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ADP regulation of insulin secretion and beta-cell apoptosis

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Cover: Photograph of insulin (green) and apoptotic Caspase-3 (red) signals in mouse islet

To my family

“To love and to be loved is the greatest happiness of existence.”

(Sydney Smith)

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ABSTRACT

The aims of this dissertation were to investigate the effects of extracellular purines on insulin secretion, and apoptosis in mouse pancreatic islets and β -cells; to examine if high glucose and free fatty acids induces β -cell apoptosis via autocrine effects of ADP acting on the P2Y₁₃ receptor; and to investigate the modulation of extracellular purines in vascular smooth muscle cells (VSMCs) through ectonucleotidase or ATP release under the effect of high glucose.

The expression of the ADP receptors P2Y₁ and P2Y₁₃ were shown in both mouse pancreatic islets and isolated β -cells using real-time PCR quantification. Insulin and glucagon secretion were measured both in mouse islets and in vivo. Results showed that ADP acting on P2Y₁ receptors stimulated insulin secretion, while acting on P2Y₁₃ receptors inhibited insulin secretion. In MIN6c4 cells, real-time PCR also revealed high expression of the ADP receptors P2Y₁ and P2Y₁₃. 2MeSADP induced calcium influx and inhibited cAMP production by activation of P2Y₁ and P2Y₁₃ respectively. ELISA and cell proliferation studies showed that 2MeSADP increased Caspase-3 activity and reduced cell proliferation. P2Y₁₃ receptor antagonist MRS2211 could fully reverse both of these effects. Western blotting showed that activation of the cAMP/PKA/CREB pathway resulted in amplification of phosphorylation of Akt/PKB, leading to resistance to apoptosis, increase of β -cell viability and proliferation rate. ATP release in MIN6c4 cells was measured by bioluminescence. High glucose and palmitate potently elevated the extracellular ATP levels. The calcium-channel blocker nifedipine, VRAC-channel inhibitor NPPB, pannexin-1 blocker carbenoxolone, or silencing of MDR1 all resulted in a substantial decrease in high glucose/palmitate induced ATP release. Furthermore, high glucose and palmitate inhibited cAMP production, reduced cell proliferation in MIN6c4 and increased activated Caspase-3 in both mouse islets and MIN6c4 cells and all these effects were dependent on the P2Y₁₃ receptor. Western blotting further showed that blocking the P2Y₁₃ receptor resulted in enhanced CREB, Bad and IRS-1 phosphorylation. Real-time PCR study of ectonucleotidase expression in primary rat aortic VSMCs revealed the expression of CD39, CD39L1 and CD73. 48 hours treatment of high glucose significantly decreased CD39 gene expression and increased CD39L1 expression.

Bioluminescence assays also revealed that high glucose caused VSMCs released of ATP and the ectonucleotidase inhibitor ARC67156 led to sustained levels of ATP.

In conclusion, we have shown that activation of the P2Y₁₃ receptor in mouse MIN6c4 cells exhibits a pronounced proapoptotic effect which is mediated by modulating the cAMP/ERKs/CREB/Akt pathway. Autocrine/paracrine effects of ADP acting on the P2Y₁₃ receptor are involved in the proapoptotic effects of high glucose and free fatty acids. P2Y₁₃ antagonist MRS2211, which increased the secretion of insulin and was able to protect the cells from ADP induced apoptosis in β -cells, could be a potential treatment of diabetes. High glucose also increases ATP release from VSMCs and accumulates more ADP by up-regulating the expression of CD39L1, thereby enhance vascular inflammation and VSMC proliferation via P2Y receptor activation.

Key words: ADP, β -cell, P2Y, insulin, apoptosis, autocrine, ectonucleotidase

LIST OF PAPERS

I. *Stefan Amisten, S Meidute-Abaraviciene, Chanyuan Tan, Björn Olde, Ingmar Lundquist, Albert S Salehi, David Erlinge.* ADP mediates inhibition of insulin secretion by activation of P2Y₁₃ receptors in mice. *Diabetologia*. 2010 Sep;53(9):1927-34.

II. *Chanyuan Tan, Albert Salehi, Siv Svensson, Björn Olde, David Erlinge.* ADP receptor P2Y₁₃ induce apoptosis in pancreatic beta-cells. *Cell Mol Life Sci*. 2010 Feb;67(3):445-53.

III. *Chanyuan Tan, Ulrikke Voss, Siv Svensson, David Erlinge, Björn Olde.* High glucose and free fatty acids induce beta-cell apoptosis via autocrine effects of ADP acting on the P2Y₁₃ receptor. *Purinergic Signalling* (in press).

IV. *Chanyuan Tan, Nelly Njukang Ajangakeo, Ihdina Sukma Dewi, Jenny Öhman.* High Glucose Induces ATP Release and Alters Ectonucleotidase Expression in Vascular Smooth Muscle Cells. (Manuscript)

List of papers not included in the thesis:

V. *Carl Högberg, Olof Gidlöf, Chanyuan Tan, Siv Svensson, Jenny Nilsson-Öhman, David Erling and Björn Olde.* Succinate independently stimulates full platelet activation via cAMP and phosphoinositide 3-kinase- β signaling. *J Thromb Haemost*. 2011 Feb;9(2):361-72.

ABBREVIATIONS

[Ca ²⁺] _i	intracellular calcium concentration
2-MeSADP	2-Methylthio-adenosine diphosphate
AC	Adenylyl Cyclase
ADP	adenosine diphosphate
ATP	adenosine 5' triphosphate
AMP	adenosine monophosphate
Bad	BCL2-associated agonist of cell death
BSA	bovine serum albumin
Ca ²⁺	calcium ion
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CO ₂	carbon dioxide
CREB	Ca ²⁺ -cAMP response element-binding protein
C _T	threshold cycle
DAG	diacylglycerol
DM	diabetes mellitus
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum

ERK	extracellular signal-regulated kinase
ENTPD1 (CD39)	ectonucleoside triphosphate diphosphohydrolase 1
ENTPD2 (CD39L1)	ectonucleoside triphosphate diphosphohydrolase 2
ENTPD3 (CD39L3)	ectonucleoside triphosphate diphosphohydrolase 3
ENTPD4 (UDPase)	ectonucleoside triphosphate diphosphohydrolase 4
ENTPD5 (CD39L4)	ectonucleoside triphosphate diphosphohydrolase 5
ENTPD6 (CD39L2)	ectonucleoside triphosphate diphosphohydrolase 6
ENTPD7 (LALP1)	ectonucleoside triphosphate diphosphohydrolase 7
ENTPD8 (NTPDase 8)	ectonucleoside triphosphate diphosphohydrolase 8
FBS	fetal bovine serum
FFA	free fatty acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLP-1	Glucagon-like peptide-1
GLUT2	Glucose transporter 2
GP	glucoprotein
GPCR	G-protein coupled receptor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HDL	high-density lipoprotein
IBMX	3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor)
IGT	impaired glucose tolerance
IL	interleukin

IP	prostaglandin I2 receptor
IP ₃	inositol 1, 4, 5-trisphosphate
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
K ⁺	potassium
K _{ATP} channel	ATP-sensitive potassium channel
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
MDR	multiple drug resistant
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide
Na ⁺	sodium
NO	nitoc oxide
O ₂	oxygen
P2X ₁₋₇	purinergic ligand-gated ion channel P2X receptor 1-7
P2Y _{1, 2, 4, 6, 11-14}	purinergic G-protein coupled P2Y receptors 1, 2, 4, 6, 11-14
p-Bad	Phosphorylated Bad
PCR	polymerase chain reaction
p-CREB	Phosphorylated CREB
p-IRS-1	Phosphorylated IRS-1
PGI ₂	prostaglandin I2
PI3-K	phosphoinositide-3 kinase

PIP2	phosphatidylinositol 4, 5-biphosphate
PIP3	phosphatidylinositol-3, 4, 5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
Q-RT-PCR	quantitative real-time polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse-transcriptase polymerase chain reaction
siRNA	Small-interfering RNA
shRNA	short hairpin RNA
STZ	streptozotocin
VRAC channels	volume-regulated anionic channels
UDP	uridine 5' diphosphate
UTP	uridine 5' triphosphate
VSMC	vascular smooth muscle cell

INTRODUCTION

G-protein coupled receptors family

Signal transduction through G-protein-coupled receptors (GPCRs) is central for the regulation of virtually all cellular functions and has been widely implicated in human disease. During the past several decades, G protein-coupled receptor (GPCR) signaling has been greatly explored and targeted for drug discovery. Today, G protein-coupled receptors (GPCRs) are targets of approximately one-third of the pharmaceutical drugs used in the clinic.

G protein-coupled receptors (GPCRs) have seven membrane-spanning helices connected by three intracellular loops and three extracellular loops with an extracellular amino terminus and an intracellular carboxy terminus¹. The heterotrimeric G proteins is a large family of membrane associated proteins that transduce signals from GPCR receptors and enzymes or ion channels, convert diverse extracellular signals (e.g. hormones, neurotransmitters and sensory signals) to intracellular “second messengers” with resultant rapid effects on hormone secretion, muscle contraction, and a variety of other physiologic functions. Each G protein comprises subunits $G\alpha$, $G\beta$ and $G\gamma$. $G\alpha$ binds guanine nucleotide, while subunits $G\beta$ and $G\gamma$ form a tightly associated dimer that occasionally is referred to as the $G\beta\gamma$ subunit.

$G\alpha$ consists of two domains: a GTPase domain that includes the sites for guanine nucleotide binding and the sites for receptor-effector interaction^{2,3}. In the inactive state, $G\alpha$ exists as a $G\alpha$ - $\beta\gamma$ heterotrimer with GDP bound to its binding pocket. Ligand-bound receptors promote GDP release and binding of ambient GTP, which results in a switch to an active conformation and dissociation from $\beta\gamma$. GTP- $G\alpha$ directly effects adenylyl cyclase (AC) or phospholipase C (PLC) and its other effectors. The turn-off mechanism is an intrinsic GTPase that hydrolyzes bound GTP to GDP^{4,5}. In mammals, there are at least 16 distinct α subunit genes, 5 distinct β subunit genes and at least 12 γ subunit genes. $G\alpha$ subunits are categorized into four groups with regard to effector coupling and sequence similarity: (i) G_s , which stimulates adenylyl cyclase, the enzyme that generates

cAMP; (ii) G_i , which inhibit adenylyl cyclase; (iii) G_q , which stimulates phospholipase C; and (iv) $G_{12/13}$ ⁶ (Figure 1).

