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Extracellular ATP protects pancreatic duct epithelial cells from alcohol-induced damage through P2Y₁ receptor-cAMP signal pathway

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Abstract

Extracellular ATP regulates cell death and survival of neighboring cells. The detailed effects are diverse depending on cell types and extracellular ATP concentration. We addressed the effect of ATP on ethanol-induced cytotoxicity in epithelial cells, the cell type that experiences the highest concentrations of alcohol. Using pancreatic duct epithelial cells (PDEC), we found that a micromolar range of ATP reverses all intracellular toxicity mechanisms triggered by exceptionally high doses of ethanol and, thus, improves cell viability dramatically. Out of the many purinergic receptors expressed in PDEC, the P2Y₁ receptor was identified to mediate the protective effect, based on pharmacological and siRNA assays. Activation of P2Y₁ receptors increased intracellular cAMP. The protective effect of ATP was mimicked by forskolin and 8-Br-cAMP but inhibited by a protein kinase A (PKA) inhibitor, H-89. Finally, ATP reverted leakiness of PDEC monolayers induced by ethanol and helped to maintain epithelial integrity. We suggest that purinergic receptors reduce extreme alcohol-induced cell damage *via* the cAMP signal pathway in PDEC and some other types of cells.

Keywords

apoptosis; alcohol; ATP; cAMP; pancreatic duct epithelial cells; purinergic receptor

Introduction

Extracellular nucleotides exert their biological activities through the P2 purinergic receptor family. P2 receptors comprise the ligand-gated ionotropic P2X receptor channels and G-protein coupled P2Y receptors, which respond to a variety of nucleotide agonists. Seven P2X (P2X_{1–7}) and eight P2Y (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y_{11–14}) receptors are

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Disclosures

There is no conflict of interest.

Author contributions

J.B.S., S.R.J., B.H., and D.S.K. developed the concept and designed research; J.B.S. and S.R.J. performed the experiments; J.B.S., S.R.J., and D.S.K. analyzed data; J.B.S., S.R.J., B.H., and D.S.K. interpreted results of experiments; J.B.S. and S.R.J. prepared figures; J.B.S. drafted manuscript; J.B.S., S.R.J., B.H., and D.S.K. edited manuscript.

expressed in mammals. Each cell type expresses multiple purinergic receptors, allowing different extracellular nucleotides to modulate diverse cellular functions such as neurotransmission, platelet aggregation, cell growth and expansion, differentiation and function of immune cells, and smooth muscle contraction (Burnstock 2006; Burnstock et al. 2013). The function of extracellular ATP in apoptosis has also been studied but its effect is controversial due to the differences in cell types and culture conditions used in each study. For example, extracellular ATP can stimulate apoptosis in primary cortical neurons (Kong et al. 2005), retinal neurons (Anccasi et al. 2013), epithelial cells (Souza et al. 2012), endothelial cells (Urban et al. 2012), and immune cells (Noguchi et al. 2008; Placido et al. 2006; Yoon et al. 2006) or inhibit apoptosis in dorsal root ganglion neurons (Arthur et al. 2006), primary cortical neurons (Chen et al. 2007), myeloid progenitor cells (Palaga et al. 2004), and PC12 (Arthur et al. 2006).

Tissues in our body experience different doses of alcohol after drinking; especially epithelial cells lining the esophagus, stomach, and intestines are exposed to high alcohol concentrations almost same as the contents in the alcoholic beverages (4 – 50 %). Other cell types experience alcohol concentration as blood alcohol content (BAC). Ethanol toxicity is well studied in a variety of tissues including liver, brain, kidney, and gastrointestinal tract (Alfonso-Loeches & Guerri 2011; Bujanda 2000; Das Kumar & Vasudevan 2008; Gramenzi et al. 2006). These studies have indicated that ethanol induces apoptosis through oxidative stress, mitochondrial dysfunction, and caspase activation (Chen et al. 2011; Lee et al. 2010; Olney et al. 2002; Ramachandran et al. 2003; Seo et al. 2013). The ethanol can be metabolized through both oxidative and non-oxidative pathways to reduce toxicity. Acetaldehyde and fatty acid ethyl ester (FAEE) among its metabolites are shown to be toxic to cells (Wilson & Apte 2003; Zakhari 2006).

We have previously reported that alcohol damages pancreatic duct epithelial cells (PDEC) *via* an apoptotic pathway (Seo et al. 2013). PDEC are relatively resistant to ethanol but damaged by quite high alcohol concentrations (> 250 mM or 1.5%). The alcohol-induced apoptosis was initiated by generation of reactive oxygen species (ROS), reduction of mitochondrial membrane potential (MMP), and activation of caspase-3. Thus, the antioxidant N-acetyl cysteine could significantly attenuate the alcohol-induced cellular responses and decreased cell death. The function of extracellular ATP on alcohol-induced apoptosis is not yet examined in PDEC.

In this study, we investigated the hypothesis that extracellular ATP modulates ethanol-induced apoptosis of PDEC. Using a series of cell biological and imaging techniques, we demonstrate that extracellular ATP dramatically reduces cytotoxicity as mediated by P2Y₁ receptor-cAMP signaling pathway.

Materials and methods

Ethics statement

Two cell lines used in this study, dog PDEC and human gallbladder myofibroblasts, were the kind gift of Dr. Sum Lee (University of Washington). They were derived 18 years ago by Oda et al. (1996) (Oda et al. 1996a). The procedures including animal euthanasia, prevention

of pain, and consent of human tissue use were approved at that time by the Animal Experiment Committee and Human Subject Review Committee at the University of Washington.

