P2X receptors regulate adenosine diphosphate release from hepatic cells

Cynthia Chatterjee · Daniel L. Sparks

Received: 27 January 2014 / Accepted: 15 July 2014 / Published online: 25 July 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Extracellular nucleotides act as paracrine regulators of cellular signaling and metabolic pathways. Adenosine polyphosphate (adenosine triphosphate (ATP) and adenosine diphosphate (ADP)) release and metabolism by human hepatic carcinoma cells was therefore evaluated. Hepatic cells maintain static nanomolar concentrations of extracellular ATP and ADP levels until stress or nutrient deprivation stimulates a rapid burst of nucleotide release. Reducing the levels of media serum or glucose has no effect on ATP levels, but stimulates ADP release by up to 10-fold. Extracellular ADP is then metabolized or degraded and media ADP levels fall to basal levels within 2–4 h. Nucleotide release from hepatic cells is stimulated by the Ca^{2+} ionophore, ionomycin, and by the P2 receptor agonist, 2'3'-O-(4-benzoyl-benzoyl)-adenosine 5'-triphosphate (BzATP). Ionomycin (10 μM) has a minimal effect on ATP release, but doubles media ADP levels at 5 min. In contrast, BzATP (10–100 μM) increases both ATP and ADP levels by over 100-fold at 5 min. Ion channel purinergic receptor P2X7 and P2X4 gene silencing with small interference RNA (siRNA) and treatment with the P2X inhibitor, A438079 (100 μM), decrease ADP release from hepatic cells, but have no effect on ATP. P2X inhibitors and siRNA have no effect on BzATP-stimulated nucleotide release. ADP release from human hepatic carcinoma cells is therefore regulated by P2X receptors and intracellular Ca^{2+} levels. Extracellular ADP levels increase as a consequence of a cellular stress response resulting from serum or glucose deprivation.

Keywords ADP · ATP · Nucleotide release · P2X receptor · P2X4 · P2X7

Abbreviations

ADP	Adenosine diphosphate
AK1	Adenylate kinase
ATP	Adenosine triphosphate
BzATP	2'3'-O-(4-Benzoyl-benzoyl)-adenosine 5'-triphosphate
NTPDase	Ectonucleotidase
P2X	Ion channel purinergic receptor
P2Y	G-protein-coupled purinergic receptor
VNUT	Vesicular nucleotide transporter

Introduction

Our research has shown that adenosine diphosphate (ADP) signaling through specific G-protein-coupled purinergic receptors (P2Y) perturbs both lipid and protein metabolism in the liver [1, 2]. Extracellular nucleotides are known to activate nuclear factor kappa B [3, 4] and trigger the release of proinflammatory cytokines [5, 6]. Elevated circulating nucleotide levels and sustained purinergic signaling may consequently promote inflammatory diseases [7, 8]. Consistent with this view, knockout of P2Y and ion channel purinergic receptors (P2X) in mice has been shown to significantly reduce inflammatory disease in the heart, liver, kidney, and other tissues [9–14].

The level of specific nucleotides in the circulation determines the net purinergic signaling consequence and appears to be centrally controlled by the liver [15]. Extracellular nucleotide levels are determined by factors that control both cellular release and extracellular metabolism/degradation of nucleotides. Nucleotides are degraded by membrane adenosine

Electronic supplementary material The online version of this article (doi:10.1007/s11302-014-9419-2) contains supplementary material, which is available to authorized users.

C. Chatterjee · D. L. Sparks (✉)
Atherosclerosis, Genetics and Cell Biology Group, University of
Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario K1Y 4 W7,
Canada
e-mail: dsparks@ottawaheart.ca

triphosphate (ATP)-metabolizing proteins and by specific ectonucleotidases, including NTPDase1, 2, 3, and 8 and CD73 [16, 17]. Knockout of the NTPDase1 (CD39) in mice causes an elevation in blood nucleotides and a metabolic syndrome phenotype (inflammation, hypertriglyceridemia, and insulin resistance) [18].

