ATP-induced \textit{in vivo} neurotoxicity in the rat striatum via P2 receptors

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The present study examined the \textit{in vivo} effects of ATP on the striatum of Sprague–Dawley rats. Intrastriatal administration of ATP produced dose-dependent striatal lesions as confirmed by cresyl violet staining. Additional immunostaining using neuronal nuclear protein (NeuN), OX-42 and GFAP antibodies revealed that ATP caused death of both neurons and glial cells. The nonmetabolizable ATP analogue ATP\textsubscript{g}S and P2X receptor agonist \(\alpha,\beta\)-methylene ATP (\(\alpha,\beta\)-MeATP) mimicked ATP effects, whereas either P2Y receptor agonist ADP or P1 receptor agonist adenosine did not. The P2 receptor antagonist reactive blue 2, but not pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid (PPADS) attenuated ATP-induced striatal injury. These results suggest that intrastriatal administration of ATP causes P2X receptor-mediated cell death in the striatum and support the hypothesis that extracellular ATP can be an important mediator of neuropathological events of brain injuries. 


**Key words:** ATP; \textit{in vivo}; P2 receptor; Striatum

**INTRODUCTION**

ATP is a neurotransmitter and/or neuromodulator in the CNS [1,2]. Extracellular ATP induces various biological responses such as excitability of spinal cord neurons [3], proliferation of astrocytes [4], inhibition of starvation-induced apoptosis in PC 12 cells [5], and modulation of neurotransmission [6]. These diverse biological actions are mediated by the activation of cell surface receptors including ligand-gated ion channels (P2X) and G-protein coupled (P2Y) receptor subtypes [7].

The cytoplasmic concentration of ATP is \(\sim 10\text{mmol/l} [8]\). Release of ATP into extracellular space has been detected following high-frequency neuronal stimulation [9], ischemia [10,11] and mechanical forces [12]. Several lines of evidence suggest that released ATP can act as an excitotoxin in certain pathological conditions, including ischemia, hypoxia, and tissue injury [1,10,13]. Ubiquitous ecto-ATPases, known as ATP hydrolyzing enzymes, regulate extracellular ATP concentration in normal condition, whereas inflammatory reactions or oxidative stress inhibits ecto-ATPases activity, resulting in the accumulation of extracellular ATP [14,15]. These results could underscore the potential neurotoxic effect of extracellular ATP in pathophysiological processes. To address this possibility \textit{in vivo}, we injected a high concentration of ATP into rat striatum because purinergic receptors are largely expressed in the striatum [7]. In the present study, we examined whether ATP could induce striatal cell death \textit{in vivo}, including neurons and glial cells, and if so, which subtypes of purinergic receptors were associated with ATP-induced toxicity by injecting a variety of purinergic receptor agonists (ATP, ATP\textsubscript{g}S for P2 receptors, ADP for P2Y receptors, \(\alpha,\beta\)-methylene ATP for P2X receptors and adenosine for P1 receptors) or antagonists (P2 receptor antagonists reactive blue 2 and pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid (PPADS)). The present data indicate that intrastriatal injection of ATP causes P2X receptor-mediated cell loss in striatum, including neurons and glial cells.

**MATERIALS AND METHODS**

**Animals:** Female Sprague–Dawley rats weighing 230–250 g were housed in groups of three in a temperature- and humidity-controlled room that was kept on an alternating 12:12 h light:dark schedule. Food and water were available \textit{ad lib} throughout the experiment. All animal experiments were conducted in accordance with the guideline set by Committee on Animal Research at the Ajou University.

**Intrastriatal microinjection:** The animals were deeply anesthetized with sodium pentobarbital (30 mg/kg; i.p.) and placed in a Kopf stereotaxic apparatus. The animals received a single stereotaxic administration of nucleotides (ATP, 0.1–2 \(\mu\)mol; \(\alpha,\beta\)-MeATP, ATP\textsubscript{g}S, ADP, 0.4 \(\mu\)mol; Sigma, St Louis, MO) and adenosine (0.4 \(\mu\)mol; Sigma) in 2 \(\mu\)l of 0.1 M phosphate-buffered saline (PBS, pH 7.4) into the right striatum (AP 0.7 mm, ML $-2.8$ mm, DV $-5.0$ mm from bregma according to the atlas of Paxinos and Watson [16]).
Some animals received a single stereotaxic coadministration of P2 receptor antagonists (reactive blue 2 and PPADS, 5, 10, 25, 50 nmol; Tocris), with 0.4 μmol ATP dissolved in 2 μl PBS into the same site of the right striatum. All injections were performed using a 26-gauge Hamilton syringe attached to an automated microinjector at a rate of 0.2 μl/min. At the completion of each injection, the needle was left in place for 5 min and then withdrawn. Wounds were sutured, and animals were allowed to recover and then returned to their cages. With an overdose of sodium pentobarbital the animals were sacrificed for further analysis at the designated time periods after injection.