Materials

CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) and cytotoxicity LDH detection assay kits were purchased from Promega Corporation (Madison, WI) and Roche Diagnostics (Mannheim, Germany), respectively. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and JC-1 dyes were from Invitrogen (Carlsbad, CA, USA); absolute ethanol (200 Proof) was from Fisher Scientific (Waltham, MA); uridine-5'-triphosphate (UTP) was from EMD Biosciences (San Diego, CA, USA); A740003, *iso*-PPADS, MRS-2365, MRS-2279, 2-methylthioadenosine 5'-triphosphate (2-meSATP), α,β -methylene-adenosine 5'-triphosphate (α,β -meATP), adenosine, and forskolin were from R&D Systems (Minneapolis, MN); adenosine-5'-triphosphate (ATP), adenosine-5'-O-3-thiotriphosphate (ATP γ S), adenosine-5'-diphosphate (ADP), 2'-3'-O-(4-benzoylbenzoyl)-ATP (BzATP), uridine-5'-diphosphate (UDP), 8-Br-adenosine-3-5-cyclic monophosphate, α,β -methyleneadenosine 5'-triphosphate (8-Br-cAMP), staurosporine, fluorescein, acetaldehyde, and fluorescein were from Sigma-Aldrich (St. Louis, MO). For Western blot analysis, rabbit anti-cleaved caspase 3 antibody was purchased from Cell Signaling (Cat. No. #9664, Cambridge, MA); polyclonal rabbit anti-human P2Y₁ (SC-20123), goat anti-actin (SC-1616), goat anti-rabbit IgG-HRP (SC-2030), and rabbit anti-goat IgG-HRP (SC-2768) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

The dog PDEC cell line was maintained at 37°C in 5% CO₂/95% air and fed two or three times weekly with Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, 2% penicillin/streptomycin solution, 1% insulin-transferrin-sodium selenite media supplement from Sigma (St. Louis, MO). For subculture, the cells in a monolayer were treated with 0.05% trypsin/EDTA at 37°C for 35 min and passaged to newly coated inserts. Cells of passage numbers 10 – 30 were used for this study. For single-cell experiments, cells were seeded on small Vitrogen-coated glass chips in a medium conditioned by the myofibroblasts and used 1 to 2 days after plating. CAPAN-1, human pancreatic duct adenocarcinoma, and tsA201, a subclone of human embryonic kidney (HEK) 293 cells were cultured at 37°C and 5% CO₂/95% air and fed two or three times weekly with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 2% penicillin/streptomycin (Blanco-Aparicio et al. 1998; Kruse et al. 2012).

Cell viability assays

CellTiter 96 Aqueous non-radioactive cell proliferation (MTS; Promega, Madison, WI) and lactate dehydrogenase (LDH; Roche Applied Science, Indianapolis, IN) assay kits were used to determine the cell viability as described in detail previously (Seo et al. 2013).

Apoptosis versus necrosis detection assay

Apoptotic or necrotic cells were measured as described previously (Seo et al. 2013). Cells were plated into a Lab-Tek chambered coverglass (Thermo Scientific) coated with Vitrogen:HBSS mixture (1:5) at a density of 1.0×10^4 cells per well in 400 μ L culture medium. After 24 h incubation, the cells were treated with 0.75 M ethanol in the presence or absence of 100 μ M ATP for 4 h. The cells were then stained with the Promokine Apoptotic/Necrotic/Healthy cell detection kit (PromoCell, Heidelberg, Germany) according to the manufacturer's instructions. The cells stained with annexin V (green) alone or together with ethidium homodimer (red) were regarded as early or late stages of apoptosis and the cells labeled with ethidium homodimer alone were counted as necrotic cells (Logue et al. 2009). For total cell number, cells stained with Hoechst33342 (blue) were counted.

Western blot analysis

Activated caspase 3 and P2Y₁ receptor proteins were detected by Western blot analysis. The cells were lysed with Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) containing EDTA-free protease inhibitor mixture (Roche Diagnostics) and then centrifuged at 12,000 rpm for 10 min at room temperature. The supernatants were separated by 4 – 12% NuPAGE gel using a running buffer and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h in TBST (10 mM Tris-HCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat dried milk and then incubated with rabbit anti-cleaved caspase-3 (1:500 dilution), rabbit anti-P2Y₁ (1:500 dilution), or goat anti-actin (1:1,000 dilution) antibodies at 4 °C for overnight. After a washing step with Tris-buffered saline with Tween 20 (TBST), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat secondary antibody (1:5,000 dilution) and visualized using the ECL system (Amersham Biosciences) followed by autoradiography. Intensity of the bands in the autoradiograms was measured using ImageJ software.

Measurement of reactive oxygen species (ROS)

Saline solution for real-time measurements contained (in mM): 137.5 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, 10 HEPES (pH adjusted to 7.3 with NaOH). Cells were incubated with 10 μ M CM-H₂DCFDA in saline solution for 30 min at 37 °C in a CO₂ incubator and washed twice. Upon oxidation, the dye becomes fluorescent 2',7'-dichlorofluorescein (DCF) in the cells irreversibly so that DCF fluorescence increases steadily even in the control condition. For convenience, we recalibrated data points using the control slope, i.e. DCF fluorescence was made flat in the control. The dye was excited at 475 nm and fluorescence signals were recorded at > 525 nm every 15 s using a charge-coupled device (CCD) camera (Evolve, Photometrics). Background fluorescence measured in a cell-free region was subtracted. Image processing and data analysis were done with MetaFluor software (Molecular Devices).

Measurement of mitochondrial membrane potential (MMP)

To monitor the mitochondrial membrane potential, cells were incubated with JC-1 dye (10 μ g·mL⁻¹) in saline solution for 30 min at 37 °C in CO₂ incubator and then washed twice

with saline solution. Mitochondrial membrane potential was determined by the relative amounts of dual emissions from mitochondrial JC-1 monomer (green) in cytoplasm or aggregates (red) inside the mitochondria. Mitochondrial depolarization causes the release of the dye into the cytoplasm so that the ratio of red/green fluorescence intensity decreases. Green monomers and red aggregates were excited at 488 and 561 nm and their fluorescence was detected at 518 – 540 and 591 – 700 nm, respectively, using a Zeiss 710 laser-scanning confocal microscope.

Quantitative Real-Time PCR (qPCR)

To investigate the expression of P2 receptor genes, total RNA was isolated from polarized PDEC with PureLink® mini kit (Invitrogen). First-strand cDNA of PDEC was synthesized using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Dog brain cDNA (Cat. No. C1734035) was purchased from Amsbio (Cambridge, MA). cDNAs were then subjected to polymerase chain reaction (PCR). Primer-pair sequences for P2 receptor subtypes and the expected size of PCR products are described in Table 1. Real-time PCR was performed with a StepOnePlus Real-Time PCR System (Agilent Technologies Inc., Santa Clara, CA) using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) and the thermocycler conditions recommended by the manufacturer. Relative gene expression was calculated by the C_t method using GAPDH as an internal control. Melting curve analysis showed a single sharp peak with the expected melting temperature (T_m) for all samples.