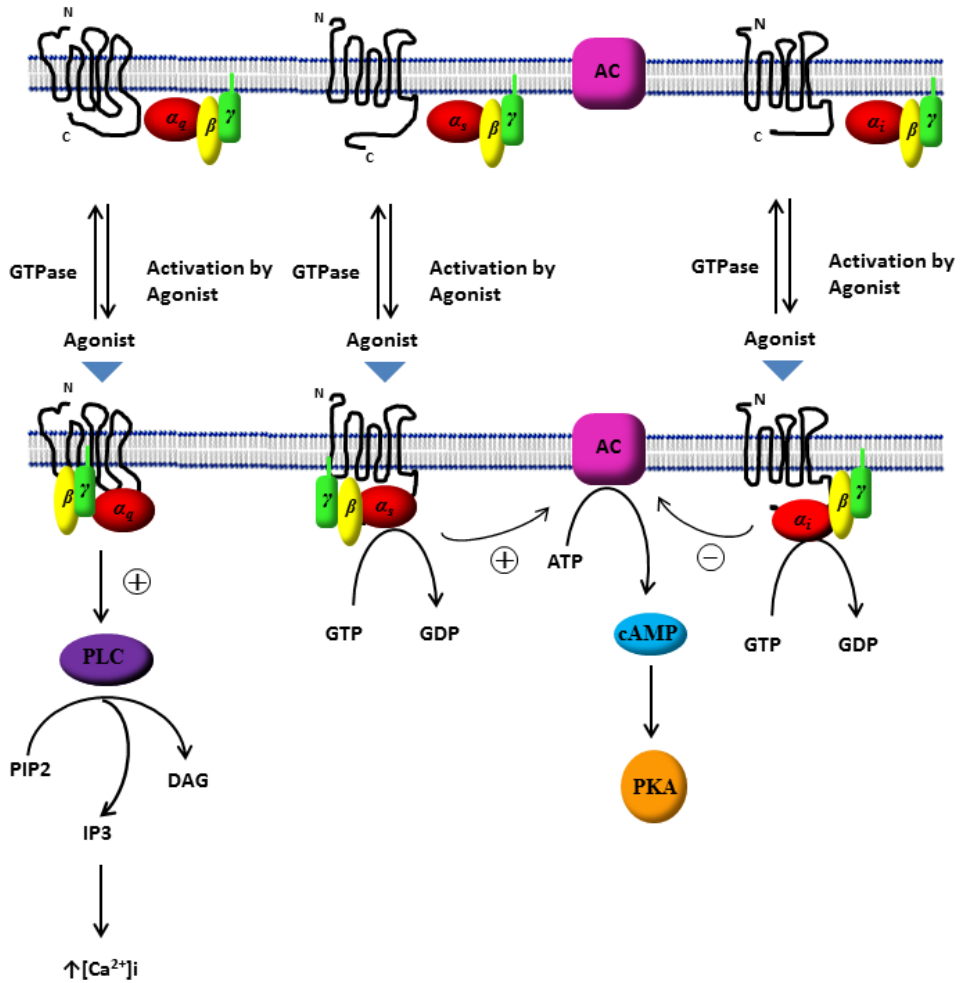


Figure 1. Model of G_s , G_i , G_q protein - coupled receptor activation.

Purinergetic signaling

In recent years it has become accepted that ATP and other nucleotides/nucleosides have extracellular roles. They are (1) released from cells; (2) they act extracellularly via specific purinergetic receptors as autocrine/paracrine regulators and neural transmitters; and (3) they are degraded by various extracellular enzymes ⁷. The following paragraphs will focus on these roles and effects.

Purines

ATP was first discovered in 1929 by the German chemist Karl Lohmann. In 1964, Paul D. Boyer proposed that ATP is synthesized through structural changes in the ATP synthase enzyme and won the Nobel Prize in Chemistry 1997.

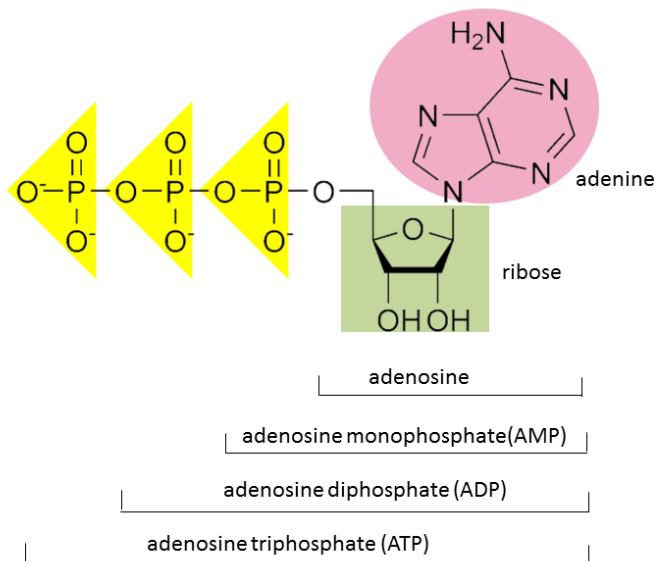


Figure 2. Molecular structure of purines. ATP consists of three phosphate groups (triphosphate), a purine base (adenine) ring and a ribose sugar. ATP could be hydrolyzed to ADP and phosphate, and ADP could be further hydrolyzed to AMP or adenosine and phosphate.

It has now been well established that ATP is a compound that plays a key role in the energy metabolism. It stores and transfers energy in all organisms and mediates numerous cellular metabolic processes like neurotransmission, neuromodulation, cell proliferation, differentiation and death in development and regeneration. ATP consists of three phosphate groups (triphosphate) and adenosine, of which composed of a purine base (adenine) ring and a ribose sugar. ATP is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Multiple metabolic processes use ATP as an energy source through converting it back into its precursors (Figure 2).

Molecular structure of purinergic receptors and their downstream signal pathways

Besides mediating energy, nucleotides also act as extracellular signalling molecule on the cell plasma membrane. Extracellular nucleotides act through purinergic receptors presently is subdivided into three subclasses. P1 or A receptors (4 subtypes) are activated by adenosine and P2X ionotropic nucleotide receptors (subtypes P2X₁₋₇) are ligand-gated ion channels, permeable for Na⁺, K⁺, and also Ca²⁺, that are activated by ATP. The final group, P2Y purine- and pyrimidine-sensitive P2Y receptors (subtypes P2Y_{1, 2, 4, 6, 11-14})⁸ are GPCRs that are linked to G_{αi}, G_{αs} or G_{αq} receptor and activated by different extracellular nucleotides (e.g., ATP, ADP, UTP, UDP, Ap(n)A), and mediate intracellular signaling preferably through activation of phospholipase C or adenylyl cyclase, thereby controlling a diversity of biological processes^{9; 10; 11}. These purinergic receptors have a widespread distribution and effects on many cells and neuron, they are therapeutic targets for many diseases, including stroke, thrombosis, osteoporosis, kidney failure, bladder incontinence, cystic fibrosis, dry eye, cancer and brain disorders¹².

Ectonucleotidases degradation

Extracellular nucleotide P2-receptor-mediated effects are both inactivated and modulated by ecto-nucleotidases. These enzymes, present in the circulation and on cell surfaces, hydrolyze extracellular ATP into ADP, AMP, and adenosine^{13; 14}.

Ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), are $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent enzymes that hydrolyzes extracellular nucleotides 5'-triphosphates (ATP) and nucleoside 5'-diphosphate (ADP)¹⁵, thereby terminate P2 receptor signaling, modulate receptor desensitization, alter specificities of the response.

Eight members of the E-NTPDase family have been cloned and functionally identified^{16, 17}, and they include cell-surface (NTPDases1-3, 8), intracellular (NTPDases4, 5, 7), and cell-surface/intracellular (NTPDase6) enzymes. The presently identified members are: CD39 (ENTPD1), CD39L1 (ENTPD2), CD39L3 (ENTPD3), UDPase (ENTPD 4), CD39L4 (ENTPD5), CD39L2 (ENTPD6), LALP1 (ENTPD7), and NTPDase 8 (ENTPD8). The most common ecto-nucleotidase expressed by the vascular endothelial cell, by monocytes and vascular smooth muscle cells is CD39. CD39 (ENTPD1; NTPDase1; apyrase) is an ecto-ATPase and ecto-ADPase, which hydrolyzes both ATP and ADP in the plasma to AMP, ultimately to adenosine¹⁸. A related protein CD39L (Like)1 (ENTPD2; NTPDase2) associated with the vasculature, is a preferential nucleoside triphosphatase ecto-ATPase, which preferentially converts ATP to ADP^{19; 20}. The enzymatic chain initiated by E-NTPDases and/or other ectoenzymes is terminated by ecto-5'-nucleotidase CD73²¹, which degrades AMP to adenosine.

Sources and Mechanism of ATP release

Intracellular ATP is generated by glycolysis and mitochondrial oxidative metabolism. External ATP is released from two distinct sources: secretory granules, nerve terminals and from damaged tissue.

Many cells exhibit regulated release of ATP, which can be mobilized within seconds in response to changing physiologic demands. The phenomenon of ATP release was first observed in peripheral and central neurons²². ATP release was later discovered in other cell types in response to receptor stimulation, cellular stress such as hypoxia, acidosis, and mechanical stimulation^{23; 24; 25}.

ATP can be released from many cells by various mechanism and pathways. Many transporters and membrane channels were demonstrated to mediate cellular release of ATP, including the pore-forming P2X₇ receptor, multidrug resistance protein,

p-glycoprotein, cystic fibrosis transmembrane conductance regulator (CFTR), volume-regulated anion channels^{26; 27; 28; 29; 30; 31}, and connexin and pannexin hemichannels^{32; 33; 34; 35}. ATP is also released through calcium-dependent vesicular exocytosis and might co-release with insulin during the exocytosis of secretory granules. When released into the extracellular space, ATP and other nucleotides function as mediators of intercellular communication.

Autocrine and paracrine of Purinergic signaling

Stimulated ATP release from mammalian cells is a newly discovered physiologic process that enables ATP and its metabolites to induce autocrine/paracrine modulation of a broad range of cell and organ functions through activation of purinergic receptors in the plasma membrane^{36; 37; 38}.

It has been shown that high glucose levels stimulate the release of ATP from several tissues and cell types such as endothelial cells, blood vessels, mesangial cells, macrophages and β -cells^{39; 40; 41; 42; 43}. The fact that glucose stimulation, of pancreatic β -cells, has been reported to generate extracellular concentrations of ATP that exceeds 25 μ M⁴² highlights the importance of this process.