Nucleotides are released from the cell through connexin hemichannels and pannexin channels and through exocytotic secretory pathways [19]. The channels are known to mediate a conductive release of ATP and UTP, while the vesicular nucleotide transporter (VNUT) transports nucleotides into dense-core granules and vesicles for Ca^{2+} -regulated exocytosis. Nucleotide release has been shown to involve P2X receptors [20, 21] and human liver cells contain mRNA for both P2X4 and P2X7 [22]. Studies have shown that activation of P2X7 and P2X4 receptors will increase intracellular $[\text{Ca}^{2+}]$ and stimulate ATP release in different cell systems [23, 24]. The gated ion channel, P2X7 receptor, has also been shown to activate both the immune and inflammatory responses [25, 26]. Fibroblasts from type 2 diabetic patients show 2–3-fold increase in ATP secretion [27] and enhanced inflammatory and cytotoxic responses through the P2X7 receptor [28].

In an attempt to clarify how extracellular nucleotide levels may be associated with hepatic cell metabolism, we have characterized nucleotide release from human hepatic carcinoma cells. A previous work has shown that cellular metabolism and extracellular nucleotide signaling are similar in primary human hepatocytes and human hepatic cell lines [29, 30]. We now show that hepatic cells rapidly release nucleotides after a change in media, and we have made the completely novel finding that hepatocarcinoma cells release ADP. Extracellular ADP levels can increase 10-fold as a consequence of cellular stress resulting from nutrient deprivation, P2X7 activation, and increased intracellular $[\text{Ca}^{2+}]$. This finding may partly explain why chronic cellular stress in metabolic disorders is associated with higher blood nucleotide levels and sustained purinergic activation of inflammatory pathways [2, 7, 8].

Materials and methods

Reagents

A438079 hydrochloride was purchased from R&D Systems Inc. (Minneapolis, MN). Ionomycin calcium salt and 2′3′-*O*-(4-benzoyl-benzoyl)-adenosine 5′-triphosphate triethylammonium salt (BzATP) were purchased from Sigma-Aldrich (Oakville, ON). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Burlington, ON). The antibodies to P2X7 (rabbit monoclonal [EPR4723] cat# ab109246), P2X4 (mouse polyclonal cat# ab168939), and adenylate kinase (AK1) (mouse monoclonal [19G4] cat#

117862) were obtained from Abcam (Cambridge, MA), while antibodies to β -actin (rabbit polyclonal cat# 4967) were purchased from Cell Signaling Technology (Danvers, MA). Affinity-purified peroxidase-linked sheep anti-mouse (cat# NA931V) and donkey anti-rabbit (cat# NA934V) antibodies were purchased from GE Healthcare Life Sciences (UK). All Stars Negative control small interference RNA (siRNA) was purchased from Qiagen (Mississauga, ON) and human P2X7, P2X4, and AK1 siRNA were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Inhibitors were of analytical grade and were solubilized in ddH₂O or dimethyl sulfoxide (DMSO).

Cells and cell culture

Human hepatic carcinoma, HepG2, cells were regularly maintained in DMEM (5 mM glucose) containing 10 % FBS and 1 % penicillin/streptomycin. Passages 4–10 were used and cells that were 80 % confluent were treated with the indicated concentrations of chemical reagents, serum, and glucose for various times as indicated.

Quantification of nucleotide release

Nucleotide release from liver cells was quantified using the bioluminescent EnzyglightTM ADP Assay Kit from BioAssay Systems (Hayward, CA) and according to the manufacturer’s recommended protocols. The assay utilizes luciferase to convert ATP and D-luciferin to oxyluciferin and light in a first reaction, where light intensity is a direct measure of ATP concentration. ADP is then converted to ATP, in a second reaction, and then ATP and ADP concentrations were quantified by subtraction. After treatment, liver cell media aliquots were harvested from $\sim 5 \times 10^6$ cells/well and then frozen at -20°C . Samples were thawed immediately prior to the assay and 20 μL /well was transferred to a 96-well black luminometer plate. The luciferin-luciferase reagents were added to the samples and the luminescence was quantified in a luminometer (Promega Glomax, 96-well plate reader). Media nucleotide levels were unchanged after RT storage for 2 h or -20°C storage for 6 months.

Knockdown of human P2 receptors and adenylate kinase

HepG2 cells were transiently transfected with All Stars Negative control siRNA or P2X7, P2X4, and AK1 siRNA sequences by reverse transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 12-well plates. In brief, complexes were prepared per manufacturer’s specifications with a Lipofectamine 2000-to-siRNA volume-to-mole ratio of 2 μL :40 pmol in 200 μL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). Lipofectamine-siRNA complexes were added to the cells immediately after the cells

were seeded at a density of 500,000 cells/well in a volume of 1 mL of normal growth media containing 10 % FBS in the absence of penicillin/streptomycin. Cell media and lysate samples were harvested at the indicated time points for immunoblot analysis. Transfection of the control and test siRNA caused no cytotoxic effects.

