell membrane is permeable to Hoechst 33342, which gives the cells a blue fluorescence. However, intact cells are impermeable to YOPro-I and propidium iodide; but these dyes can permeate damaged cells, resulting in green and red fluorescence, respectively. Therefore, the ratio of the cell number of green- or red-dyed cells/the cell number of blue-dyed cells was calculated to evaluate cell viability during experiments. There were no differences in the ratios between AMP- and AMP N_1 -oxide-treated cells or between the agent- and vehicle-treated cells (data not shown), suggesting that suppression of cell growth, and not enhanced cell death, was responsible for the reduced cell number. Our previous observation that AMP N_1 -oxide inhibits cell proliferation of cultured PC12 cells prior to

neuronal differentiation (7) may support this idea. Therefore, it is likely that both AMP and AMP N_1 -oxide stimulate cell proliferation of NSCs via adenosine A_{2A} receptor signaling; however, AMP N_1 -oxide might suppress cell proliferation via some unknown or unidentified mechanism. AMP N_1 -oxide-induced suppression of cell growth might be advantageous for cell differentiation by NSCs as well as for neurite-outgrowth from PC12 cells, in which latter case AMP N_1 -oxide was more potent than AMP (7).

Effects on cell differentiation

Primary NSCs maintained in the proliferation medium were transferred to the differentiation medium (FGF-2-free) containing or lacking AMP N_1 -oxide or AMP, and cultured for 5 days. For these experiments, AMP N_1 -oxide at 0.8 or 4.0 μ M was used, because AMP N_1 -oxide at 20 μ M or over severely suppressed cell growth (Fig. 1). AMP N_1 -oxide significantly increased the percentage of GFAP-positive cells (astrocytes) among total cells in a dose-dependent manner, but did not cause any change in that of Tuj1-positive cells (neurons) or CNPase-positive cells (oligodendrocytes, Fig. 2). However, AMP induced no changes in the expression of any of these differentiation markers. These results demonstrate that AMP N_1 -oxide, but not AMP, facilitated specifically the generation of astrocytes from NSCs, and suggest that AMP N_1 -oxide is one of the components of RJ that influence cell lineages. We are now testing RJ components other than AMP N_1 -oxide to learn whether they could also affect the generation of differentiated cells from NSCs. An unsaturated fatty acid, 10-hydroxy-trans-2-decenoic acid, which is a unique and characteristic component of RJ, showed neurogenesis-stimulating activity toward NSCs (6), supporting this idea.

Fig. 1 Influence of AMP and AMP N_1 -oxide on FGF-2-induced cell proliferation. NSCs growing as neurospheres were plated on poly-L-ornithine-coated 6-well plates, and cultured for 7 days in proliferation medium containing various concentrations of AMP or AMP N_1 -oxide with a change of fresh medium every other day. Cells were detached from the culture vessels by treatment with 0.1% trypsin, and the total number of cells was counted with a hemocytometer. The values are expressed as the mean $\pm$ SE ($n = 4$). Significance of differences from the value of the cells cultured with neither AMP nor AMP N_1 -oxide was determined by one-way ANOVA with Tukey's test. Significance, ** $p < 0.01$

Intracellular signaling of AMP N_1 -oxide

It is known that transcription factors STAT3 and Smad1, which are activated by leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2), respectively, form a complex with the co-activator p300 and that this complex synergistically generates astrocytes from NSCs (4, 14). Furthermore, a recent report indicated that LIF induces the expression of BMP2 via STAT3 activation, which expression leads to the consequent activation of Smad1 to promote generation of astrocytes from NSCs (2), suggesting that activation of STAT3 is a crucial step for astrogenesis by NSCs. Therefore, we estimated the phosphorylation status of STAT3 in

