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P2 receptor stimulation induces amyloid precursor protein production and secretion in rat cortical astrocytes

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ABSTRACT

Amyloid precursor protein (APP) is ubiquitously expressed in a variety of tissues but is predominantly expressed in the brain. The expression of APP has been well studied in neurons but little is known about its presence in astrocytes. The study presented here shows that purinergic signaling is involved in the production and secretion of APP in primary cultures of rat cortical astrocytes. Extracellular ATP caused an increase in APP production and release in a time- and concentration-dependent manner and was inhibited by antagonists of P2 receptors. Further agonist and antagonist studies revealed involvement of P2Y2 and P2Y4 receptors in nucleotide-stimulated production and release of APP. In addition, signaling studies with various protein kinase inhibitors demonstrated that blockade of mitogen-activated protein kinases, but not Akt, inhibited nucleotide-stimulated APP expression and release. These results indicate that APP production and secretion can be regulated by activation of P2Y2/4 receptors coupled to protein kinase signaling pathways and suggest that astrocytes can be a potential source of APP.

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Amyloid precursor protein (APP) is a large transmembrane protein consisting of a long N-terminal extracellular domain and a small intracellular cytoplasmic domain. It is present in a variety of tissues but is predominately expressed in the brain [24]. Local accumulation of APP can create a possible source for overproduction of beta-amyloid peptides, which are generated by the proteolytic processing of APP and are the primary component of amyloid plaques found in the brains of Alzheimer's disease (AD) patients. Studies have shown the presence of APP-positive astrocytes in AD or injured brains [19,22]. In addition, recent studies have highlighted the fact that astrocytes can be an important source for APP [9,30]. APP regulation is controlled by multiple stimulatory signals, including thrombin, interleukin-1beta, and nerve growth factor [8,13,20], however little is known about factors that induce expression of APP from rat cortical astrocytes.

Nucleotides are released by various cell types [11] under both physiological and pathological conditions. They can exert both short-term effects such as neurotransmission and long-term effects such as proliferation, differentiation, and apoptosis on many cell types, including astrocytes [5]. Extracellular ATP stimulates P2-type purinergic receptors, which are classified into ligand-gated ion channels (P2X1–7) or metabotropic heptahelical G protein-coupled receptors (P2Y1,2,4,6,11–14) [4]. Astrocytes express both P2Y and P2X receptors [21], and these receptors are linked to protein kinase signaling cascades, including protein kinase B/Akt and the mitogen-activated protein kinases (MAPKs) [26], that mediate gene expression [3,7]. The results presented here show that extracellular nucleotides, through activation of specific P2Y receptors, regulate APP expression and release in rat cortical astrocytes and is selectively mediated by several MAPK signaling pathways.

Primary rat cortical astrocyte cultures were obtained from neonatal rat (Fischer) cerebral cortices as previously described [27]. Cells were seeded onto uncoated 35 mm tissue culture plates (Falcon) and were not replated before use. Confluent 3-week-old cultures maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech) containing 10% horse serum (Gibco) were made quiescent by incubation in DMEM containing 0.5% horse serum for 48 h before treatment. Stock solutions of nucleotides and analogs (Sigma) as well as purinergic receptor antagonists (Sigma, Tocris) and signal transduction inhibitors (Tocris) were divided into single-use aliquots and stored at −80 °C.

After treatment, media were collected and concentrated using Amicon Ultra-4 centrifugal filters (Millipore). Cells were rinsed in ice-cold phosphate buffered saline (Sigma), lysed in Laemmli sample buffer, and protein concentrations were determined by the modified Lowry protein procedure [23]. Samples containing equal

Abbreviations: 2MeSADP, 2-methylthio-ADP; 8PSPT, 8-(*p*-sulfophenyl)-theophylline; AD, Alzheimer's disease; APP, amyloid precursor protein; BzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; DAPI, 4′-6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NECA, *N*-ethylcarboxamidoadenosine; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; PPADS, pyridoxalphosphate-6-azophenyl-2′-4′-disulfonice acid; RB2, reactive blue 2; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SB, SB202190; SP, SP600125.