Immunoblot analysis

After treatment for the indicated time points, cells were washed twice with ice-cold PBS. Cells were lysed in NP-40 lysis buffer (Biosource, Camarillo, CA) supplemented with 1 mM PMSF and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). Cell protein concentrations were determined by the BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Cell lysate samples containing equal total protein (30 µg) were separated by SDS-PAGE and analyzed by Western blot using specific antibodies to P2X7, P2X4, AK1, and β -actin. Blots were exposed using the Alpha Innotech FluorChemTM HD Imager, and band intensities were quantified with the Alpha Ease FCTM software.

Statistical analysis

The significant difference between group mean values was evaluated using the SigmaStat 3.5 software (Systat Software Inc., San Jose, CA) to perform a one-way analysis of variance on ranks by a pairwise multiple comparison using the Student-Newman-Keuls post hoc test. Values are shown as mean $\pm$ SD for three independent experiments and $P < 0.05$ was considered significant.

Results

Extracellular nucleotides in liver cell media

Nucleotide levels in the media of confluent human hepatic carcinoma cells were quantified with an EnzyglightTM bioluminescent assay and shown to be in low nanomolar concentration, with nucleotide levels averaging ~20 nM (Fig. 1). Studies on other human cell lines have shown similar extracellular nucleotide levels for resting cells [31]. A change in nutrient-complete cell media (10 % FBS and 5 mM glucose) had no effect on extracellular nucleotide levels (Fig. 1). A change to serum-reduced media (1 % FBS and 5 mM glucose) had no effect on ATP levels, but resulted in a 5-fold increase in ADP accumulation at 5 min (Fig. 1). Glucose deprivation had a similar effect. The change to a glucose-deficient media had no effect on ATP levels, but was associated with a 2-fold increase in ADP release over that observed with FBS depletion (Fig. 2a, b). ADP levels reached maximal levels at 5 min

and then decreased by ~50 % over 2 h. This suggests that media ADP is metabolized or degraded over time. We then evaluated the importance of AK1 expression on nucleotide release and showed that knockdown of AK1 with siRNA had no effect on media nucleotide levels (Supplemental Fig. 1).

Effect of intracellular Ca^{2+} on nucleotide release

A previous study has shown that nucleotide secretion is sensitive to intracellular Ca^{2+} levels and stimulated by Ca^{2+} ionophores, i.e., ionomycin [23]. DMSO-solubilized ionomycin (10 µM) had a minimal effect on [ATP] (Fig. 3a), but increased ADP release from hepatic cells ~2-fold at 5 min, compared to control cells (Fig. 3b). Of note, we found that the ionomycin carrier, DMSO (1 %), stimulates both ATP and ADP release from hepatic cells on its own (Fig. 2 vs Fig. 3, controls). The P2 receptor agonist, BzATP, has been shown to act through P2X receptors to increase intracellular [Ca^{2+}] and stimulate ATP release [23, 24]. BzATP (10 µM) greatly increased media ATP and ADP levels at 5 min, by ~100- to 300-fold (Figs. 4b and 5b). BzATP also stimulates ADP release and accumulation over 2 h, in parallel to ATP clearance from the media.

Effect of P2X receptor expression on ATP release

Gene silencing experiments were undertaken to determine the importance of P2X receptors on nucleotide secretion. Suppression of P2X receptor expression had a significant effect on ADP release. Knockdown of P2X7 or P2X4 protein expression (by 50 and 70 %, respectively) with siRNA significantly reduced the release of ADP by about 30–40 %, but had a little effect on ATP levels (Figs. 4a and 5a). In contrast, knockdown of P2X7 and P2X4 receptor expression by gene silencing had no effect on BzATP-induced nucleotide release at 5 min (Figs. 4b and 5b). Treatment with the P2X receptor inhibitor, A438079, had similar consequences to P2X receptor gene silencing. Treatment of hepatic cells with A438079 (100 µM) also reduced ADP release at 5 min, but had no effect on BzATP-induced nucleotide release (Supplemental Fig. 2).