Diabetes

Pathology and characters of diabetes

Today, around 250 million people world-wide are living with diabetes and by 2025 this total is expected to increase to over 380 million. Diabetes mellitus is caused by the body not being able to control its blood sugar level through insulin and it has been recognized as a growing world-wide epidemic. Clinical investigations have established that gluco- and lipotoxicity are responsible for the progression and complications of diabetes and underscored the role of hyperglycemia in the pathogenesis of the disease. Biochemical investigations have

revealed a large number of mechanisms responsible for the toxicity of high glucose and lipid concentrations⁴⁴.

Regulation of blood glucose

Glucose is the energy supplier for most tissues. Insulin and glucagon are the major hormones that regulate the blood glucose concentration.

Glucose-lowering hormone insulin is produced and secreted by pancreatic β -cells. Insulin secretion is initiated when levels of blood sugar rise, and causes the tissues to increase glucose uptake and the liver to convert more glucose into glycogen by glycogenesis, thus to decrease blood sugar levels. A defect in insulin secretion or insulin action results in impaired glucose tolerance (IGT), which causes hyperglycemia and progress towards diabetes mellitus.

Glucagon, which has a function opposed to insulin, is produced and secreted by pancreatic alpha cells. If the blood glucose level falls, the pancreatic alpha cells release glucagon and increase glucose level in the circulation. This hormone increases glucose concentration by acting on liver cells, which convert glycogen storage into glucose by glycogenolysis. Glucose is then released into the blood stream, thereby increasing blood sugar levels.

The pancreatic endocrine system

Pancreas

The pancreas performs both endocrine and exocrine functions. The endocrine tissue of the pancreas consists of specialized hormone-producing cells called the islets of Langerhans. The islets of Langerhans are dispersed throughout the organ and comprise α , β , δ and PP cells, which secrete the hormones glucagon, insulin and amylin, somatostatin and pancreatic polypeptide, respectively⁷.

Recently, there are increasing evidence showing that both endocrine and exocrine cells express purinergic receptors, which influence processes such as insulin secretion and epithelial ion transport. It has been shown that β -cells express different molecular subtypes of P2 and adenosine receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2X subtypes and A₁ receptors) and glucagon-secreting α cells also express P2 and adenosine receptors (P2X₇, A₂ receptors)^{45; 46; 47; 48; 49}. Nucleotides released by pancreatic cells acting on membrane purinergic receptors exert a physiological autocrine or paracrine modulation in pancreatic islets.

Purinergic signaling is involved in regulation of insulin release

Rising blood glucose levels triggers insulin secretion in β -cells. The glycolytic phosphorylation of external glucose causes a rise in the cytosolic ATP:ADP ratio. This rise leads to the closure of ATP-sensitive potassium channels (K_{ATP} channel) in the plasma membrane and a decrease of potassium outflow⁵⁰ resulting in depolarization of the plasma membrane, which thereby induces opening of voltage-dependent calcium channel (Ca²⁺), leading to an influx of extracellular calcium and elevation of cytosolic calcium, that in turn triggers exocytosis of insulin secretory granules (Figure 3). In presence of glucose, the secretion of insulin from β -cell is pulsatile⁵¹, and this property is related to rhythmic oscillations of intracellular calcium concentration⁵². The pulsatility of the secretion is essential in avoiding the down-regulation of the insulin receptor⁵³ and the development of insulin-resistance⁵⁴.

A large number of studies indicate that external ATP and ADP also have key roles in β -cell function and insulin secretion mediated by activating cell-membrane purinergic receptors of both ionotropic and metabotropic subtypes^{55; 56; 57; 58; 59} and by interacting with intracellular Ca²⁺ signals. ATP was first proposed at 1997 to play a role in the coordination of β -cell calcium signals and synchronization of calcium-dependent events⁶⁰. β -cells could both receive neural and secretory ATP signals with coordinating effects on the Ca²⁺ oscillations, ATP generates [Ca²⁺]_i transients which activate exocytosis, resulting in intermittent release of ATP⁶¹. This ATP signal message, combined with gap junction coupling, is propagated to adjacent cells and thereby affects Ca²⁺ oscillations in adjacent cells. The subtype of

receptors implicated in these effects needs further study. In some studies, ADP has been shown to increase insulin release via P2Y receptors both in vitro and in vivo^{55; 56; 57}. However, ADP has also been shown to be able to reduce insulin release in mice^{58; 59}. The reason for these discrepancies is probably due to involvement of several different purinoreceptors^{59; 61; 62; 63; 64; 65; 66; 67; 68}.

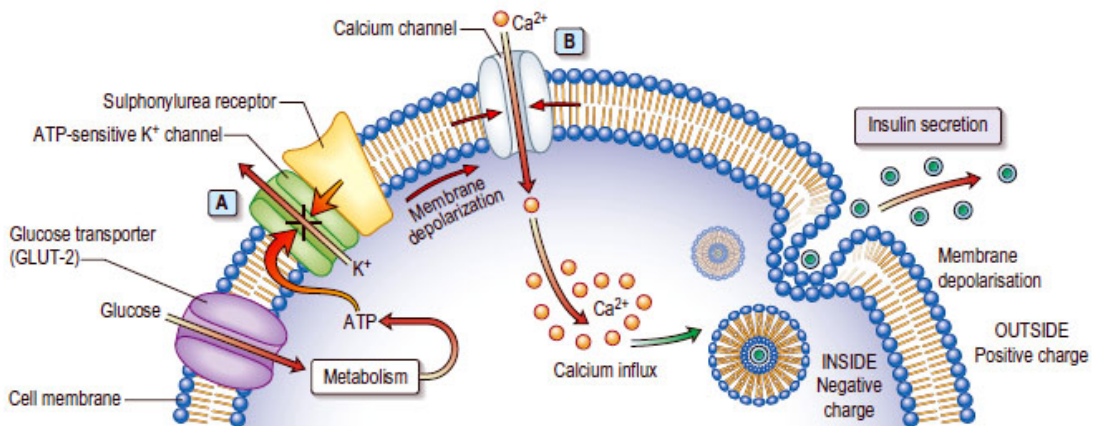


Figure 3. Local factors regulate Insulin secretion from a beta-cell of the islet of pancreas. Glucose enters the cell via the GLUT-2 transporter. Inside the cell there is metabolism with the generation of ATP. This causes the ATP-sensitive K⁺ channel to close, as shown in A. Closure of this channel leads to cell membrane depolarization which in turn allows calcium ions to enter the cell via another calcium channel; shown in B. Increased intracellular calcium activates calcium dependent phospholipid protein kinase and leads to exocytosis of insulin granules. We thank the figure from

<http://medicinexplained.blogspot.com.es/2011/08/insulin-secretion-local-regulation.html>

Apoptotic signaling in pancreatic beta-cells

β-cell mass and apoptosis

Type 2 diabetes is characterized by a progressive decline in β-cell function and chronic insulin resistance. During the development of type 2 diabetes, there is a decline in pancreatic β-cell mass, which has been suggested as one of the mechanisms behind the failure to maintain the increased demand for insulin. This decrease in β-cell mass could be caused by either disruption of β-cell formation or increased β-cell apoptosis⁶⁹. Thus, efforts to prevent β-cell apoptosis during the pathogenesis of diabetes are necessary to ameliorate the course of type 2 diabetes.

Purinergic signaling and apoptosis

Apoptotic cells are characterized morphologically by compaction of the nuclear chromatin, shrinkage of the cytoplasm and production of membrane-bound apoptotic bodies, and characterized biochemically by fragmentation of the genome and cleavage or degradation of several cellular proteins⁷⁰. Apoptosis plays a critical role in development as well as in several different disease states. On the other hand, many studies have shown that purines and pyrimidines are important signaling molecules that affect proliferation, differentiation, and cell death by interacting with purinergic (P2) receptors^{71; 72; 73; 74}. It is further suggested that activation of P2 receptor is linked to protein kinase A (PKA) and the extracellular signal-regulated kinase 1 and 2 (ERK1/2), pathways important for proliferation and apoptosis^{74; 75; 76; 77}. However, the influence of extracellular ADP and ATP on apoptosis has not been much investigated in β-cells. A recent publication reports that ATP itself has little or no effect on β-cell apoptosis⁷⁸. However, in glia cells, the ADP receptor agonist, ADPβS, enhanced DNA fragmentation and cell death⁷⁹. Stress-associated ATP release acts as a danger signal that controls apoptosis and induces phagocytosis to injured cells^{80; 81; 82; 83}.

Diabetic cardiovascular disease

Vascular diabetic complications are a major threat to public health and affect a majority of diabetic patients despite effective blood glucose control. Chronic inflammation, hypercoagulability and vascular smooth muscle cell (VSMC) proliferation are common features of diabetic vascular diseases, and the purinergic signaling system have been demonstrated to participate in these processes^{84, 85}.

It is also possible that P2-receptors could influence lipid regulation. The P2Y₁₃ receptor is a key regulator of hepatic high-density lipoprotein (HDL) endocytosis and control cholesterol catabolism by the body. Blocking P2Y₁₃ receptors could increase the endocytosis pathway of high-density lipoprotein (HDL)-cholesterol and thereby protective against atherosclerosis⁸⁶.

AIMS OF THIS THESIS

The main goal of this thesis is to understand the role of purinergic signaling in vascular diabetic disease and the expression and function of purine receptors using pancreatic insulinoma-cell line MIN6c4 and mouse isolated islets as the experimental model systems. Basically, the studies have focused on solving the following issues.

- to investigate if endogenous release of purines regulate insulin secretion (**Paper I**)
- to determine the involvement of P2Y receptors in insulin secretion (**Paper I**).
- to examine the role of P2Y₁₃ receptor in the regulation of the cell survival for β -cells (**Paper II**).
- to explore the mechanisms of β -cell ATP release stimulated by high glucose and palmitate (**Paper III**).
- to study the involvement of autocrine/paracrine activation of P2Y₁₃ receptors in the detrimental effects on β -cell function and survival caused by high glucose and saturated fatty acids (**Paper III**).
- to elucidate the mechanisms and modulation of hyperglycemia mediated pro-inflammatory response in VSMCs (**Paper IV**).
- to elucidate the ATP release and expression pattern of ectonucleotidases in VSMC under high glucose stimulation (**Paper IV**).

MATERIALS AND METHODS

Cells and tissue culture conditions

In Paper I, II and III, MIN6c4 cells were used. In Paper I and III, mouse pancreatic islets were used. In Paper I, the mouse pancreatic β -cells were used. In Paper IV, rat primary aortic Vascular Smooth Muscle Cells (VSMCs) were used. All experiments were performed according to local ethical guidelines.