Knockdown of P2Y₁ receptor using siRNA

To knock down P2Y₁ receptor, we used chemically synthesized double-stranded siRNA with 21-nt duplex RNA and 2-nt 3' dTdT overhangs purchased from Dharmacon (GE Healthcare, CO). The sense (target) sequence in P2Y₁ is 5'-CUU CUA CUA CUU CAA CAA GAC-3'. For control siRNA, we used 5'AAG UGG ACC CUG UAG AUG GCG-3' (Ahn et al. 2003). For the siRNA experiment, PDEC were transfected with siRNA samples for 4–5 h, using Lipofectamin3000 transfection reagents (Invitrogen) as according to the manufacturer's instructions. After 3 days, expression level of P2Y₁ proteins and cell viability were examined.

Measurement of cAMP using fluorescence resonance energy transfer (FRET)

For real-time monitoring of intracellular cAMP production, cells were transfected with Epac1-camps plasmid (1 µg/ 35 mm dish), kindly provided by M. Lohse (Germany), using X-tremeGENE transfection reagents (Roche applied science) as described previously (Jung et al. 2010). After 1–2 days, fluorescence was excited at 405 nm, and emission from CFP and YFP was detected from the whole cytoplasm at 420–480 nm (F_{CFP}) and 560–615 nm (F_{YFP}) using a confocal microscope (LSM 710; Carl Zeiss, Inc.). The F_{CFP} and F_{YFP} signals were corrected for background. To determine bleed-through corrections, we measured F_{CFP} and F_{YFP} for cells expressing CFP alone and YFP alone. 38% of the CFP signal appears in YFP channel and no YFP signals in CFP channel. Therefore, the final FRET ratio was calculated as $(F_{YFP}-0.38 \cdot F_{CFP})/F_{CFP}$. A decrease of the FRET ratio represents an increase in intracellular cAMP concentration since cAMP binding to the Epac1 domain causes a conformational change for CFP-YFP separation.

Measurement of leakiness of PDEC monolayers

PDEC were cultured on Vitrogen-coated membrane to form tight epithelial monolayers over 3–4 days after plating as described previously (Oda et al. 1996b; Seo et al. 2013). A PDEC monolayer was installed into a home made chamber and the tightness of the monolayer was estimated by perfusing a saline solution containing 10 μ M fluorescein to the luminal side and detecting the leak of the dye to the serosal side. Fluorescein at the serosal compartment was excited at 488 nm and fluorescence emission was detected at 492–622 nm using Zeiss 710 confocal microscope.

Statistical analysis

Numerical values in the text and figures are given as mean $\pm$ SEM. n denotes the number of analyzed wells, cells, or monolayers from at least two independent experiments. All statistical analysis was performed using unpaired two-tailed Student's t -test in Microsoft Excel or ANOVA with multiple comparisons in GraphPad Prism. A p value of < 0.05 was considered significant.

Results

ATP Protects Against Ethanol-induced Damage of PDEC

First, we tested the cytotoxic effects of ethanol and its metabolites using the MTS assay. Consistent with our previous study (Seo et al. 2013), PDEC was relatively resistant to ethanol. With acute treatments (4 - 24 h), the cells were damaged only by very high doses of ethanol (>500 mM, 2.9%). However even more moderate ethanol concentrations (125 - 250 mM, 0.75 - 1.5% v/v) could induce significant cell damage with a longer (72 h) incubation (Fig. 1a). Looking at ethanol metabolites, acetaldehyde, an oxidative metabolite, induced cell damage at 0.1 to 100 mM (Fig. 1b). In contrast, fatty acid ethyl esters (FAEE), non-oxidative metabolites, did not damage PDEC up to 30 mM (Fig. 1c), suggesting that ethanol toxicity in PDEC might be mediated mainly by acetaldehyde.

Next, we examined whether extracellular ATP modifies ethanol-induced cell damage. For this and the following experiments we used a high concentration of ethanol (750 mM) and short incubation time (4 h) to induce a significant probability of cell death while avoiding possible effects of ATP on cell proliferation (Huang et al. 1989). Cell viability monitored with the MTS assay and cell damage detected with lactate dehydrogenase (LDH) leakage indicated dose-dependent toxic effects of ethanol treatment. Remarkably these deleterious actions were significantly mitigated in the presence of 100 μ M ATP (Fig. 2a, c). ATP shifted the dose-dependency of cell damage, reflecting a protective effect for all ethanol concentrations. ATP at concentrations above 10 or 30 μ M protected PDEC significant as judged by cell viability (Fig. 2b) or by LDH leakage (Fig. 2d), respectively. The effective ATP concentration was higher when toxicity was assessed by plasma membrane damage (LDH leakage) than by the viability assay (MTS). Note that the treatment time was 24 h for the LDH assay (Fig. 2b) and 4 hr for the MTS assay (Fig. 2d).

To check for similar ATP protective effects in other cell lines including human pancreatic epithelial cells (CAPAN-1) and human embryonic kidney cells (tsA201), we first performed

the cell viability assay with ethanol in the cell lines. Ethanol induced cell damage in both cell lines, but their sensitivity to ethanol was different. tsA201 cells were more sensitive than CAPAN-1 cells (Fig. 3a, b). However, interestingly, a reduction of ethanol-induced cell damage by ATP was also observed with other cell lines including human pancreatic epithelial cells (CAPAN-1) and human embryonic kidney cells (tsA201) (Fig. 3c, d). In addition, we examined the effect of ATP on acetaldehyde-induced damage. Again ATP was protective (Fig. 4).

Because ethanol induces apoptosis in PDEC (Seo et al. 2013), we used apoptosis and necrosis cell detection assays to determine whether ATP can block ethanol-induced apoptosis (Fig. 5a, b, details in Materials and Methods). The percentage apoptotic cells ($72 \pm 17\%$) induced by ethanol was similar to that induced by staurosporine, a standard inducer of apoptosis. Of note, in the presence of ATP, ethanol-induced apoptosis was significantly reduced ($7.2 \pm 1.7\%$). We also examined whether ATP attenuates the activation of caspase 3 by ethanol using an antibody specific for the cleaved and active form of caspase 3 (Fig. 5c, d). In the corresponding Western blot, ethanol activated caspase 3 (17 or 19 kDa), an effect again mitigated by ATP, at 100 and 300 μM . Thus, ATP appears to prevent ethanol-induced cell damage in PDEC by blocking generation of apoptotic signals.