Discussion

Extracellular nucleotide concentration is normally maintained at low concentrations to limit purinergic signaling [32]. Nucleotide levels are controlled by cellular release and extracellular degradation. The extracellular nucleotide milieu thereby acts through specific P2X and P2Y receptors to promote a purinergic signaling response. Acute and short-lived nucleotide signaling is required for normal cellular function, while

Fig. 1 Serum deprivation stimulates nucleotide release. Hepatic carcinoma cells were incubated in nutrient-complete (10 % FBS, 5 mM glucose) DMEM overnight, and then the media was changed to DMEM containing 5 mM glucose and 10 or 1 % FBS. Media aliquots were sampled over a 120-min period and media ATP and ADP concentrations were determined by bioluminescence assay. Values are expressed as mean±SD of three independent experiments. * $P<0.01$ vs control, ** $P<0.001$ vs control

chronic purinergic signaling appears to have negative effects on insulin receptor (IR- β) signaling [1, 33] and cellular inflammatory pathways [7, 8].

We have shown that extracellular nucleotides play an important regulatory role in both insulin receptor signaling and cellular autophagy and thereby regulate protein secretion from human hepatic carcinoma cells [1]. The factors that regulate nucleotide release and metabolism by hepatic cells were therefore evaluated. ADP levels in the cell media are normally maintained at nanomolar concentration and equimolar to media ATP levels, but can increase by orders of magnitude with a stress response. A change in cell media normally has a

minimal effect on extracellular nucleotide levels, unless the media is deficient in nutrients, i.e., serum or glucose. Serum (FBS) or glucose deprivation promotes minimal change in media ATP levels, but up to a 10-fold increase in ADP release (Figs. 1, 2, and 3). ADP release appears to be a novel finding, as most other studies have only measured and reported effects on cellular ATP release [19]. ADP release is also stimulated by the calcium ionophore, ionomycin, which had a little effect on ATP levels (Fig. 3). Both the cellular stress response and calcium ionophores may therefore stimulate ADP release by increasing intracellular $[Ca^{2+}]$. Consistent with this view, DMSO was also shown to stimulate ADP release (Fig. 3, control vs Fig. 2), and DMSO is known to increase intracellular $[Ca^{2+}]$ by triggering the release of Ca^{2+} stores [34]. Media nucleotide levels were shown to decrease over time (Figs. 1, 2, 3, 4, and 5), which suggests that hepatic cell media may contain nucleotidase activity. Extracellular nucleotide levels therefore appear to be a product of both release and degradation.

Intracellular ATP is equally distributed between the nucleus, mitochondria, and cytosol in hepatic cells [35], while ADP levels are normally very low. This suggests that ADP release from hepatic cells is likely to involve a regulated pathway. Exocytic nucleotide release may involve the VNUT, which transports nucleotides into dense-core granules and vesicles for Ca^{2+} -regulated exocytosis [19]. In this study, we show that ADP release is stimulated by nutrient deprivation, P2X7 receptor activation, and increased intracellular $[Ca^{2+}]$. P2X7 and Ca^{2+} pathways are known to stimulate the exocytic release of nucleotides in other cell lines [20, 21] and may act through VNUT secretory pathways [36]. Nutrient deprivation is also known to increase intracellular Ca^{2+} [37], which may suggest that serum and glucose deprivation stimulate a Ca^{2+} -dependent exocytic release of ADP. ADP release may also be

Fig. 2 Glucose deprivation stimulates nucleotide release. Hepatic cells were incubated in nutrient-complete media overnight and then the media was changed to FBS-deficient DMEM containing 0, 5, and 25 mM glucose. Media aliquots were sampled over a 120-min period and media

ATP (a) and ADP (b) concentrations were determined by bioluminescence assay. Values are expressed as mean±SD of three independent experiments. * $P<0.001$ vs control

Fig. 3 Calcium ionophores stimulate ADP release. Hepatic cells were incubated overnight and then the media was changed to FBS-deficient DMEM. Cells were treated with the Ca^{2+} ionophore, ionomycin (10 μM), in 1 % DMSO. Media aliquots were sampled over a 120-min period and

media ATP (a) and ADP (b) concentrations were determined by bioluminescence assay. Values are expressed as mean $\pm$ SD of three independent experiments. * P <0.01 vs control

facilitated by membrane channels, i.e., connexin hemichannels and pannexin, as described for ATP [38] or through membrane ATPases, such as $\text{F}_1\text{-ATPase}$ and ABC transporters [39]. BzATP is a potent agonist of ATP release from hepatic cells (Figs. 4 and 5) and can promote ATP release through pannexin channels [38]. In hepatic cells, BzATP primarily affects ATP release and promotes increasing ADP levels in parallel to the clearance of ATP from the media (Fig. 4b). This

suggested that ADP may be a product of the degradation of newly released ATP. BzATP may increase ADP levels in a similar fashion to stress and ionomycin, by increasing intracellular $[\text{Ca}^{2+}]$ and may also act through membrane pannexin channels to stimulate ATP release.