Fig. 2 Effects of AMP and AMP N_1 -oxide on the expression of differentiation markers of neurons, astrocytes, and oligodendrocytes. Dissociated cell suspensions of NSCs were plated on coverslips coated with poly-L-ornithine. The cells were maintained for 2 days in the proliferation medium, and then transferred to the differentiation medium containing various concentrations of AMP or AMP N_1 -oxide and cultured for another 5 days. Thereafter the cells were fixed and then reacted with antibody against Tuj1 (Aa, Ba), GFAP (Ab, Bb) or CNPase (Ac, Bc) and subsequently incubated with the appropriate Alexa Fluor 488-conjugated secondary antibody. **A:** Photographs of cells expressing the differentiation marker of neurons (a), astrocytes (b) or oligodendrocytes (c) after treatment with 4.0 μ M AMP N_1 -oxide. Scale bar indicates 50 μ m. **B:** Ratio of the cells expressing each cell marker. The number of antigen-positive cells was counted, and the ratio of positive cells to total cells was calculated. The values are expressed as the mean $\pm$ SE ($n = 4$). Significance of differences from the value of non-treated cells (control) was determined by one-way ANOVA with Tukey's test for each antigen. Significance, * $p < 0.05$, ** $p < 0.01$.

AMP- or AMP N_1 -oxide-treated NSCs. As shown in Fig. 3, AMP N_1 -oxide, but not AMP, conspicuously

Fig. 3 Phosphorylation of transcription factor STAT3 by AMP N_1 -oxide. NSCs growing as neurospheres were plated on poly-L-ornithine-coated 6-well plates, and cultured for 2 days in the proliferation medium (FGF-2-containing medium). Next, the cells were exposed to 0.8 or 4.0 μ M AMP or AMP N_1 -oxide in the differentiation medium for 4 days, and then harvested. Cell protein (5 μ g each) was subjected to 7.5% SDS-PAGE, and both STAT3 and phosphorylated STAT3 were detected by Western blotting using specific antibodies. The immunoreactivity was visualized, and the intensity was densitometrically calculated. **A:** Photographs of typical Western immunoblot patterns of STAT3 protein and phosphorylated STAT3. **B:** Ratio of the intensity of phosphorylated STAT3 to that of STAT3 protein is expressed as the fold-increase of the value over that of the corresponding non-treated cells (control). The values are expressed as the mean $\pm$ SE ($n = 4$). Significant differences from the value of the non-treated cells (control) were determined by one-way ANOVA with Tukey's test. ** $p < 0.01$.

induced phosphorylation of STAT3, demonstrating that the induction of astrogenesis by AMP N_1 -oxide was correlated with the activation of STAT3.

Cytokines/neurotrophic factors sharing gp130 as a signal transducer, such as LIF and ciliary neurotrophic factor (CNTF), can activate STAT3 (8, 13). gp130 and ligand-specific chains possess no intrinsic tyrosine kinase domain but are associated with members of the Jak family of cytoplasmic tyrosine kinases. The Jak kinases are activated after ligand-induced homo- or heterodimerization of gp130. This activation appears to link the cell-surface receptors to the nuclear genes through tyrosine phosphoryla-

tion and activation of transcription factor STAT3 (14). Thus, STAT3 is a key molecule for astrogenesis by NSCs that mediates the expression of astrocyte-specific genes. Although the mechanism of STAT3 activation by AMP N_1 -oxide is unknown at present, the following 3 possibilities may be considered: AMP N_1 -oxide may 1) induce the synthesis of LIF and/or CNTF, 2) cause the dimerization of gp130, or 3) activate Jak kinases. Furthermore, it is reported that astrogenesis by NSCs increases with a decrease in cytosine methylation of the STAT3 promoter region during aging (14), providing another possibility, *i.e.*, DNA demethylation facilitated by AMP N_1 -oxide.

Our present results suggest that AMP N_1 -oxide may be a good tool for protection against and therapy for certain brain injuries, because astrocytes play important roles in brain development and the response to injury (15). Further evaluation of the physiological importance of AMP N_1 -oxide-induced astrogenesis in the brain is thus warranted.

Acknowledgements

This work was supported by a grant from the Japan Royal Jelly Fair Trade Council.

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