ATP Inhibits Ethanol-induced ROS Generation and Mitochondrial Depolarization

Are the cellular events triggered by ethanol in PDEC also blocked by ATP? We previously demonstrated that ethanol stimulates the production of ROS and depolarizes the membrane potential of mitochondria in pancreatic duct cells (Seo et al. 2013). Consistent with our previous study, ethanol induced a 5.9 ± 0.8 fold increase in ROS (measured with CM-H₂DCFDA dye; control group: 1.0 ± 0.6 fold) (Fig. 6a, b). ATP significantly reduced this ethanol-induced ROS generation (3.2 ± 0.7 fold).

Next, we measured mitochondrial membrane potential (MMP) using JC-1 dye (details in Materials and Methods) and found that ethanol induced membrane depolarization ($42 \pm 3\%$ compared to control) (Fig. 6c, d), and ATP countered the ethanol-induced depolarization. These data indicate that ATP protects against severe ethanol damage by blocking ethanol-induced ROS generation and mitochondrial depolarization.

P2Y₁ Receptor Mediates Protective Effects of ATP in Ethanol-induced Cell Damage

It is well established that ATP is a physiological ligand of purinergic G protein-coupled P2Y receptors and of ATP-gated P2X receptor ion channels. To investigate which receptor(s) are involved in the protective effects of ATP, we checked the gene expression levels of P2Y and P2X receptor subtypes in PDEC, with the exception of the P2Y₁₃ receptor, for which the dog cDNA sequence is not available. Quantitative RT-PCR (qPCR) results indicate that among purinergic type 2 receptors, P2Y₁, P2Y₂, P2Y₁₁, P2X₂, and P2X₄ mRNA was highly expressed in dog PDEC (Fig. 7a), albeit at different levels. In addition, to evaluate the primer sets for purinergic receptors, we repeated qPCR analysis with dog brain cDNA. As shown in Fig. 7b, P2Y₆, P2X₂, P2X₄, and P2X₅ mRNA was highly expressed in dog brain but expression of P2Y₄ and P2X₆ mRNA was not detected.

To determine which receptor(s) are involved in the protective effects of ATP in PDEC, we used various synthetic agonists of P2Y and P2X receptors (Fig. 8). Pharmacological studies revealed protective effects for ATP γ S (nonhydrolyzable ATP analog) and ADP (agonist for P2Y₁, P2Y₁₁, P2Y₁₂, and P2Y₁₃), but not UDP (P2Y₆ agonist) and UTP (P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ agonist) (Fig. 8a, b). Among the P2X receptor agonists, BzATP (P2X₇ and P2Y₁₁ agonist and partial P2X₁ and P2Y₁ agonist) and 2-meSATP (P2Y₁, P2X₁, P2X₂, P2X₃, and P2X₅ receptor agonist), but not α,β -meATP (P2X₁, P2X₃, and P2X₄ agonist), significantly protected PDEC from ethanol cytotoxicity (Fig. 8c, d). In addition, the ATP protection was not attenuated by *iso*-PPADS (P2X₁, P2X₂, P2X₃, and P2X₅ receptor antagonist) or A740003 (P2X₇ receptor antagonist) (Fig. 9a). Further, we tested adenosine. Adenosine is produced from the degradation of adenine nucleotides by exonucleases and stimulates type 1 purinergic receptors (Burnstock 2006) and is known to protect pancreatic and hepatic grafts from mitochondrial damage during low temperature graft maintenance used in liver transplantation and experimental pancreas transplantation (Palombo et al. 1991). However, up to 300 μ M adenosine did not protect PDEC from ethanol toxicity (Fig. 9b). Collectively these results suggested that P2Y₁ receptors can mediate ATP protective effects.

To confirm a key role of P2Y₁, we further used pharmacology and knock-down experiments. The protective effect of ATP was reduced by the selective P2Y₁ antagonist, MRS-2279 (Fig. 10a), and the ATP effect was reproduced with a selective agonist of P2Y₁, MRS-2365, which in turn was attenuated by the P2Y₁ antagonist (MRS-2279) (Fig. 10b). Finally we tried knockdown experiments using siRNA designed for P2Y₁ (Fig. 11a - d). Western blot analysis revealed that P2Y₁ receptors are reduced by about 40 % in the dish of siRNA-treated cells compared to cells transfected with scrambled siRNA (Fig. 11a, b). The cells with P2Y₁ knockdown were more sensitive to ethanol (Fig. 11c, d) and were not protected by ATP (Fig. 11d).

Both PKA-dependent and PKA-independent Pathways Are Important for cAMP-mediated Protective Effects of ATP

Protein kinase A (PKA) and protein kinase C (PKC) pathways mediate the major downstream actions of purinergic receptors, and key roles of cAMP/PKA signals in ethanol toxicity and apoptosis have been reported in several cell types (Insel et al. 2012; Moore et al. 1998). Therefore we examined potential mediation through PKA using forskolin (to stimulate cAMP production) and 8-Br-cAMP (a membrane-permeable cAMP analog) and PKC using phorbol 12-myristate 13-acetate (PMA). Both forskolin and 8-Br-cAMP countered ethanol-induced PDEC damage in a dose-dependent manner (Fig. 12a, b). Additionally H89, a PKA inhibitor, significantly lowered protection against ethanol damage by ATP but the reduction was not complete (Fig. 12c). In contrast, PMA did not protect against ethanol-induced damage (data not shown). To verify whether the purinergic receptor increased cAMP and, subsequently, cAMP-activated PKA, we monitored the intracellular cAMP level using a FRET-based Epac-1 probe (Nikolaev et al. 2004). As expected, ATP stimulated intracellular cAMP production (Fig. 12d). MRS-2365, the selective agonist of P2Y₁ receptor, also increased intracellular cAMP to a similar extent as ATP, and that effect was attenuated by MRS-2279 (P2Y₁ antagonist). Finally, we tested the effect of both H89

and MRS-2279 on ethanol-induced ROS generation and mitochondrial depolarization (Fig. 12e, f). When the P2Y₁ receptor was inhibited with MRS-2279, the ATP protective effect on ethanol-induced ROS production was completely blocked (Fig. 12e). Inhibiting PKA with H89 also reduced the ATP protective effect on ROS production, although the reduction was not complete. The partial block by H89 was observed with cell viability as well (Fig. 12c). Inhibiting P2Y₁ receptors with MRS-2279 reduced the ATP protective effect on ethanol-induced mitochondrial depolarization as well. However, that ATP protective effect was not lost by inhibiting PKA with H89 (Fig. 12f). These data suggest that the mechanism for ATP protection of PDEC from ethanol involves both PKA-dependent and PKA-independent cAMP signaling from stimulated P2Y₁ receptors.