Studies have shown that nucleotide release may involve both G-protein-coupled P2Y receptors [31] and P2X receptors [20, 21]. Nucleotide release is affected by intracellular $[\text{Ca}^{2+}]$

Fig. 4 P2X7 receptor inactivation blocks ADP release. Hepatic cells were transfected with either negative control (si-Ctrl) or P2X7 siRNA and incubated for 72 h. Hepatic cells were incubated overnight and then the media was changed to FBS-deficient DMEM and media aliquots were sampled from P2X7 siRNA-treated cells (a). In a separate series of

experiments, P2X7 siRNA-transfected cells were treated with 10 μM of the P2 receptor agonist, BzATP, and media aliquots were sampled (b). Media ATP and ADP concentrations were determined by bioluminescence assay and values are expressed as mean $\pm$ SD of three independent experiments. * P <0.001 vs control or BzATP, ** P <0.05 vs control

Fig. 5 P2X4 receptor inactivation blocks ADP release. Hepatic cells were transfected with either negative control (si-Ctrl) or P2X4 siRNA and incubated for 72 h. Hepatic cells were incubated overnight and then the media was changed to a FBS-deficient DMEM and media aliquots were sampled from P2X4 siRNA-treated cells (a). In a separate series of

experiments, P2X4 siRNA-transfected cells were treated with 100 μM of the P2 receptor agonist, BzATP, and media aliquots were sampled (b). Media ATP and ADP concentrations were determined by bioluminescence assay and values are expressed as mean±SD of three independent experiments. * $P < 0.01$ vs control

and P2X7 [39]. The gated ion channel, P2X7 receptor, has been shown to stimulate nucleotide release and activate both the immune and inflammatory response [25, 26]. Most of the studies that have reported the effect of P2X7 on nucleotide release have stimulated this receptor with the agonist, BzATP. To confirm the view that BzATP stimulates nucleotide release by activating P2X receptors, we evaluated the effects of specific siRNA, targeted to silence P2X7 and P2X4 gene expression. Knockdown of these receptors significantly inhibited ADP release from hepatic cells (Figs. 4a and 5a), confirming the involvement of P2X receptors in ADP release. However, reducing P2X7 and P2X4 expression with siRNA had no effect on BzATP-induced nucleotide secretion at 5 min (Figs. 4b and 5b). Similar results were observed when hepatic cells were treated with the P2X receptor inhibitor, A438079. This inhibitor also blocked ADP release but had no effect on BzATP-mediated nucleotide release (Supplemental Fig. 2). This unexpected finding suggests that BzATP may act through some other P2 receptor to promote nucleotide release. Earlier reports suggest that P2Y receptors may play important roles. P2Y receptors can also activate pannexin channels [40] and BzATP is known to activate P2Y receptors [41] and stimulate ATP release from hepatic cells [42].

In summary, this work shows that nutrient deprivation, P2X activation, or the calcium ionophore, ionomycin, causes a stress response in hepatic cells that results in stimulation of ADP release and accumulation in the cell media. We have

previously shown that extracellular ADP blocks insulin receptor signaling and thereby stimulates cellular autophagy and perturbs protein secretion [1]. This work may therefore illustrate how nutrient deprivation initiates a cellular response to starvation through an autocrine and paracrine control of purinergic signaling. Elevated levels of extracellular nucleotides, and ADP in particular, may consequently disturb cellular metabolism and promote pathologic consequences to cellular metabolism.