MIN6c4 cell culture

Mouse MIN6 pancreatic β -cell line subclone MIN6c4, a generous gift from Professor Jun-Ichi Miyazaki, Osaka, was grown in Dulbecco's modified Eagle's medium (DMEM) containing with Glutamax-1 and 25 mM glucose (Invitrogen, Paisley, UK) supplemented with 15% (v/v) heat-inactivated Fetal Bovine Serum (FBS, Invitrogen), 60 μ M β -mercaptoethanol, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were maintained in a 37°C incubator with 5% CO₂.

Isolation and culture of mouse islets

Good islet of Langerhans isolation is one of the most important factors for reliable islet studies, and islet isolation has been a fundamental technique in the field of diabetic investigation for almost half a century. A classical procedure includes three steps: collagenase perfusion, pancreas digestion and islet purification. Islets were isolated according to previously described protocol⁸⁷ (Paper I and III) by retrograde injection of a collagenase solution via the bile-pancreatic duct. After sacrificing the animals by cervical dislocation and removal of sternum, the liver was exposed to allow access to the biliary tract. The biliary tract was clamped using a micro vascular clip. A 5mL syringe with a G30 needle was filled with collagenase (Roche, collagenase P 20 U/mL) dissolved in HBSS. Through the common bile duct

located on the duodenum, cold collagenase solution was slowly injected into the pancreas. The pancreas was excised and islets isolated by digestion for 20 min at 37°C. Tissues were dispersed by manual shaking and islets were washed 3×5 min in cold HBSS with 10mM HEPES prior to hand picking under a stereomicroscope at RT.

Purified islets that were to be used for total RNA extraction were collected directly in 500 µl TRIzol (Invitrogen, Paisley, UK) and stored at -80 °C. Purified islets intended for islet treatment were equilibrated for further treatment.

Isolation of mouse β -cells

In Paper I, β -cells were purified using a method previously used for enterochromaffin-like-cell purification⁸⁸ with some modifications. Freshly isolated mouse pancreatic islets (5,000 islets) were dispersed using trypsin digestion (0.9 mg/ml, Boehringer Mannheim, Mannheim, Germany) and omission of calcium in the presence of EDTA. After dispersion, the β -cell preparations were further purified by repeated counterflow elutriation using first a standard chamber and then a Sanderson chamber (Beckman, Palo Alto, CA, USA). The enriched cells from the standard chamber were collected at 25 ml/min at 380-560 g. They were purified further in a Sanderson chamber and collected at 18 ml/min and 800 g. This cell preparation consisted of about 80 % β -cells. The β -cell preparation was then subjected to density gradient centrifugation refer to the Material and Method section of Paper I. The purity of each β -cell preparation was assessed by RIA measurement of insulin, glucagon, somatostatin and pancreatic polypeptide per mg protein⁸⁹. At least 10 samples (tubes) of the cells were examined for each hormone. The final cell preparation consisted of around 95±8 % β -cells. The β -cells were then collected in 1,000 µl TRIzol (Invitrogen) and stored at -80 °C.

Isolation of rat primary aortic VSMCs and culture

Primary rat aortic VSMCs were a generous gift from Karolina Turczynska, Lund University. Male Sprague-Dawley rats were euthanized by CO₂ and the thoracic aorta was dissected. Fat and connective tissue were removed under sterile conditions in calcium free Hank's balanced salt solution supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. The vessels were subsequently incubated for 30 min at 37°C in serum free DMEM medium containing 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation). The adventitia was then pulled off using forceps and the aorta was incubated for 3-4 hours at 37°C in serum free DMEM cell culture medium containing 2 mg/ml collagenase type 2 and 0.1mg/ml elastase (Sigma-Aldrich). VSMCs were grown in DMEM:Glutamax-1 containing 5.5 mM glucose (Invitrogen, Paisley, UK) in the presence of 10 % heat-inactivated FBS; Invitrogen, Paisley, UK), 60 µmol/l β-mercaptoethanol, 50 U/ml penicillin, and 50 µg/l streptomycin under 5 % CO₂ and 95 % air at 37°C. Explanted cells were used up to passage 7.

Gene expression analysis

A real-time reverse transcriptase polymerase chain reaction (Q-RT-PCR) is a laboratory technique based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. There are two types of real-time PCR: Probe-based Taqman real-time PCR and Intercalator-based SYBR Green real-time PCR. In this thesis, we used the SYBR Green method, in which SYBR Green binds to newly synthesized double-stranded DNA. Binding of SYBR Green, to DNA, generates a fluorescent shift that is used in quantification of the amplification product. We used this SYBR Green Q-RT-PCR method to detect absolute or relative gene expression level by detecting the threshold cycle (Ct) during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplified curve.

RNA isolation and cDNA synthesis

After wash with PBS, Mouse islet, β -cell and MIN6c4 cells were harvested and total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. RNA concentrations were determined using a nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). RNA purity was checked by measurement of the $A_{260/280\text{nm}}$ ratio, which was routinely in the range of 1.8-2.0. First-strand cDNA was synthesized from 3 μg total RNA and random hexamer primers was performed using the RT Kit (Applied Biosystems, CA, USA) or RevertAid[™] H Minus First Strand cDNA Synthesis Kit (Fermentas, CA, USA) according to the manufacturer's instructions.

Design of primers for real-time PCR

A specific set of gene-specific primers for SYBR Green assays were chosen to obtain a product close to 200 bp (Table. 1). Primers were designed using VectorNTI software (Invitrogen, Informx, UK) with NCBI EntrezGene reference sequences as templates and synthesized by Eurofins MWG.

Table. 1

Targeted gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>P2Y₁</i>	GTTCAAGCAGAATGGAGACAGG	GGAAACCCCTCACTCAGGTGG
<i>P2Y₁₂</i>	AGTATTCCCCTGGAGACACTCATATC	GAGAACCTGGGTGATCTTGTAGTC
<i>P2Y₁₃</i>	GCCTTTCAAAAATCCTTTCCGA	TGTTTTTGGAAAAGCCGTCT
<i>CD39</i>	GATCATCACTGGGCAGGAGGAAGG	AAGACACCGTTGAAGGCACACTGG
<i>CD39L1</i>	GCTGGGTGGGCCGGTGGATACG	ATTGAAGGCCCGGGGACGCTGAC
<i>CD39L3</i>	CGGGATCCTTGCTGTGCGTGGCATTTCTT	TCTAGAGGTGCTCTGGCAGGGAATCAGT
<i>NTPDase8</i>	CTTCTGGCCTCACCATGCTC	GATTCCCGGTCCTTCTACCTG
<i>CD73</i>	CCCCGGGGCCACTAGCACCTCA	GCCTGGACCACGGGAACCTT
<i>GAPDH</i>	GGTCATCCCAGAGCTGAACG	TTGCTGTTGAAGTCGCACGGA
<i>Beta-actin</i>	CTACCTCATGAAGATCCTCACCGA	CGCTCATTGCCAATGGTGAT
<i>Mdr1a(Abcb1a)</i>	CATGACAGATAGCTTTGCAAGTGTAG	GGCAAACATGGCTCTTTTATCG

Quantitative Real-time reverse transcriptase polymerase chain reaction

Real-time reverse transcriptase polymerase chain reactions (Q-RT-PCR) were used to assess purinergic receptor expression patterns in pancreatic islets, β -cells and MIN6c4 cells (Paper I, II). Real-time quantitative PCR was performed in a LightCycler (Roche, Basel, Switzerland) and reactions were carried out in glass capillaries in 10 μ l reactions containing 1 μ l cDNA template, 3 mM MgCl₂, 0.5 μ M of forward and reverse primers and 1 \times LightCycler DNA Master SYBR Green I mix (Roche, Basel, Switzerland). The PCR enzyme was activated by initial 600 sec incubation at 95°C, followed by 45 PCR cycles (1 sec denaturation at 95°C, 6 sec annealing at primer specific temperatures and 23 sec elongation at 74°C). After 45 cycles, a melting curve analysis was performed, where SYBR Green I fluorescence emission was measured during gradually increasing temperature (from 55°C to 95°C). The amplification products of primer pairs produced single top melting curves. The amplification efficiency of primers that passed the melting curve test was evaluated through amplification of serially diluted template cDNAs (1:1 to 1:16). The amplification efficiency (E) is determined by the formula $E=10^{(-1/k)}$, where k is the slope of the linear function of the crossing point values plotted against the log₂ transformed dilution steps of each template cDNA. The ideal primer set has an amplification efficiency of E=2. A primer specific E value ($E_{\text{target gene}}$) was used when calculating the relative expression of each target gene relative to GAPDH, a well-known housekeeping genes ($E_{\text{HK gene}}$) according to the Δ CT method⁹⁰. The following formula is used to calculating the relative expression of each target gene relative to GAPDH:

$$\text{Relative expression of target gene} = \frac{(E_{\text{target gene}})^{-\Delta\text{CT}_{\text{target gene}}}}{(E_{\text{HK gene}})^{-\Delta\text{CT}_{\text{HK gene}}}}$$

Q-RT-PCR were also used to assess Mdr-1 and ectonucleotidases gene expression patterns in MIN6c4 Mdr-1 silent cells, MIN6c4 control cells and rat primary VSMCs by performing in an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) (Paper III, IV). These quantitative real-time PCR (Q-RT-PCR) reactions were carried out in MicroAmp Fast 96-Well Reaction Plate

(Applied Biosystems, CA, USA) in 20 µl reactions containing 1 µl cDNA template, 0.2 µM of forward and reverse primers and 1×Faster SYBR Green Master Mix (Applied Biosystems, CA, USA). The PCR enzyme was activated by initial 20 sec incubation at 95°C, followed by 40 PCR cycles (3 sec denaturation at 95°C, 30 sec annealing and elongation at 60 °C). Results were expressed using the comparative Ct method as described in User Bulletin 2 (Applied Biosystems) with Beta-actin as the housekeeping gene, a well-known housekeeping gene. The fold change in the level of target mRNA between untreated and treated cells was then expressed as $2^{-\Delta\Delta C_t}$ with $\Delta\Delta C_t \pm$ S.E. A melting curve is allowed to verify the specificity of the amplification. Briefly, the $\Delta\Delta C_t$ values were calculated as following:

Comparative expression of target gene in treated and untreated cells = $2^{-\Delta\Delta C_t}$

$$\Delta\Delta C_t = (C_{t \text{ gene of interest}} - C_{t \text{ HK gene}})_{\text{treated}} - (C_{t \text{ gene of interest}} - C_{t \text{ HK gene}})_{\text{untreated}}$$

Protein expression analysis

Protein extraction

MIN6c4 cells, growing in 6-well plates were incubated for 30 min in the presence of different test agents. MIN6c4 cells were lysed in ice-cold SDS buffer containing a cocktail of protease inhibitors. After sonication for 15 sec and heating for 5 min at 99°C, cellular lysate was centrifuged. The protein concentration was determined by Dc protein assay (BIO-RAD) according to the manufacturer's instructions.