ATP Protects Against Damage of PDEC Monolayers by Ethanol

The epithelial barrier is an essential property of epithelial monolayers. We therefore investigated the effect of ATP on monolayer integrity using a leakage assay with fluorescein (Fig. 13). Control monolayers did not allow passage of the dye across the monolayer (slope of fluorescence increase: $0.01 \pm 0.003 \text{ AU} \cdot \text{min}^{-1}$). When PDEC monolayers were incubated with ethanol for 4 h, the permeability of the dye increased markedly ($1.5 \pm 0.2 \text{ AU} \cdot \text{min}^{-1}$), reflecting serious compromise of the monolayer barrier. For reference, the curve labeled “Disk Membrane” plots dye permeation when there are no cells on the supporting disk filter. The ethanol-induced leakage was significantly reduced by ATP treatment ($0.33 \pm 0.18 \text{ AU} \cdot \text{min}^{-1}$).

Discussion

This study demonstrates that ATP treatment effectively protects PDEC and epithelial monolayers from damage by toxic concentrations of alcohol (Fig. 14). Based on pharmacological screening and siRNA knock-down experiments, we found that the protective ATP effect is likely mediated by P2Y₁ receptors and both PKA-dependent and PKA-independent cAMP signaling. Cell biological studies suggest that the activation of purinergic receptors not only attenuated alcohol-induced ROS generation and mitochondrial depolarization but also reduced the number of alcohol-induced apoptotic cells and the activation of caspase-3.

We have previously reported that alcohol induces apoptosis of PDEC through ROS generation, mitochondrial depolarization, and caspase-3 activation and that alcohol damage was inhibited by antioxidant treatment (Seo et al. 2013). This study now establishes that extracellular application of ATP reduces these ethanol-induced apoptotic signaling pathways (Fig. 5, 6). The protective effect of ATP on viability was observed at concentrations above 10 μM ATP (Fig. 2b). This is a (patho)physiologically relevant concentration range because cells can reach higher levels if the neighboring cells undergo necrotic cell death and release intracellular ATP (typically in the millimolar range) (Elliott et al. 2009). However, other studies have also shown that extracellular ATP activates ROS production in several cell types such as macrophages (Noguchi et al. 2008), PC12 cells (Sun & Chen 1998), human intestinal epithelial cells (Souza et al. 2012) and reduces mitochondrial membrane potential in granulosa luteal cells (Park et al. 2003). We speculate that these contrasting results may

be due to different assay conditions such as use of high ATP concentrations (up to mM), culture conditions, or cell types. Alternatively the intracellular signals activated by ATP are variable, depending on the types of expressed purinergic receptors. For example, human intestinal epithelial cell lines were found to express mainly P2Y₁, P2Y₆, and P2Y₁₂ receptors (HCT8 cells, Coutinho-Silva et al. 2005). and P2Y₆ receptor (RAW264.7 cells, Zhang et al. 2011). Even though PANC-1 and CFPAC-1 are human pancreatic duct cell lines, their expression level of purinergic receptor and their intracellular Ca²⁺ signaling responses to various ligands are variable (Hansen et al. 2008). In addition, high ATP concentration (> 0.5 mM) and various incubation times (5 min ~ 24 h) are used in the previous studies (Noguchi et al. 2008; Park et al. 2003; Souza et al. 2012), possibly activating different subtypes of purinergic receptors, such as P2X₇ that is often related to apoptosis (Miller et al. 2011).

Ethanol toxicity can be mediated by its metabolites such as acetaldehyde and FFAEE. Previously, we demonstrated that PDEC can metabolize ethanol, and that its oxidative metabolite, acetaldehyde, is also involved in ethanol toxicity. Briefly, PDEC express metabolic genes capable of ethanol oxidation including alcohol dehydrogenase 4 (ADH4), ADH5, catalase, acetaldehyde dehydrogenase 1A1 (ALDH1A1), and ALDH2 and generate acetaldehyde upon ethanol treatment. In addition, inhibition of both ALDH1 and ALDH2 with disulfiram and daidzin increase ethanol cytotoxicity in PDEC through accumulation of intracellular acetaldehyde presumably. In this study we also observed that acetaldehyde induces cell damage of PDEC at 100 μ M as in our previous study (Fig. 1b) (Seo et al. 2013) and more interestingly the acetaldehyde-induced cell damage is efficiently attenuated by extracellular ATP treatment (Fig. 4). However, it seems that PDEC are less sensitive to FFAE than acetaldehyde, since various FFAEs did not induce significant cell damage of PDEC up to 30 mM. Therefore, ethanol toxicity in PDEC is presumably via acetaldehyde instead of FFAE.

We screened P2Y₁ receptor as the candidate ATP receptor by pharmacological and molecular cell biological approaches. Expression of P2Y₁ mRNA is higher among the P2Y receptors in PDEC (Fig. 7a). In addition, the ATP protective effect is mimicked by ADP and a P2Y₁ agonist and reduced by both P2Y₁ antagonist and knock-down of P2Y₁ receptor (Fig. 8–11). Similarly another group has observed that extracellular ATP protects against oxidative stress-induced damage of astrocytes via P2Y₁ receptor (Shinozaki et al. 2005). The functional role of P2Y₁ receptor in apoptosis is still controversial. For example, activation of P2Y₁ receptor protects against apoptosis of a myeloid progenitor cells (Palaga et al. 2004) but stimulates apoptotic signals in prostate cancer cells (Wei et al. 2011) and astrocytoma cells (Mamedova et al. 2006).