References

- Chatterjee C, Sparks DL (2012) Extracellular nucleotides inhibit insulin receptor signaling, stimulate autophagy and control lipoprotein secretion. *PLoS ONE* 7:e36916
- Sparks DL, Chatterjee C (2012) Purinergic signaling, dyslipidemia and inflammatory disease. *Cell Physiol Biochem* 30:1333–1339
- von Albertini M, Palmethofer A, Kaczmarek E, Koziak K, Stroka D, Grey ST, Stuhlmeier KM, Robson SC (1998) Extracellular ATP and ADP activate transcription factor NF-kappa B and induce endothelial cell apoptosis. *Biochem Biophys Res Commun* 248:822–829
- Aga M, Watters JJ, Pfeiffer ZA, Wiepz GJ, Sommer JA, Bertics PJ (2004) Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-kappa B signaling pathways in murine RAW 264.7 macrophages. *Am J Physiol Cell Physiol* 286:C923–C930
- Khakh BS, North RA (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442:527–532

6. Trautmann A (2009) Extracellular ATP in the immune system: more than just a “danger signal”. *Sci Signal* 2:e6
7. DiVirgilio F, Solini A (2002) P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol* 135:831–842
8. Sellers MB, Tricoci P, Harrington RA (2009) A new generation of antiplatelet agents. *Curr Opin Cardiol* 24:307–312
9. Hohenstein B, Renk S, Lang K, Daniel C, Freund M, Leon C, Amann KU, Gachet C, Hugo CP (2007) P2Y1 gene deficiency protects from renal disease progression and capillary rarefaction during passive crescentic glomerulonephritis. *J Am Soc Nephrol* 18:494–505
10. Hechler B, Freund M, Ravanat C, Magnenat S, Cazenave JP, Gachet C (2008) Reduced atherosclerotic lesions in P2Y1/apolipoprotein E double-knockout mice: the contribution of non-hematopoietic-derived P2Y1 receptors. *Circulation* 118:754–763
11. Ji X, Naito Y, Weng H, Endo K, Ma X, Iwai N (2012) P2X7 deficiency attenuates hypertension and renal injury in deoxycorticosterone acetate-salt hypertension. *Am J Physiol Renal Physiol* 303:F1207–F1215
12. Ayata CK, Ganai SC, Hockenjos B, Willim K, Vieira RP, Grimm M, Robaye B, Boeynaems JM, Di VF, Pellegatti P, Diefenbach A, Idzko M, Hasselblatt P (2012) Purinergic P2Y(2) receptors promote neutrophil infiltration and hepatocyte death in mice with acute liver injury. *Gastroenterology* 143:1620–1629
13. Sun S, Xia S, Ji Y, Kersten S, Qi L (2012) The ATP-P2X7 signaling axis is dispensable for obesity-associated inflammasome activation in adipose tissue. *Diabetes* 61:1471–1478
14. Hoque R, Sohail MA, Salhanick S, Malik AF, Ghani A, Robson SC, Mehal WZ (2012) P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice. *Am J Physiol Gastrointest Liver Physiol* 302:G1171–G1179
15. Fustin JM, Doi M, Yamada H, Komatsu R, Shimba S, Okamura H (2012) Rhythmic nucleotide synthesis in the liver: temporal segregation of metabolites. *Cell Rep* 1:341–349
16. Kukulski F, Levesque SA, Sevigny J (2011) Impact of ectoenzymes on p2 and p1 receptor signaling. *Adv Pharmacol* 61:263–299
17. Deaglio S, Robson SC (2011) Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Adv Pharmacol* 61:301–332
18. Enjyoji K, Kotani K, Thukral C, Blumel B, Sun X, Wu Y, Imai M, Friedman D, Csizmadia E, Bleibel W, Kahn BB, Robson SC (2008) Deletion of cd39/entpd1 results in hepatic insulin resistance. *Diabetes* 57:2311–2320
19. Lazarowski ER (2012) Vesicular and conductive mechanisms of nucleotide release. *Purinergic Signal* 8:359–373
20. Manohar M, Hirsh MI, Chen Y, Woehrle T, Karande AA, Junger WG (2012) ATP release and autocrine signaling through P2X4 receptors regulate gammadelta T cell activation. *J Leukoc Biol* 92:787–794
21. Gutierrez-Martin Y, Bustillo D, Gomez-Villafuertes R, Sanchez-Nogueiro J, Torregrosa-Hetland C, Binz T, Gutierrez LM, Miras-Portugal MT, Artalejo AR (2011) P2X7 receptors trigger ATP exocytosis and modify secretory vesicle dynamics in neuroblastoma cells. *J Biol Chem* 286:11370–11381