SDS-PAGE and Western blotting

Western blotting is an analytical technique for detection of specific proteins in a given sample. Protein samples from MIN6c4 cells were detected using standard Western Blot procedures. Lysate protein samples, representing 20 µg of total protein, were separated on a Precase 12 % SDS-polyacrylamide gel (Lonza, USA) and transferred to Immobilon-P transfer membranes (Millipore Corporation, Mass,

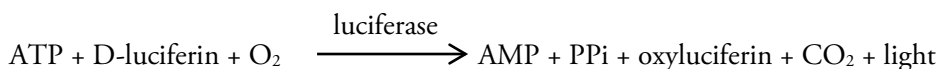
USA). The membranes were blocked with TBS-T containing 5 % non-fatty milk and probed over night at 4°C with or without different primary antibodies. After five washes, the membranes were incubated 60 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary anti-rabbit antibodies and the membranes were visualized on using SuperSignal West FemtoMaximum Sensitivity Substrate (Thermo, U.S.A). The intensities of bands were quantitated by scanning densitometry (Fluor-S Multi-Imager).

All the different signaling Western blotting experiments were performed on at least 3 different independent experiments. To verify the amount of protein loaded and to calibrate the integration of protein band, a 37-kDa band was found in the parallel detection of GAPDH for MAP kinases detection and total Akt (60-kDa), CREB (43-kDa), Bad 823-kDa or IRS-1 (180-kDa) as the calibrators individually for phosphorylated-Akt, phosphorylated-CREB, phosphorylated-Bad and phosphorylated-IRS-1 in Paper II and III.

Cell biological studies of the P2Y₁₃ receptor

ATP release analysis

In Paper I, III and IV, ATP release was measured using an adaptation of the luciferase method previously described by Taylor et al. ⁹¹. Firefly luciferase catalyses the following reaction:



This allows cellular release of ATP to be monitored by measuring the light intensity. Briefly, 3.5×10^4 of MIN6c4 cells/well or 8×10^4 of rat primary VSMCs were plated in each 35 mm Petri dish and were allowed to grow in 25 mM glucose DMEM containing 15 % FBS for 24 h or 72 h. After replacing with 1 ml serum free DMEM containing 5.6 mM glucose and cultivating the cells for another 24 h, 1 ml $2 \times$ ATP SL reagent (Biothema, Sweden) in serum free DMEM containing 5.6 mM glucose, was added and the Petri dish was incubated for 30 min at RT in

the dark. After recording the baseline luciferase activity in the Petri dish for 1 min in a GloMax 20/20 Luminometer (Promega Corporation, Madison, WI, USA), treatment was added and luminescence was recorded.

Insulin and glucagon secretion analysis

Enzyme linked immunosorbent assays (ELISA), also known as enzyme immunoassays (EIA), are tests designed to detect antigens or antibodies by producing an enzyme triggered color change. The sandwich ELISA we employed in this thesis is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate wells. A simple washing step removes unbound enzyme labeled antibody and the bound conjugate is detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. The reaction is stopped by addition of acid to give the absorbance determined in a plate reader.

In Paper I, insulin and glucagon secretion from islets in vitro were measured. The freshly isolated islets were pre-incubated for 30min at 37°C in KRB, pH 7.4, supplemented with 10 mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.1% BSA and 4 mM glucose before addition of the agents to be tested. Each incubation vial contained 12 islets in 1.0 ml buffer solution and was gassed with 95 % O₂-5 % CO₂ to obtain constant pH and oxygenation. After pre-incubation, the medium was changed and supplemented with various receptor agonists and antagonists. The islets were then incubated for 1 h at 37°C in a metabolic shaker (30 cycles per min). Aliquots of the medium were then removed for the assay of insulin and glucagon.

Insulin secretion was also measured from MIN6c4 cells in Paper III. 3×10^5 MIN6c4 cells were cultured in each 35 mm Petri dish in 2 ml of 25 mM glucose DMEM containing 15 % FBS for 72 h. The medium was replaced with 1 ml of Krebs-Ringer bicarbonate HEPES buffer for 30 min. The buffer was exchanged for a KRBH containing 16.7 mM glucose or 100 μ M palmitate. Samples were

collected every 30 s and replaced with an equal amount of fresh buffer. The insulin contents of the samples were determined using an ELISA kit (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions.

Intracellular cAMP concentration in MIN6 cells

In Paper I, II and III, Intracellular cAMP concentration in MIN6 cells was measured by using the competitive ELISA assay based on the competition between free cAMP and a cAMP-acetylcholinesterase (AChE) conjugate (cAMP tracer) for a limited number of cAMP-specific rabbit antibody binding sites. This rabbit antibody-cAMP (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then the reagent contains the substrate to AChE is added to the well. The absorbent is detected spectrophotometrically. In our study, MIN6c4 cells (4×10^5 cells/well) were cultured in a 24-well plate for 3 days. After washing with OptiMEM and addition of 25 μ M Rolipram, the cells were incubated for 20 min in OptiMEM medium containing the test compounds. Extracting the monolayer with 200 μ l 0.1 M hydrochloric acid terminated the incubations. The cell lysates were centrifuged at 1000 rpm for 10 min at 4°C. cAMP levels in cells extract were measured by this competitive ELISA using Cyclic AMP EIA kit (Cayman Chemical, USA) according to manufacturer's instructions.

cAMP real-time luciferase analysis in MIN6 cells by transient transfection of cAMP reporter

In Paper III, real time of cAMP production was analysed by using transient transfection of MIN6c4 cells with the experimental plasmid Glosensor L9, a kind gift from Dr. Neal Cosby, Promega. 5×10^5 cells of MIN6c4 cells were plated in a 35 mm Petri dish and cultivated to get a confluence of 30-70 %. The Glosensor A1 plasmid was transfected into the MIN6c4 cells using Targefect-PCL (Targeting Systems, El Cajon, CA, USA) according to the manufacturer's instructions. 48 h

after transfection the cell culture medium was replaced with 1ml low glucose DMEM (containing 10 mmol/l HEPES, pH 7.5) prepared cAMP reagent medium (pH 7.5) which containing 10 mmol/l HEPES and 2 % (volume/volume) Glosensor cAMP reagent (Promega Corporation, Madison, WI, USA). After equilibration, for 2 h at RT, the baseline luciferase activity was recorded for 3 min in a GloMax 20/20 Luminometer (Promega Corporation, Madison, WI, USA). The test substances were then added and the luminescence was recorded measured for another 10 min.

Functionality studies of the P-glucose protein (p-gp) through lentiviral Mdr-1 gene silencing in MIN6 cells

RNA interference (RNAi) plays a variety of roles in biology. A small hairpin RNA or short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence target gene expression via RNAi pathway, where it interferes with the expression of a specific gene. Expression of shRNA in cells is typically obtained by delivery of plasmids or through viral vectors. It efficiently knocks down the gene expression by binding to the key sequences on messenger RNA of proteins of interest. This method was used in Paper III, for efficient silencing of Mdr-1 gene. In brief, MIN6c4 cells were seeded in 12 well dishes and grown to obtain 50 - 60 % confluence. The medium was then replaced with 1 ml fresh medium, containing Polybrene (5 µg/ml), and shRNA lentiviral particles (Santa Cruz Biotechnology) to give a MOI of approximately 0.05. The cells were incubated for 24 h at 37°C when the medium was replaced with selection medium containing 1.5 µg/ml puromycin. The puromycin selection was continued until a stable puromycin resistant cell population was obtained.

Fluorescent measurements of $[Ca^{2+}]_i$ concentration

$[Ca^{2+}]_i$ concentration was measured in Paper II, MIN6c4 cells were plated in 96-well plates at a density of 4×10^5 cells/well and allowed to grow for 2 days. The

medium was removed and 20 μ l FLUO-4 loading buffer, containing 4.5 μ M FLUO-4 AM and 0.01 % Pluronic in HBSS (Hank's BSS), was added per well. After incubating 1 h at 37 °C the loading buffer was removed and the cells were washed once with 50 μ l HBSS. 50 μ l HBSS +/-antagonists was added and the cells were incubated for 30 min at RT. Fluorescence (excitation 485 nm, emission 535 nm) was recorded, in a VICTOR microplate reader (PerkinElmer, Finland), after injection of 25 μ l 10 μ M 2MeSADP/well.

Apoptosis detection on the P2Y₁₃ receptor using MIN6 cells

Caspase-3 activity analysis in MIN6 cells

The activation of caspase-3, which cleaves a number of different proteins, including poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C δ and actin, has been shown to be important for the initiation of apoptosis⁹². In Paper II and III, Caspase-3 activity was determined using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes) according to the manufacturer's instructions. Briefly, after incubation with test substances for 36 h MIN6c4 cells were washed with PBS and lysed and Caspase-3 activity in the extracts was measured by fluorometric assay. Fluorescent product of the substrate Z-DEVD-rhodamine 110 generated by Caspase-3 in the cell extract was detected by a microplate fluorometer (VICTOR, PerkinElmer, Finland) with excitation of 496 nm and emission of 520 nm. Background fluorescence was determined by including a specific Caspase-3 inhibitor (Ac-DEVD-CHO) in the reaction mixtures. The results were correlated to the protein concentration.

Cell viability analysis

The effects of a sustained application of P2-receptor agonist or antagonist on cell viability of MIN6c4 cell line were studied using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay detecting the

cellular mitochondrial capacity to convert MTT tetrazolium salt to formazan. The MIN6c4 cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well in a volume of 200 μ l of cell culture medium per well. After 24 h, test substances were added and the plates were kept in the CO₂ incubator for 3 days, then 20 μ l of the MTT reagent (5 mg/ml in PBS buffer) was added to each well, and the plates were incubated in a CO₂ incubator at 37°C for an additional 4 h. The medium was aspirated and replaced with 200 μ l of Dimethyl Sulfoxide Solubilization Solution (DMSO) to dissolve the insoluble purple formazan product. The absorbance was quantified by measuring at 490 nm in a microplate reader (VICTOR, PerkinElmer, Finland).

Cell survival and proliferation

MIN6c4 cells were seeded in 24-well plates at a concentration of 1×10^4 cells per well in DMEM cell culture medium. The cells were counted every two days using a Bürkert chamber under the microscope and the cell proliferation curve was drawn according to the cell numbers.