Involvement of cAMP signal in ethanol toxicity has been suggested in other cell types. For example, chronic ethanol treatment reduced adenylyl cyclase activity in human erythroleukemia cells (Rabbani & Tabakoff 2001), and activation of PKA reduced ethanol sensitivity in *Drosophila*, rodents, and humans (Moore et al. 1998). These reports suggest that the cAMP-PKA pathway is critically related to ethanol toxicity. In fact, it has been reported that cAMP is a mediator to either stimulate or inhibit apoptosis signaling in numerous cell types including cardiac myocytes, neurons, leukocytes, vascular endothelial

cells, lung carcinoma cells, gastrointestinal epithelial cells, and pancreatic islet β -cells (Insel et al. 2012). In our study, the cAMP signal pathway mediates protective ATP effects against ethanol-induced apoptosis of PDEC (Fig. 12). Puzzling is that the identified putative target receptor of ATP, the P2Y₁ receptor is not generally considered to be coupled to G_s protein for activation of adenylyl cyclase to generate cAMP. Rather, this receptor commonly couples to G_q to activate PKC. However, our studies show that a P2Y₁ receptor agonist (MRS-2365) increase intracellular cAMP production as ATP did and this stimulation is inhibited by a selective receptor antagonist (Fig. 12d). In addition, other groups have also reported the possibility that P2Y₁ may regulate intracellular cAMP levels even without coupling to G_s protein (Guerra et al. 2004). The exact coupling mechanism is not yet clear but, possibly, Ca²⁺ signals generated by the receptors activate Ca²⁺-dependent adenylyl cyclase (Guerra et al. 2004; Willoughby & Cooper 2007; Halls & Cooper 2011). Another possibility is that activation of P2Y₁ receptors stimulates cAMP production by trans-activating other receptors linked to the cAMP signal pathway. For example, it has been reported that P2Y₁ receptors regulate EGF receptors in epithelial cells (Buvinic et al. 2007) and interact with A₁ adenosine receptors in cotransfected cells, human astroglial cells, and rat brain tissues (Fredholm et al. 2003; Tonazzini et al. 2008; Yoshioka et al. 2002; Yoshioka & Nakata 2004). Nevertheless our data suggest a crucial role of the cAMP signal pathway in the protection by ATP in alcohol-induced damage.

We now speculate on possible roles of ATP-dependent protection against apoptosis. Firstly, some epithelial cells lining the gastrointestinal tract experience extreme doses of alcohol during its intake (4 - 50 %). Our *in vitro* assay revealed that ethanol concentrations almost that high (500 mM, 3 %) damaged cells significantly during a single 4 h exposure. Since ATP also protected CAPAN (human PDEC) and tsA201 kidney epithelial cells, the same mechanism might protect epithelia and other types of cells in the tract during frequent and heavy drinking (a hypothesis for test in the future studies).

Secondly, actions of ATP could retard development of alcohol-induced pancreatitis. Pancreatic ducts are responsible for luminal bicarbonate secretion and for confining digestive enzymes inside the ducts during their transport from acinar cells toward the duodenum. A strong barrier function and low paracellular permeability are essential to both functions (Rotoli et al. 2004; Steward et al. 2005). This and other studies have shown that ethanol increases the permeability of PDEC monolayer gradually leading to loss of tight junctions and epithelial denudement following cell death (Harvey et al. 1989; Rotoli et al. 2004; Seo et al. 2013) (Fig. 13). The ethanol-induced ductal leakage may contribute to the development of pancreatitis because the effect of any leaked auto-activated digestive enzymes back into the intersitium will be further amplified by self-digestion and pathological inflammation of pancreatic tissues (Rotoli et al. 2004; Schmid-Schonbein & Hugli 2005). Interestingly, the ethanol-induced leakiness of PDEC monolayer is also significantly reduced by ATP treatment (Fig. 13). Hence, we can suggest that activation of purinergic receptors in PDEC might be a self-defense mechanism to block the progression of severe pancreatitis where a significant amount of ATP is released from damaged pancreatic acinar cells or neighboring ductal cells. As might be expected, lowering the ethanol concentration to 125 mM (0.75% ethanol and still much higher than the legal BAC for driving in North America) damages PDEC little in 4 h but needs 72 h for a significant cell

death (Fig. 1). Naturally, damage induced by alcohol is a function of concentration and duration, so even blood levels of alcohol might slowly trigger apoptotic cell death during years of heavy drinking (the major cause of chronic pancreatitis).

Thirdly, ATP rescues cell death involving induction of ROS and failure of mitochondrial function, suggesting that purinergic signaling potentially might alleviate other insults leading to oxidative stress such as radiation, temperature shock, and hydrogen peroxide (Katschinski et al. 2000; Pourzand & Tyrrell 1999; Wijeratne et al. 2005; Shinozaki et al. 2005). For example, recently it has been described that 2MeSADP and ATP γ S increase the expression of genes related to antioxidant defense including catalase and superoxide dismutase 2 in astrocytes (Forster & Reiser 2016). In addition, these purine agonists increase the total level of glutathione so that rat brain astrocytes are protected against hydrogen peroxide toxicity.

In summary, our data show protective effects of P2Y₁ receptor on ethanol-induced apoptosis of PDEC for the first time. Demonstration of underlying intracellular signals allows us to predict that activation of other GPCRs coupled to cAMP signals would have the same beneficial effect.

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Abbreviations used in this paper

ADH	alcohol dehydrogenase
ALDH	acetaldehyde dehydrogenase
FAEE	fatty acid ethyl ester
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MMP	mitochondrial membrane potential
PDEC	pancreatic duct epithelial cells
PKA	protein kinase A
PKC	protein kinase C
P2 receptor	purinergic type 2 receptor
PMA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species

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Fig. 1.

Cytotoxicity of ethanol and its metabolites in PDEC. **a** PDEC were treated with different concentrations of ethanol for 4, 24, and 72 h, and cell viability was tested with the MTS assay. **b** Treatment with acetaldehyde for 4 h induced significant cell damage. **c** Cells were exposed to the indicated fatty acid ethyl ester (FAEE) concentrations for 24 h. OAEE, oleic acid ethyl ester; PAEE, palmitic acid ethyl ester; POA, palmitoleic acid; POAEE, palmitoleic acid ethyl ester. The values are expressed relative to the control group. Symbols and lines are experimental data and smooth curves from GraphPad Prism, respectively. $n = 3 - 6$ for each condition, # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with the control group.

Fig. 2.

ATP reduces ethanol-induced cell damage in PDEC. **a** Cells were treated with different concentrations of ethanol with or without 100 μ M ATP for 4 h. Cell viability was tested with the MTS assay. The values are expressed relative to the control group. **b** Protective effects were observed at various concentrations of ATP. Cells were treated with 750 mM ethanol. **c** and **d** After a 24 h exposure to the indicated ethanol and ATP concentrations, the activity of LDH in the conditioned cell-culture medium was measured. The results are mean $\pm$ SEM and representative of two independent experiments. Symbols and lines in Figure 2a and c are experimental data and smooth curves from GraphPad Prism, respectively. $n = 4 - 6$ for each condition, # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with the control group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the ethanol-treated group.

Fig. 3.