22. Emmett DS, Feranchak A, Kilic G, Puljak L, Miller B, Dolovcak S, McWilliams R, Doctor RB, Fitz JG (2008) Characterization of ionotrophic purinergic receptors in hepatocytes. *Hepatology* 47: 698–705
23. Gutierrez-Martin Y, Bustillo D, Gomez-Villafuertes R, Sanchez-Nogueiro J, Torregrosa-Hetland C, Binz T, Gutierrez LM, Miras-Portugal MT, Artalejo AR (2011) P2X7 receptors trigger ATP exocytosis and modify secretory vesicle dynamics in neuroblastoma cells. *J Biol Chem* 286:11370–11381
24. Brandao-Burch A, Key ML, Patel JJ, Amett TR, Orriss IR (2012) The P2X7 receptor is an important regulator of extracellular ATP levels. *Front Endocrinol (Lausanne)* 3:41
25. DiVirgilio F, Boeynaems JM, Robson SC (2009) Extracellular nucleotides as negative modulators of immunity. *Curr Opin Pharmacol* 9:507–513
26. Bours MJ, Dagnelie PC, Giuliani AL, Wesselius A, Di VF (2011) P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. *Front Biosci (Schol Ed)* 3:1443–1456
27. Solini A, Chiozzi P, Morelli A, Passaro A, Fellin R, Di VF (2003) Defective P2Y purinergic receptor function: a possible novel mechanism for impaired glucose transport. *J Cell Physiol* 197:435–444
28. Solini A, Chiozzi P, Morelli A, Adinolfi E, Rizzo R, Baricordi OR, Di VF (2004) Enhanced P2X7 activity in human fibroblasts from diabetic patients: a possible pathogenetic mechanism for vascular damage in diabetes. *Arterioscler Thromb Vasc Biol* 24:1240–1245
29. Schofl C, Ponczek M, Mader T, Waring M, Benecke H, von zur MA, Mix H, Cornberg M, Boker KH, Manns MP, Wagner S (1999) Regulation of cytosolic free calcium concentration by extracellular nucleotides in human hepatocytes. *Am J Physiol* 276:G164–G172
30. Pandey NR, Renwick J, Misquith A, Sokoll K, Sparks DL (2008) Linoleic acid-enriched phospholipids act through peroxisome proliferator-activated receptors alpha to stimulate hepatic apolipoprotein A-I secretion. *Biochemistry* 47:1579–1587
31. Sumi Y, Woehrle T, Chen Y, Yao Y, Li A, Junger WG (2010) Adrenergic receptor activation involves ATP release and feedback through purinergic receptors. *Am J Physiol Cell Physiol* 299:C1118–C1126
32. Dwyer KM, Deaglio S, Gao W, Friedman D, Strom TB, Robson SC (2007) CD39 and control of cellular immune responses. *Purinergic Signal* 3:171–180
33. Mistafa O, Hogberg J, Stenius U (2008) Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells. *Biochem Biophys Res Commun* 365:131–136
34. Morley P, Whitfield JF (1993) The differentiation inducer, dimethyl sulfoxide, transiently increases the intracellular calcium ion concentration in various cell types. *J Cell Physiol* 156:219–225
35. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H (2009) Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci U S A* 106:15651–15656
36. Takai E, Tsukimoto M, Harada H, Sawada K, Moriyama Y, Kojima S (2012) Autocrine regulation of TGF-beta1-induced cell migration by exocytosis of ATP and activation of P2 receptors in human lung cancer cells. *J Cell Sci* 125:5051–5060
37. Colombaioni L, Frago LM, Varela-Nieto I, Pesi R, Garcia-Gil M (2002) Serum deprivation increases ceramide levels and induces apoptosis in undifferentiated HN9.10e cells. *Neurochem Int* 40: 327–336
38. Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, Scemes E (2008) P2X7 receptor-Pannexin1 complex: pharmacology and signaling. *Am J Physiol Cell Physiol* 295:C752–C760
39. Lohman AW, Billaud M, Isakson BE (2012) Mechanisms of ATP release and signalling in the blood vessel wall. *Cardiovasc Res* 95: 269–280
40. Locovei S, Wang J, Dahl G (2006) Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium. *FEBS Lett* 580:239–244
41. Suplat-Wypych D, Dygas A, Baranska J (2010) 2', 3'-O-(4-benzoylbenzoyl)-ATP-mediated calcium signaling in rat glioma C6 cells: role of the P2Y(2) nucleotide receptor. *Purinergic Signal* 6: 317–325
42. Feranchak AP, Roman RM, Schwiebert EM, Fitz JG (1998) Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. *J Biol Chem* 273:14906–14911