**Histology and immunohistochemistry:** After a post-injection period of 4, 8 or 24 h or 7 days, the animals were perfused transcardially with a saline containing 0.5% sodium nitrite and heparin (10 U/ml) followed by a cold 4% paraformaldehyde in PB. Brains were removed from the skull and post-fixed overnight in the same fixative and then transferred into 30% sucrose solution in 0.1 M PB at 4°C until they sank. Serial coronal sections of striatum were cut at 40 μm and collected as free-floating sections in 0.1 M PB. Some sections mounted on gelatin-coated slides were stained for Nissl substance using cresyl violet and examined under a bright-field microscope. The extent of histological lesion areas of striatum were measured using a cool CCD image analysis system and were analyzed by comparing the averaged regional cross-sectional area of the lesion in the nine cresyl violet-stained sections anterior and posterior to the center of the injection site [17]. Immunohistochemistry was performed on free-floating sections prepared from the brains of ATP injected rats as described previously [18]. Briefly, free-floating sections were quenched with 3% hydrogen peroxide in PBS for 10 min. The sections were incubated in PBS containing 3% normal goat serum and 0.3% Triton X-100 (Sigma) for 30 min at room temperature and then incubated overnight with following primary antibodies: anti-neuron-specific nuclear protein (NeuN; monoclonal; 1:500; Chemicon, Temecula, CA) for neurons, mouse anti-glial fibrillary acidic protein (GFAP; monoclonal; 1:500; Sigma) for astrocytes, and mouse anti-CD11b surface antigen (OX-42; monoclonal; 1:500; Serotec, Oxford, UK) for microglia. Sections were rinsed three times with PBS, incubated for 1 h with biotinylated secondary antibodies (1:200; Vector Laboratories; Burlingame, CA), followed by avidin–biotin–peroxidase complex (ABC; 1:200; Vector Laboratories) for 1 h and visualized with 3,3'-diaminobenzidine (DAB; Sigma) and hydrogen peroxide. Sections were washed in 0.1 M PB, mounted on
gelatin-coated slides with Permount and analyzed under bright-field microscopy.

**Statistical analysis:** All data are given as means ± s.e.m. The statistical significance of differences was assessed using ANOVA, followed by Student–Newman–Keuls’ analyses or Student’s two-tailed t-test (SPSS for windows: Standard Version). Statistical significance was defined as $p < 0.05$ for all analyses.

**RESULTS**

Histological examination of cresyl violet-stained sections demonstrated *in vivo* that at 24 h after unilateral injection of ATP into the striatum, ATP produced cell death in the striatum ipsilateral to the injection side (Fig. 1a). Highly magnified photographs showed healthy and large cells in the striatum contralateral to the injection side (Fig. 1b), whereas a number of morphologically distinct dying, shrunken cells were seen in the striatum ipsilateral to the injection side (Fig. 1c). Additional immunostaining using NeuN antibody for neurons, OX-42 antibody for microglia, and GFAP antibody for astrocytes revealed that the types of damaged cells consisted of neurons, microglia, and astrocytes (Fig. 1d–i). Treatment with ATP yielded dose-dependent increases in lesion areas. Treatment with 0.1 or 0.2 $\mu$mol ATP did not cause striatal damage (Fig. 2a). In marked contrast, however, 24 h after intrastriatal treatment with 0.4–2 $\mu$mol ATP a significant increase in lesion areas that ranged from 3.27 ± 0.11 mm$^2$ ($p < 0.05$) to 6.51 ± 0.77 mm$^2$ ($p < 0.05$) was produced, compared with the PBS-treated control (0.25 ± 0.01 mm$^2$; Fig. 2a). In addition, ATP-induced cell death on the striatum occurred at an early stage (Fig. 2b). The lesion area was 1.96 ± 0.10 mm$^2$ ($p < 0.05$ compared to control) at 4 h and reached a maximum at 8 h (3.63 ± 0.05 mm$^2$, $p < 0.05$ compared with 4 h). Thereafter, lesion areas did not differ from the values obtained at 24 h (3.14 ± 0.18 mm$^2$, $p < 0.05$ compared with control) or 7 days (3.12 ± 0.12 mm$^2$, $p < 0.05$ compared with control) after treatment with 0.4 $\mu$mol ATP. No control animals receiving PBS had discernible lesions in their striatum at any time point (ranging from 0.20 ± 0.01 mm$^2$ to 0.48 ± 0.05 mm$^2$).