ATP inhibits ethanol-induced cell damage in CAPAN-1 and tsA201 cells. CAPAN-1 (**a**) and tsA201 (**b**) cells were treated with different concentrations of ethanol for 4 h, and cell viability was tested with the MTS assay. CAPAN-1 (**c**) and tsA201 (**d**) cells were incubated with 1,500 and 500 mM ethanol for 4 h, respectively, in the presence or absence of indicated ATP concentrations. After the treatments, cell viability was measured with MTS assay. The results are mean $\pm$ SEM and representative of two independent experiments. Symbols and lines in Figure 3a and b are experimental data and smooth curves from GraphPad Prism, respectively. $n = 3 - 6$ for each condition, $^{\#} p < 0.05$, $^{\#\#} p < 0.01$, and $^{\#\#\#} p < 0.001$ compared with the control group, and $^* p < 0.05$ and $^{***} p < 0.001$ compared with the ethanol-treated group.

Fig. 4.

ATP inhibits acetaldehyde-induced cell damage. PDEC were incubated with 10 mM acetaldehyde in the presence or absence of 100 μ M ATP for 4 h. After the treatments, cell viability was examined with a MTS assay. The results are mean $\pm$ SEM and representative of two independent experiments. $n = 10$ for each condition, ### $p < 0.001$ compared with the control group, and *** $p < 0.001$ compared with the acetaldehyde-treated group.

Fig. 5.

ATP protects against ethanol-induced apoptosis. **a** Cells were treated for 4 h with 750 mM ethanol in the presence or absence of 100 μM ATP. After treatment, the cells were stained with annexin V-FITC (green, apoptotic cells), ethidium homodimer III (red), and Hoechst 33342 (blue) for 15 min. Control; 750 mM Ethanol; 1 μM staurosporine to trigger apoptosis; 750 mM Ethanol and 100 μM ATP. Scale bar is 20 μm. **b** The percentage of apoptosis in cells treated as in A ($n = 4$ for each condition from two separate experiments). **c** PDECs were treated for 4 h with 750 mM ethanol in the presence or absence of 100 μM ATP and subsequently analyzed by Western blot analysis to detect activated-caspase 3 and actin proteins. **d** Caspase 3 activation was quantified by calculating its ratio relative to actin ($n = 3$ for each condition from two experiments). ^{##} $p < 0.01$ and ^{###} $p < 0.001$ compared with the control group. ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$ compared with the ethanol-treated group.

Fig. 6.

ATP suppresses ethanol-induced ROS generation and mitochondrial depolarization. **a** and **b** ROS measured with CM-H₂DCFDA. Cells were exposed to 750 mM ethanol with or without 100 μ M ATP during the indicated period. The areas under the curves and above the 1.0 level were calculated during drug treatments (3 to 12 min) and normalized to that of the control. **c** and **d** Mitochondrial membrane potential (MMP) estimated with the JC-1 dye. Decrease of the ratio (F561/F488) indicates MMP depolarization. CCCP was used as a positive control. For the bar graph (**d**), the average value of MMP was calculated during drug treatments (3 to 12 min). The results are mean $\pm$ SEM and representative of two independent experiments. $n = 3 - 21$ for each condition, $^{##} p < 0.01$ and $^{###} p < 0.001$ compared with the control group. $^{*} p < 0.05$ and $^{***} p < 0.001$ compared with the ethanol-treated group.

Fig. 7.

Purinergic receptor profile of dog PDEC and brain. Quantitative RT-PCR (qPCR) analysis on expression of P2Y and P2X receptors in PDEC (**a**) and brain tissue (**b**). Data were normalized using GAPDH expression, and values are shown as means $\pm$ SEM ($n = 6$ for PDEC; and $n = 3$ for dog brain). Expression levels below the detection limit of the qPCR assay are marked with 'ND' (not detectable).

Fig. 8.

Pharmacological screening of purinergic receptors involved in protective ATP effects. Cells were treated with 750 mM ethanol for 4 h in the presence or absence of varying agonist concentrations for P2Y (a, b) or P2X (c, d) receptors. Cell viability was measured after treatment. The values were calculated relative to the control group. Symbols are experimental data. Lines are smooth curves from GraphPad Prism (ADP, UTP, UDP, 2-meSATP, and α,β -meATP) or the Hill equation fitted by Igor software (ATP, ATP γ S, and BzATP). $n = 4 - 6$ for each condition, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the ethanol-treated group.

Fig. 9.

P2X and P1 receptors do not mediate the ATP protective effect on ethanol-induced cell damage. **a** PDEC were treated with 750 mM ethanol in the presence or absence of 100 μ M ATP for 4 h with or without 30-min pretreatment with *iso*-PPADS (P2X₁, P2X₂, P2X₃, and P2X₅ receptor antagonist) or A740003 (P2X₇ receptor antagonist) to assess involvement of P2X receptors. **b** Cells were treated with 750 mM ethanol with various adenosine concentrations for 4 h. Cell viability was determined with the MTS assay after the treatments ($n = 3$ for each condition and ^{###} $p < 0.001$ compared with the control group).

Fig. 10.

Pharmacological studies with a selective antagonist and an agonist of P2Y₁ receptors. **a** Cells were treated with 750 mM ethanol in the presence or absence of 100 μM ATP, with or without 30 min pretreatment with 100 μM of the P2Y₁ receptor antagonist MRS-2279. In this figure, $n = 4 - 6$ for each condition, ^{###} $p < 0.001$ compared with the control group. ^{***} $p < 0.001$ compared with the ethanol-treated group. ^{ξξξ} $p < 0.001$ compared with the ATP-treated group. **b** PDEC were treated with 750 mM ethanol for 4 h in the presence or absence of 10 μM of the P2Y₁ agonist MRS-2365, with or without 30 min pretreatment with 100 μM of the P2Y₁ antagonist MRS-2279. After the treatments, cell viability was determined with the MTS assay ($n = 3$ for each condition, ^{###} $p < 0.001$ compared to the control group, ^{***} $p < 0.001$ compared to the ethanol-treated group, and ^ξ $p < 0.05$ compared with the MRS-2365-treated group).

Fig. 11.