Immunofluorescence

Mouse islet treatment for immunofluorescence study

After isolation, mouse isolated islets in Paper III were equilibrated overnight at 37°C in a humidified incubator holding 5 % CO₂ in 5.6 mM glucose baseline medium (RPMI 1640 glutamax medium (GIBCO), 10 % FBS (GIBCO), 50 U/mL penicillin and 50 μ g/mL streptomycin (A2213, BioChrom AG, Berlin Germany). Equilibrated islets were divided into treatment groups and moved to treatment wells containing treatment medium. Islets were incubated in treatment wells for 24h in a humidified incubator holding 5 % CO₂. Treated islets were fixed in stefaninnis fixative (0.2 % picric acid, 2 % formaldehyde in 0.1 M phosphate buffer, pH 7.2) for 30 min, rinsed 2 \times 10 min in Tyrode's solution containing 10 %

sucrose prior to embedding in cryomount (Histolab, Gotenburg) and stored at -80°C.

Immunofluorescence study of Caspase-3 activity in mouse isolated islets

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope. This technique uses the specificity of fluorescent dyes linked antibodies target to their specific biomolecule of the antigen within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. In Paper III, immunofluorescence was used to study the Caspase-3 activity in mouse isolated islets. The embedded islets were cut into 10 µm cryo sections and mounted onto slides. Slides were washed in PBS containing 0.25 % triton X100 and stained using antibodies against activated Caspase-3 (Cell signaling prediluted #8120) over night at 4°C. Slides were washed once in PBS containing 0.25% TritonX100. Antigens were visualized using 1:1000 diluted Dylight 594 donky anti-rabbit in PBS containing 0.25 % Triton X100 and 0.25% BSA, incubated at room temperature for 1 hour. Hoechst staining was performed as a counter stain to visualize the nucleus of cells. Islets were visualized using fluorescent microscope (Olympus BX42) and pictures were captured using a camera (Olympus XC30). Pictures were analyzed using Image J cell counter application (rsbweb.nih.gov/ij) and total numbers of cells as well as Caspase-3 activated cells were counted. The frequency of Caspase-3 activated cells were calculated and normalized to the control value of the same animal.

Immunofluorescence study for VSMCs identification

In Paper IV, immunofluorescence was used to confirm the identification of VSMCs. Primary rat VSMCs were seeded on cover slips in culture. The cells were rinsed with PBS, fixed in 4 % formaldehyde, subsequently permeabilized with 0.2 % Triton-X prior to 2 hours of blocking with 2 % bovine serum albumin. Cells were incubated with a monoclonal primary antibody (anti-smooth muscle actin; 1:400; Sigma-Aldrich) over night at 4 °C. After multiple washing steps, the cells were

incubated with secondary goat anti-mouse antibody (Cy5; 1:500) for 2 hours at room temperature. Nuclei were visualized using Sytox Green (1:3000). Image acquisition was performed using a Zeiss LSM Pascal confocal microscope using a 40x objective.

Drugs and antibodies

Lentiviral Mdr-1 siRNA (m) and scrambled control siRNA were from Santa Cruz Biotechnology, INC (USA). The cyclic AMP kit was obtained from Cayman Chemical (USA). The targfect-PCL kit was purchased from BioSite (USA). The EnzChek® Caspase-3 Assay Kit #2 was purchased from Molecular Probes (USA). Radioimmunoassay kits for insulin and glucagon determination were obtained from Diagnostika (Falkenberg, Sweden) and from Eurodiagnostica (Malmö, Sweden). 2MeSADP and MRS2211 were purchased from Tocris (UK), Collagenase (CLS 4) was obtained from Worthington Biochemicals, Freehold, NJ, USA. Bovine serum albumin was from ICN Biochemicals, High Wycombe, UK. IBMX (isobutylmethylxanthine), an inhibitor of phosphodiesterase, and all other drugs and chemicals, unless otherwise stated, were from Sigma Chemicals, St Louis, MO, USA or Merck AG, Darmstadt, Germany either of analytic or laboratory grade.

MAPKs kit, anti-phospho-SAPK/JNK, anti-phospho-p38, anti-phospho-ERK1/2, anti-GAPDH, anti-phospho-Akt, anti-Akt, anti-phospho-CREB, anti-CREB antibodies, anti-phospho-Bad, anti-Bad, anti-phospho-IRS-1, anti-IRS-1 and Caspase-3 antibodies were all obtained from Cell Signaling Technology (TX, USA).

Statistical methods

Results were expressed as mean and standard error of the mean (SEM) unless otherwise stated. The level of significance for the difference between sets of data was assessed by Student's unpaired t-test using Graph-Pad InStat, Version 5.0 (GraphPad Software, SanDiego, CA, U.S.A.). For immunofluorescence Caspase-3

activity in mouse islets, statistical analysis' was performed using one way ANOVA followed by Dunnett's post hoc test. Statistically significant differences were considered at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) (two-tailed test).

Animals & Ethics

In Paper I, female mice of the NMRI strain (B&K Universal, Sollentuna, Sweden), weighting 25-30 g were used for in vitro and in vivo studies. They were fed by a standard pellet diet (B&K Universal) and tap water ad libitum. The study conforms to the Guide for the Care and Use of Laboratory Animals, US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee of Lund University, Sweden.

In Paper III, eight female C57B6 mice from Charles River (Sulzfeld, Germany) were used in the experiments. Animals had free access to standard chow and water. Experimental designs as approved by animal ethics committee, Lund and Malmö, Sweden. Animals were used in accordance with the European community council Directive (86/609/EEC and 2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:534).

RESULTS AND DISCUSSION

Paper I: ADP mediates inhibition of insulin secretion by activation of P2Y₁₃ receptors

Glucose regulates insulin, glucagon secretion and ATP release

In the first part of paper I, we focused on discovering the effects of extracellular purines on insulin and glucagon secretion in isolated mouse islets by using apyrase. As we know, apyrase degrades ATP and ADP to AMP and adenosine. 20mM of glucose resulted in an expected increase of insulin secretion, and an expected decrease of glucagon secretion in isolated mouse islets. The increased secretion of insulin and the decreased secretion of glucagon were both absent when apyrase (1 U/ml) was added to the islets in the presence of 20 mM glucose. This could possibly be explained by enzyme-mediated generation of adenosine which acting on A₁ receptor and thus inhibit insulin secretion and stimulate glucagon secretion.

Then we tested if glucose could cause ATP release by using the MIN6c4 mouse insulinoma β -cell line. 16.6 mM glucose resulted in a significant increase in ATP release. Equimolar concentrations of mannitol did not affect ATP release. But ATP release was blocked by the calcium-channel inhibitor nifedipine. These results not only showed that glucose regulates insulin, glucagon secretion in mouse islets but also showed that glucose induces ATP release into the extracellular space in MIN6c4 cells.

P2Y receptors are expressed in mouse islets and in β -cells

In the second part of paper I, we wanted to determine if the P2Y receptors are involved in the effects of insulin and glucagon regulation by extracellular purines. We quantified the mRNA levels for all purinergic P2Y receptors both in intact mouse pancreatic islets and in isolated β -cells. In mouse pancreatic islets and mouse β -cells, the ADP receptors P2Y₁ and P2Y₁₃ are both highly expressed when normalized to the reference gene GAPDH, while P2Y₁₂ or other P2Y ATP

receptors could not be detected (Figure 4). Consequently, extracellularly released ATP can only stimulate islet ADP receptors P2Y₁ or P2Y₁₃ after conversion into ADP. These results suggest that either one or both of P2Y₁ and P2Y₁₃ ADP receptors might be involved in the regulation of glucose in insulin, glucagon secretion and ATP release.

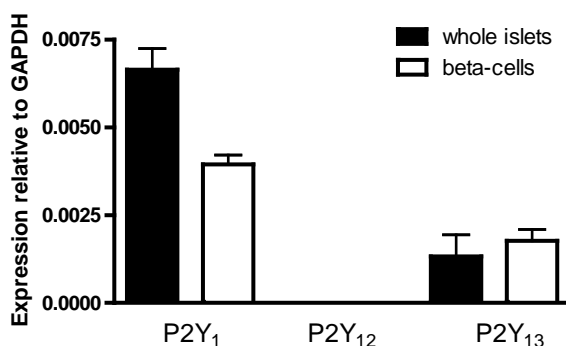


Figure 4. Expression of mouse ADP receptors P2Y₁, P2Y₁₂ and P2Y₁₃ in pancreatic islets and β -cells from the mouse. Black columns, whole islets; white columns, isolated β -cells.

ADP regulates insulin and glucagon secretion

To study if ADP regulates insulin and glucagon secretion and further determines which receptor mediates this regulation, we carried out the pharmacological experiments of insulin and glucagon secretion on mouse islets and β -cells using the stable ADP analogue 2MeSADP as the agonist, and the antagonist MRS2211 to specifically block P2Y₁₃ receptor or MRS2179 to specifically block P2Y₁ receptor. 10 μ M of 2MeSADP could activate both P2Y₁ and Y₁₃ receptors, but it did not have any effect on insulin secretion at 8.3 mM glucose. Applying 10 μ M of MRS2179 to pancreatic islets in the presence of 10 μ M 2MeSADP inhibited insulin release, while 10 μ M of MRS2211 in the presence of 10 μ M 2MeSADP significantly stimulated insulin release. The results showed that ADP play a dual role on β -cells, stimulating insulin release via P2Y₁ receptors and inhibiting insulin release via P2Y₁₃ receptors (Figure 5).

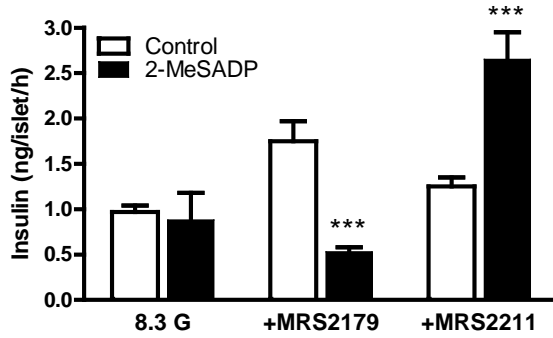


Figure 5. Effect of the stable ADP analogue 2-MeSADP on insulin secretion in mouse islets at 8.3 mM glucose. In the presence of the P2Y₁ antagonist MRS2179, 2-MeSADP reduced insulin secretion. In the presence of the P2Y₁₃ antagonist MRS2211, insulin secretion was increased.