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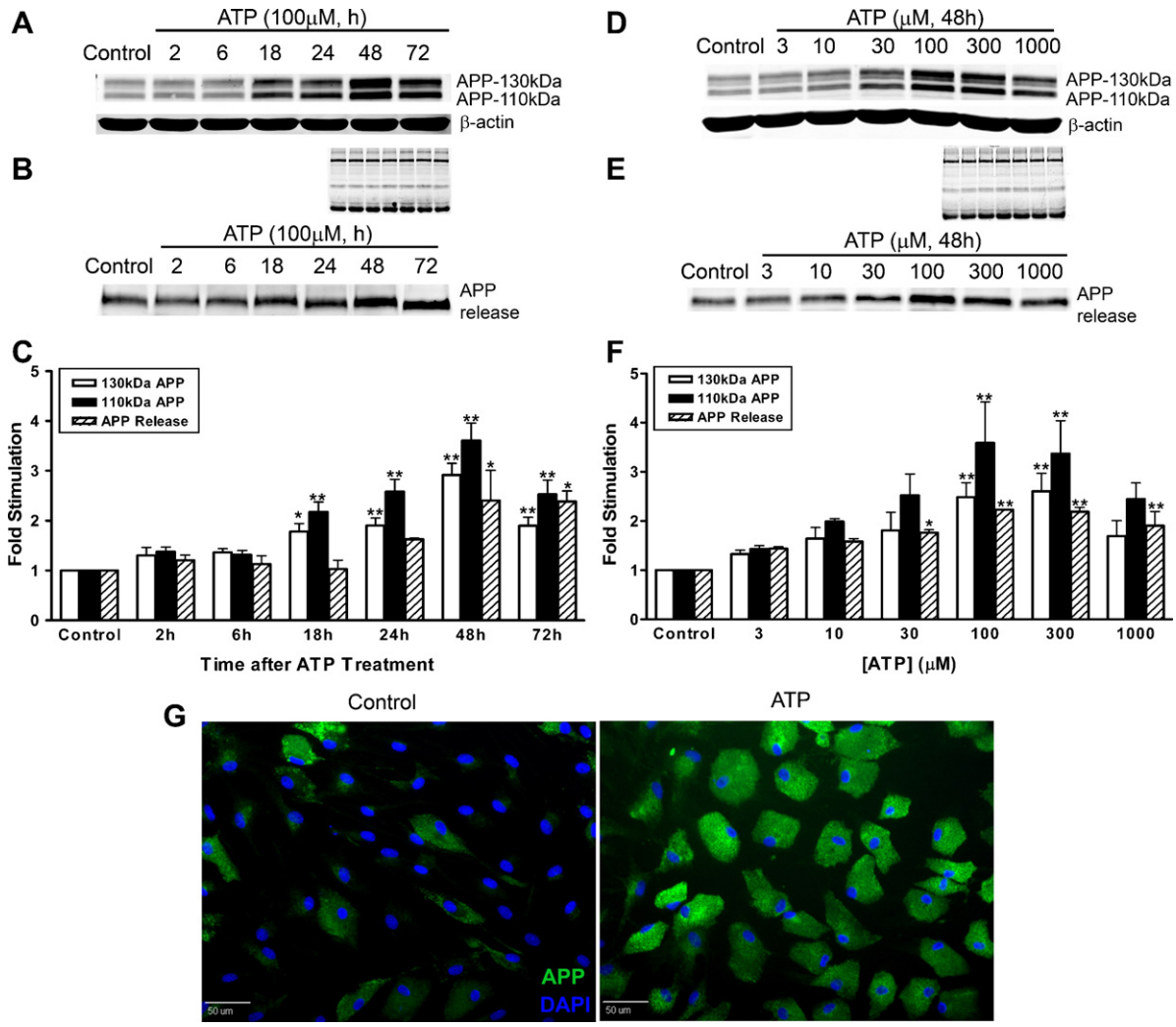

Fig. 1. APP production and secretion is stimulated by extracellular ATP. Immunoblotting and quantitation of data in the bar graph show that extracellular ATP caused an increase in APP production and secretion in a time- (A–C) and dose-dependent (D–F) manner when compared to vehicle-treated controls ($^{*}p < 0.05$, $^{**}p < 0.01$, $n = 7$). The slight downward trend of APP at 1 mM ATP was not significantly different from APP levels at 100 μM and 300 μM ATP ($p > 0.05$). Data are represented as mean ± SEM. β-actin is a loading control. Inset is a Coomassie blue staining showing equal loading of media. (G) Immunocytochemistry of cultured rat astrocytes. ATP-treated cells (100 μM, 48 h) exhibited strong APP expression compared with vehicle-treated control. Green, APP; blue, DAPI. (Scale bar: 50 μm).

amount of protein were subjected to SDS–PAGE (7.5% acrylamide gel) and mouse anti-APP (1:1000, Millipore) and mouse anti-β-actin (1:100,000, Sigma) were used for immunoblotting, which was conducted as previously described [27]. For quantification, ratios of APP to β-actin were calculated, and data are expressed as fold stimulation (mean ± SEM) compared either to controls or nucleotide-treated cells as stated in the figure legends.

Immunocytochemistry was performed 48 h after ATP treatment as previously described [27] with rabbit anti-APP (1:100, Cell Signaling) or anti-GFAP (1:400, DAKO). Cells were viewed with an Olympus motorized reflected fluorescence System IX81 and Slidebook software (Intelligent Imaging Innovations). Gain and exposure levels were set for control cultures and kept constant for all other cultures within an experiment.

The number of experimental replications is given in the figure legends; experiments were conducted with cultures from different seedings. Data were analyzed with the Student *t* test for two groups or repeated-measures analysis of variance for multiple groups, followed by post hoc comparisons (Dunnett's test) using the Instat software package (GraphPad).

To determine ATP effects on APP expression, time-course experiments were first conducted. Astrocyte were treated with 100 μM extracellular ATP, and after various time points media were collected and cells were lysed and APP levels were determined by immunoblotting. The 22C11 clone of the anti-mouse APP detects three isoforms of APP: 130 kDa, 110 kDa and 120 kDa secreted APP. The term "APP expression" refers to both the 130 kDa and 110 kDa isoforms. Extracellular ATP treatment significantly increased expression of 130 kDa and 110 kDa APP isoforms from 18 h to 72 h, with the highest increase observed at 48 h (Fig. 1A and C). In addition, APP release was significantly increased at 48 h and 72 h after extracellular ATP treatment (Fig. 1B–C). Immunoblot analysis also showed that expression of both APP isoforms and release depended on the concentration of extracellular ATP; treating cultures with 100 μM or 300 μM extracellular ATP for 48 h resulted in a significant increase in APP level (Fig. 1D–F). Immunocytochemical experiments confirmed the effect of extracellular ATP on APP expression. Treating astrocytes with 100 μM extracellular ATP for 48 h led to a robust increase in APP immunoreactivity when compared to vehicle-treated cultures (Fig. 1G). Additional staining was performed to see if changes in GFAP expression were observed with extracellular ATP treatment. After treatment with ATP (100 μM, 48 h) an increase in GFAP immunoreactivity was observed