Because extracellular ATP is rapidly degraded to ADP and adenosine, the effects of ATP analogues ($\alpha$,$\beta$-MeATP, P2X receptor selective agonist; ATP$\gamma$S, a non-metabolizable analogue of ATP; ADP, P2 receptor agonist; adenosine, P1 receptor agonist) were also assessed 24 h after the intrastriatal injection of 0.4 $\mu$mol analogues. The effect of ATP$\gamma$S on the production of the striatal lesion areas (8.42 ± 0.59 mm$^2$, $p < 0.01$ compared to the control; $p < 0.05$ compared to ATP) was the most potent (Fig. 3). Smaller, but statistically significant, lesion areas were also observed following ATP (3.14 ± 0.18 mm$^2$) or $\alpha$,$\beta$-MeATP (4.62 ± 0.54 mm$^2$) treatment. Administration of ADP or adenosine into the striatum failed to produce striatal damage, similar to the control (Fig. 3).

To evaluate the contribution of P2 receptors to the ATP-mediated striatal damage, various doses of P2 receptor antagonists (PPADS and reactive blue 2) were intrastriatally co-administered with ATP and striatal damage was verified by measuring the lesion areas 24 h post-injection. Administration of 25 or 50 nmol reactive blue 2 reduced the striatal lesion areas by 48% ($p < 0.05$) and 30%, respectively (Fig. 4). In contrast, PPADS was unable to alter the ATP effects, at the doses used in our study.

**DISCUSSION**

The results presented here firstly demonstrate that intrastriatal administration of ATP causes cell death in the striatum *in vivo*. ATP-induced neurotoxicity in the striatum was mediated by P2 receptors. Intrastriatal injection of ATP also produced glial cell loss in the lesion area.

ATP is present at a concentration of 10 mmol/1 within the cytoplasm of most cells and blood platelets contain 1 M ATP in the dense granule [19]. In the normal condition, the
extracellular ATP level is detected at low levels and modulated by ectoATPases. However, increases in extracellular ATP levels were observed in the pathophysiological conditions, such as ischemia, tissue injury and hypoxia [10,11,13], probably leading to a higher amount of extracellular ATP than in a normal state. Loss of ectoATPase activity in the pathological conditions provided additional evidence for the accumulation of extracellular ATP [14,15]. Several studies also reported that extracellular ATP produced cell death in endothelial cells or thymocytes [20–22]. In this regard, all of the observations are comparable to our present results showing the first evidence for in vivo neurotoxicity of exogenous ATP.

Since ATP is rapidly metabolized by extracellular ectoATPase to yield ADP and adenosine [23], it is possible to speculate about the involvement of ATP metabolites in ATP-induced cell death. To test this hypothesis, ADP, adenosine and ATPγS were intrastriatally injected. Neither ADP or adenosine caused striatal injury, whereas ATPγS mimicked the cytotoxic effect of ATP. This is presumably due to the fact that ATPγS is a non-metabolizable ATP analogue and acts as an ectoATPase inhibitor, resulting in increases of ATP concentration in extracellular space [24]. Our data, therefore, strongly supports the hypothesis that uncontrolled extracellular ATP could be cytotoxic.

In the present study, we found that α,β-MeATP, a selective P2X agonist, but not ADP, a selective agonist of P2Y receptor, produced significant striatal damage within 24h. These results indicate the possible involvement of P2X receptors in ATP-induced neurotoxicity. In addition, reactive blue 2, not PPADS, protects striatum from ATP-mediated cytotoxicity. Unfortunately, however, P2 receptor antagonists such as reactive blue 2, PPADS, and suramin do not discriminate well, if at all, between P2X or P2Y receptors due to a lack of selectivity. Similar to this result, Collo et al. [25] demonstrated that P2X2 and P2X5 receptors are sensitive to suramin and PPADS, whereas P2X4 and P2X6 receptors are insensitive. Additionally, suramin and PPADS, but not reactive blue 2, completely inhibited ATP-induced depolarization in the nucleus locus coeruleus [26]. Taken together, our results raise the possibility that ATP-induced neurotoxicity on the striatum in vivo is mediated by reactive blue 2-sensitive and PPADS-insensitive P2X receptor subtypes under our experimental conditions. Alternatively, antagonism could be masked since PPADS and reactive blue 2 have the property of acting as an ecto-ATPase inhibitor, resulting in the inhibition of ATP degradation [27]. If this is the case, ATP-induced neurotoxicity in vivo can be inhibited, potentiated, or sustained by the balance between dual actions of these compounds, either receptor antagonist and/or ecto-ATPase inhibitor.

Finally, analysis by immunocytochemical staining indicated that glial cells in the striatum were also vulnerable to ATP-induced cytotoxicity. Although further studies are required to characterize the exact mechanism(s) underlying ATP-induced toxic action on glial cells, this cytotoxicity is, at least in part, mediated by activation of purinergic receptors since glial cells express P2 receptor subtypes such as P2X and P2Y receptors [28].
CONCLUSION

Our findings suggest that extracellular ATP via P2 receptors could be, in part, responsible for neuropathological processes in the CNS, including trauma and ischemia.

REFERENCES


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