Endogenously released ADP regulates insulin release via G α i-coupled P2Y₁₃ receptors

To further test if endogenously released ADP regulates insulin release via P2Y₁₃ receptors, the P2Y₁₃ receptor antagonist MRS2211 (10 μ M) was added to mouse islets in the presence of both intermediate (8.3 mM) and high (20 mM) glucose. MRS2211 increased both insulin and glucagon secretion at intermediate and at high glucose (Figure 6).

a



b

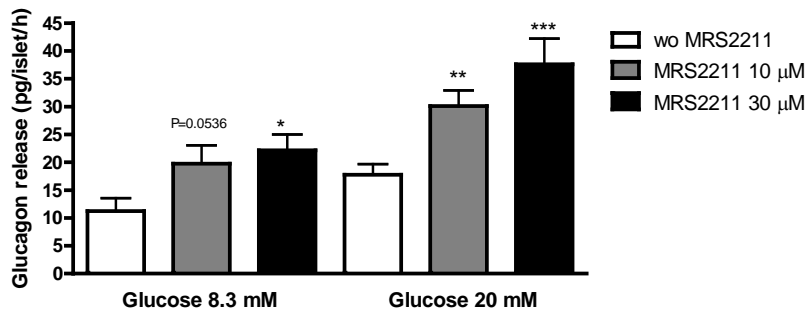


Figure 6. Effect of the P2Y₁₃ antagonist MRS2211 on insulin (a) and glucagon (b) secretion in mouse islets at different glucose concentrations.

In vivo study further showed that MRS2211 markedly enhances insulin and significantly attenuates glucagon secretion following an i.p. injection of glucose (Figure 7). P2Y₁₃ is a G α i-coupled receptor. The mechanism of G α i-coupled P2Y₁₃ receptor activation is to intracellularly decrease cAMP levels. cAMP, which is used for intracellular signal transduction in many different organisms, could potentially elevate insulin secretion upon a simultaneous increase in [Ca²⁺]_i via activation of protein kinase A as well as a cAMP-mediated direct effect on the exocytotic machinery^{93; 94}. We also study the mechanism underlying the stimulatory effect of the P2Y₁₃ agonist on insulin secretion by examining the effect of 2MeSADP on cAMP production. We found that 10 nM of 2MeSADP decreased the cAMP level by half, while the P2Y₁₃ antagonist MRS2211 was able to reverse this effect.

All these results suggest an autocrine role for ADP acting via P2Y₁₃ receptors and inhibiting insulin secretion. The P2Y₁₃ antagonist MRS2211 was shown to increase glucose-stimulated insulin release and lower glucose levels in vitro and in vivo, suggesting that it might be of value for the new treatment of type 2 diabetes.

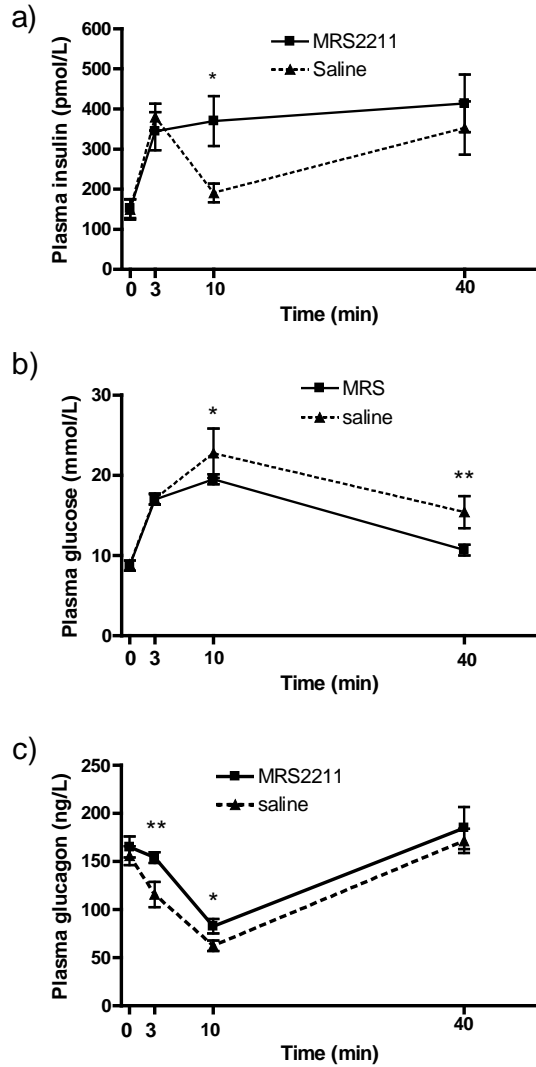


Figure 7. Effect of the P2Y₁₃ antagonist MRS2211 on (a) insulin (b) glucose and (c) glucagon secretion *in vivo*.

Paper II: ADP receptor P2Y₁₃ induce apoptosis in pancreatic β -cells

Functional P2Y receptors are expressed in MIN6c4 mouse insulinoma pancreatic β -cell line

The work in Paper II was intended to further examine the role of ADP specific purinergic P2Y receptors in β -cell apoptosis. To do so, we use the mouse insulinoma β -cell line MIN6 as our primary model system. First, the relative expression of P2Y ADP receptor mRNA in MIN6 cell was quantified by real-time PCR. Results show that there are no P2Y ATP receptors genes but three ADP receptors genes P2Y₁, P2Y₁₂ and P2Y₁₃ could be detected. Among these P2Y ADP receptor subtypes, the P2Y₁₃ had the highest expression (Figure 8). As a consequence, extracellular released ATP can only stimulate P2Y receptors after conversion to ADP that acts on these three receptors on β -cells.

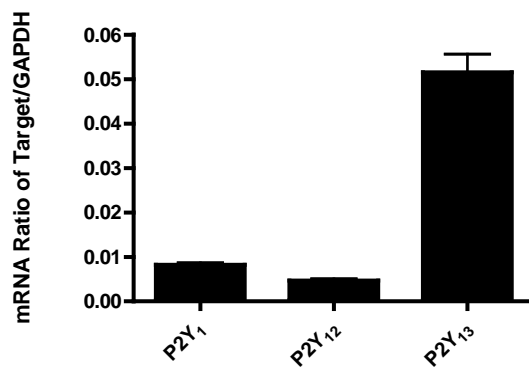


Figure 8. mRNA quantification of P2Y ADP receptors in MIN6c4 cells.

Secondly, we investigate the functionality of the G_q and G_i linked ADP-sensitive receptors, P2Y₁, Y₁₂ and Y₁₃ in MIN6 cells. It is well known that stimulation of G_{αq} linked P2Y₁ receptor mobilize Ca²⁺ from intracellular stores, and stimulation of G_{αi} linked P2Y₁₂ and Y₁₃ receptor decrease cAMP production. GLP-1 (glucagon-like peptide-1) stimulates cAMP production through activation of the GLP-1 receptor. We then checked the effect of 2MeSADP on GLP-1 stimulated

cAMP production. 2MeSADP significantly reduced the cAMP accumulation by 59 %. The specific antagonist of P2Y₁ receptor, MRS2179, significantly reduced the cAMP production by another 71 % compared to the baseline control. In contrast, blocking the P2Y₁₃ receptor by MRS2211 during 2MeSADP stimulation resulted in a four times increase in cAMP levels. Moreover, the P2Y₁₂ receptor specific antagonist, AR-C69931MX, did not have any significant effect on cAMP level. These results not only confirm that 2MeSADP inhibited GLP-1 stimulated cAMP production through Gi-couple P2Y₁₃ receptor, but also indicate that ADP exerts opposing regulatory effects on cAMP level via different receptor subtypes.

To confirm the presence and functionality of Gq-coupled ADP receptors P2Y₁, intracellular Ca²⁺ level in β-cell was also measured. Blocking P2Y₁ receptor by MRS2179 during 2MeSADP stimulation resulted in a decrease in intracellular Ca²⁺ level in β-cells. This inhibition proves the presence of functional P2Y₁ ADP receptors in MIN6 cells and that the intracellular calcium level is regulated by this receptor.

ADP induce apoptotic effect through acting on P2Y₁₃ receptor

Caspase-3 is a key mediator of apoptotic pathways in mammalian cells. To investigate whether 2MeSADP could influence Caspase-3 level and to what extent this effect could be modulated by P2Y₁₃, we cultured MIN6c4 cells for 36 h to study the effect of 2MeSADP in the absence or presence of MRS2211 on Caspase-3 activity.

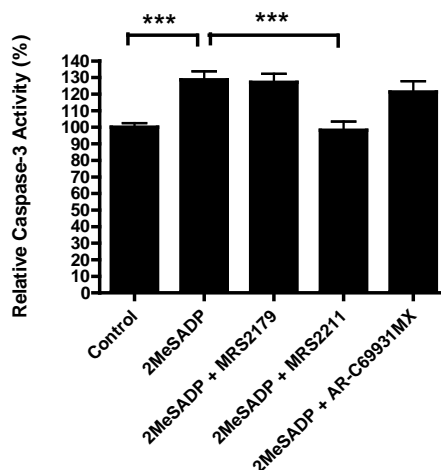
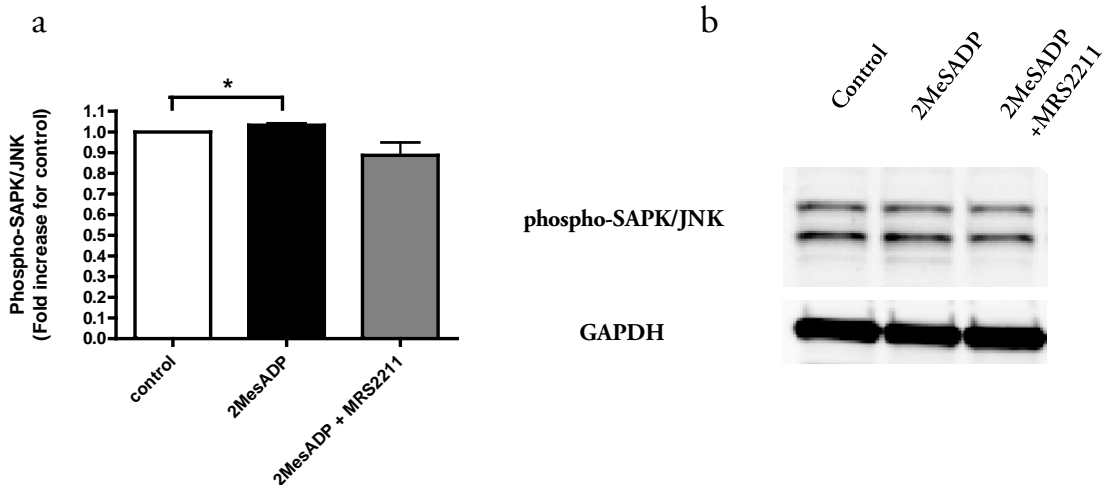


Figure 9. Effects of ADP receptor ligands on Caspase-3 activity in MIN6c4 cells.