P2Y₁ receptors mediate the protective effects of ATP. Reduction of P2Y₁ receptor proteins by siRNA knockdown (**a - d**). Cells were treated with transfection reagent and with P2Y₁ siRNA, scrambled siRNA or without siRNA (control) and lysed 3 days later. Western blot analysis was performed to detect expression of P2Y₁ proteins in PDEC (**a**). The amount of P2Y₁ protein was quantified by calculating its ratio relative to actin (**b**). $n = 3$ for each condition from two experiments, * $p < 0.05$ compared with the control. Cells transfected with P2Y₁ siRNA or scrambled siRNA were treated for 4 h with different concentrations of ethanol (**c**) or with 750 mM ethanol in the presence or absence of 100 μ M ATP (**d**) and then cell viability was analyzed with the MTS assay. The values are expressed relative to the control group. The results are mean $\pm$ SEM. Symbols and lines in Figure 11c are experimental data and smooth curves from GraphPad Prism, respectively. $n = 6$ for each condition, ### $p < 0.001$ compared with the control group. ** $p < 0.01$ and $\xi\xi\xi$ $p < 0.001$ compared with the ethanol-treated group of control siRNA. N.S, not significant.

Fig. 12.

cAMP signaling is necessary and sufficient for the protective ATP effects. Cells were treated with 750 mM ethanol in the presence or absence of forskolin ($n = 3$) (a), or 8-Br-cAMP ($n = 3$) (b). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the ethanol-treated group. c Cells were treated with ethanol in the presence or absence of ATP with or without a 30 min pretreatment with 10 μ M H89 ($n = 3$). *** $p < 0.001$ compared with the control group. * $p < 0.05$ and ** $p < 0.01$ compared with the ATP-treated group. d Intracellular cAMP level measured with Epac-1 camps FRET probe. Up on the graph signifies increasing cAMP. 100 μ M ATP ($n = 4$); 10 μ M MRS-2365 ($n = 6$), agonist of P2Y₁ receptor; 100 μ M MRS-2279 ($n = 7$), antagonist of P2Y₁ receptor. Effects of H89 and MRS-2279 on the protective ATP

effect in ethanol-induced ROS generation (e) and -mitochondrial depolarization (f). Cells were stained with CM-H₂DCFDA or JC-1 dye and treated with 750 mM ethanol in the presence or absence of 100 μ M ATP with or without preincubation in 10 μ M H89 for 3 min or 100 μ M MRS-2279 for 30 min. The change of ROS or MMP value was analyzed as Fig. 6b and d, respectively. The results are mean $\pm$ SEM and representative of two or three independent experiments. $n = 5 - 18$ for each condition, ^{###} $p < 0.001$ compared with the control group, ^{**} $p < 0.01$ and ^{***} $p < 0.001$ compared with the ethanol-treated group, and ξ $p < 0.05$, $\xi\xi$ $p < 0.01$, and $\xi\xi\xi$ $p < 0.001$ compared with the ATP-treated group.

Fig. 13.

ATP decreases ethanol-induced leakage of PDEC monolayers. **a** PDEC monolayers were treated for 4 h with 750 mM ethanol in the presence or absence of 100 μ M ATP, and then their permeability was determined from the appearance of fluorescein on the apical side after perfusing fluorescein into the luminal side of the monolayers. Untreated control monolayers had a low permeability reflecting a conserved barrier function ($n = 4$), and coated disk membranes without cells represent the maximum permeability ($n = 4$). Monolayers treated with ethanol had an elevated permeability to fluorescein ($n = 4$). Monolayers cotreated with ethanol and ATP ($n = 3$) showed a reduced leakage. **b** Average rate-of-change of fluorescein permeability. The rate was estimated from the slope between 2 and 8 min ($^{##} p < 0.01$ compared with the control group and $^{**} p < 0.01$ compared with the ethanol-treated group. AU, arbitrary unit).

Fig. 14.

Working model for the role of purinergic receptors in ethanol-induced cell death in PDEC. Short-term treatment with a high concentration of ethanol kills the cells by generating reactive oxygen species (ROS), depolarization of mitochondrial membrane potential (MMP), release of cytochrome C, and activation of caspases (Seo et al. 2013). Our new results suggest that extracellular ATP activates P2Y₁ receptors and elicits both PKA-dependent and PKA-independent cAMP signal pathway that protect cells by blocking the cellular mechanisms leading to apoptosis.

Table 1

Primer sets used for RT-PCR analysis

Gene	GenBank Accession number	Forward primer	Reverse primer	Exp. size (bp)
P2RY1	NM_001193673	CCA CGC CAA CCT GTA CGG CA	CAC GAC GAG CCA CAC GAG CAG	145
P2RY2	XM_542321	TTT GCC CGA GAC GCC AAG CC	GAC GTC AGT TCT GTC CCA CCT GT	93
P2RY4	XM_003640257	GTT CTC TCT GCA CCA TCG CCG	TGT AGA CCA CAC TGA GAA TG	138
P2RY6	XM_542320	TGC TAC GAC CTG AGC GCA CC	CGT CCT GGC GGC ACA GAC GAT G	142
P2RY11	NM_001204441	GCC GTT TCC AGG CAG CCT TTC T	GCT GAG AAG ACC ACG GCG GG	136
P2RY12	NM_001003365	GGC TGG GAA TGG CAG TCT GTG T	GAT CAG AAA TGA CTG TGT TC	182
P2RY14	XM_542838	ATT CCA GCA CCA CGC AGC CTC	TTG AGC AGG ATC CCC GCG AC	112
P2RX1	XM_548344	GGA GCA TCC AGA AGG GGG TAC A	GAA GTT CTC GGC CTC TTG GAG	202
P2RX2	XM_534633	CAA GAC AAA CAA CAG CAC AAC C	TGT GGC CAG ATT AAT GAT GGT G	121
P2RX3	XM_540614	GGG ACG TGG TCA AGT TTG CG	CTC TTC TCC GAA ACG CCA TCC	160
P2RX4	XM_003639907	GCA TTC AGA TCA ACT GGA ACT G	CTC AGC ACC AGT CAG GTC AC	161
P2RX5	XM_003639268	GGT CCA TGG TCA GCT GGA CG	GGC AAA CCT GAA GTT GTA CCC	194
P2RX6	XM_543562	TGT GGG CGG ACC CTT CGA C	CGC AGC CTG GTA GTA GGC TA	179
P2RX7	EU334661	CTG GAC AAT CAG AGG AGA TG	TTC CGA CAG CAC AGC TCT TC	145
GAPDH	AB038240	CGG CCC CTC TGG GAA GAT GTG G	CCT TGG CAG CGC CAG TGG AAG	80

The expected transcript size (base pair, bp) is indicated in the right column.