As shown in Figure 9, 10 μ M of 2MeSADP was capable of inducing a significant increase in Caspase-3 activity in MIN6c4 cells. This effect was markedly suppressed by 10 μ M of MRS2211. Moreover, P2Y₁ and P2Y₁₂ antagonist did not have significant effect on Caspase-3 level during 2MeSADP stimulation. This result indicates a pro-apoptotic effect of 2MeSADP through P2Y₁₃ receptor.

Apoptotic signal-transduction pathway of P2Y₁₃ activation

We next studied the signal transduction of P2Y₁₃ activation. Activation of MAPKs (mitogen-activated protein kinases) are well known to be involved in directing cellular responses to a diverse array of stimuli and thus regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis⁹⁵. It also has been shown that ERK kinases are important for cell survival, SAPK/JNKs and p38 are stress responsive and involved in apoptosis⁹⁶. To assess whether P2Y₁₃ receptor signal transduction affect these pathways, we exposed MIN6c4 cells to 10 μ M 2MeSADP in the absence or presence of MRS2211 for 30min and monitored MAPK phosphorylation using Western Blotting.



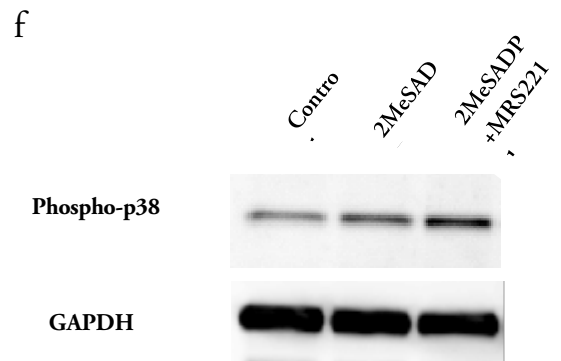
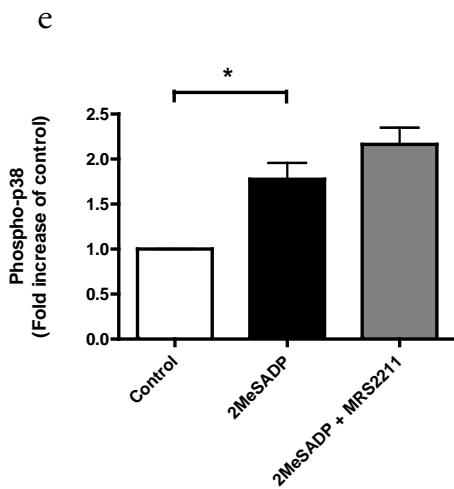
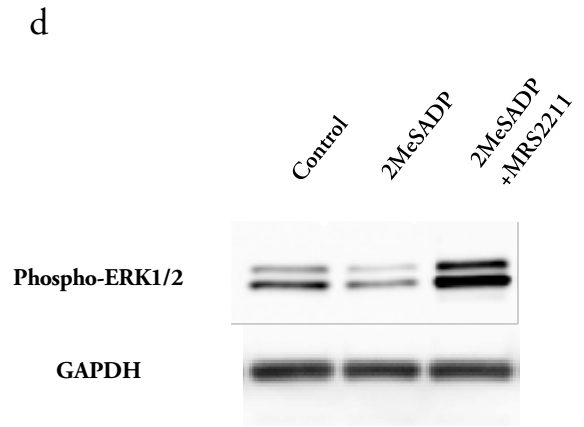
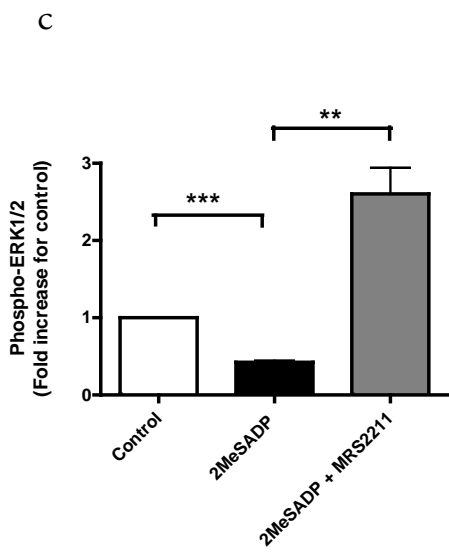


Figure 10. Effects of ADP receptor ligands on the MAP kinases phosphorylation status of MIN6c4 cells. Western blot analysis using antibodies against phosphorylated MAPkinases. (a-b) SAPK/JNK. (c-d) p44/42. (e-f) p38. MIN6c4 cells were incubated for 30min in medium containing PBS control, 2MeSADP (10 μ M) with or without MRS2211 (10 μ M).

When cells were treated with 2MeSADP for 30min, very little ERK phosphorylation was observed (Figure 10 a). However, MIN6C4 cells exposed to 2MeSADP plus MRS2211 resulted in an increase of ERK phosphorylation, suggesting a marked protective capacity of this P2Y₁₃ receptor blocking (Figure 10 b). We also found that 2MeSADP inhibited ERK while stimulating SAPK/JNK and p38, thus giving rise to a combined pro-apoptotic signal (Figure 10 c). Pharmacological intervention indicated P2Y₁₃ being responsible for the ERK inhibition while P2Y₁ stimulated p38. These results indicated a pro-apoptotic effect of 2MeSADP through P2Y₁₃ receptor.

Survival and differentiation signal-transduction pathway of P2Y₁₃ activation

Akt has previously been shown to be involved in β -cell survival. CREB (cAMP response element-binding protein) is a transcription factor plays a role in β -cell survival and differentiation. We carried out Western Blotting on MIN6c4 cells to determine the role of P2Y₁₃ receptor signal transduction in Akt and CREB activation. Application of 10 μ M 2MeSADP to the incubation medium resulted in a suppression of Akt activation. However, MRS2211 markedly enhanced both Akt (Figure 11 a-b) and CREB (Figure 11 c-d) phosphorylation during 2MeSADP stimulation. These results further implicated that inhibition of P2Y₁₃ receptor signal transduction promotes the survival effect. However, there is no significant difference after incubation with 10 μ M of 2MeSADP compared to control. This might be affected by P2Y₁ activation, since activation of P2Y₁ receptor could increase in the intracellular concentration of Ca²⁺ and Ca²⁺- regulated signaling pathways have been identified that trigger CREB activation.

P2Y₁₃ receptor activation mediated MIN6c4 cells growth and proliferation

To investigate the functional consequences of P2Y₁₃ activation in pancreatic β -cells, MIN6c4 cells were seeded at a low cell density in the presence or absence of different stimulants. As measured by the MTT assay 3 days after treatment, the growth of MIN6c4 cells was inhibited by treatment with 10 μ M of 2MeSADP. However, when cells were incubated with 10 μ M MRS2211, the effect was inverted and cells proliferated more efficiently. Determination of the cell growth

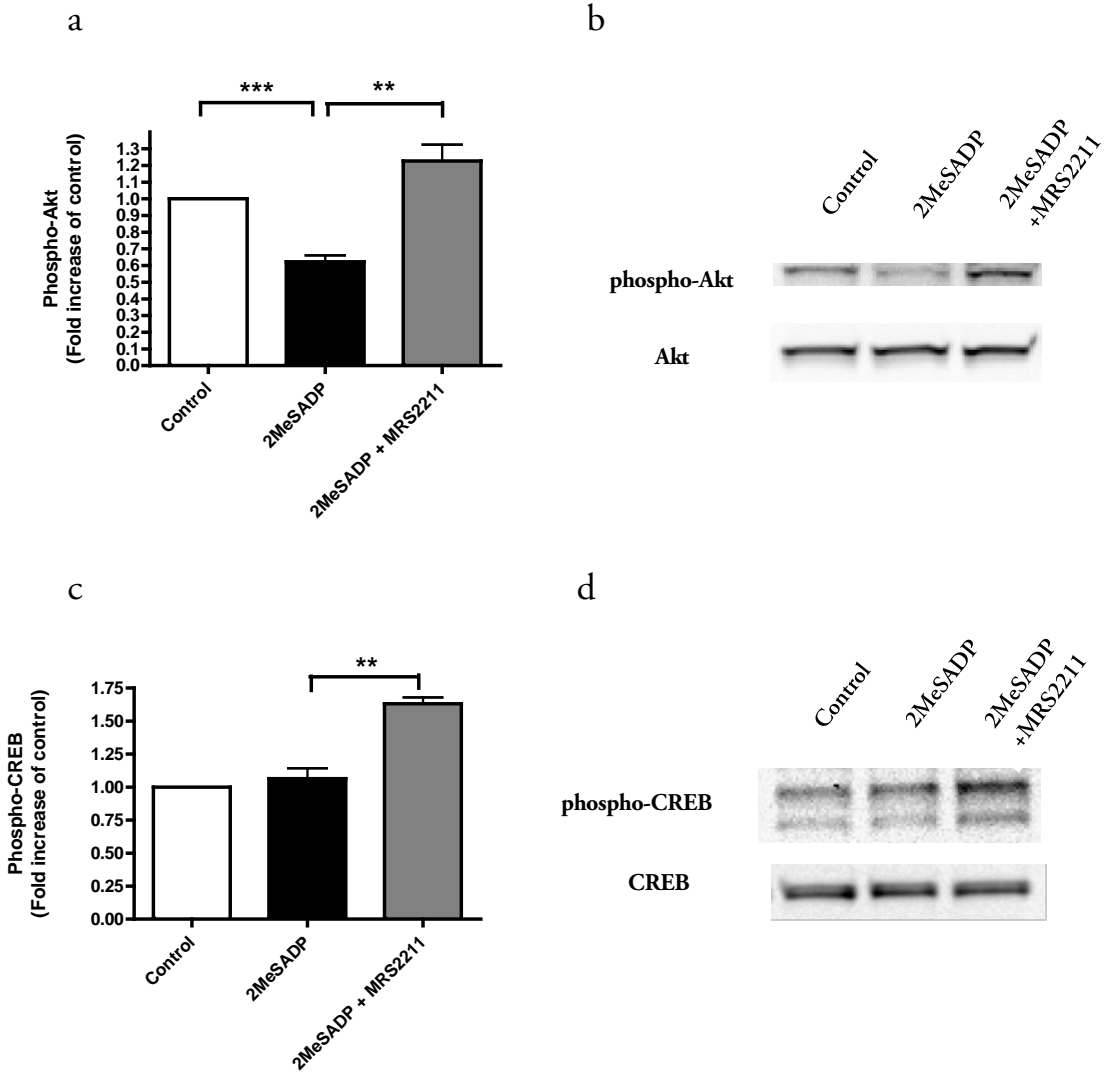


Figure 11. Effects of ADP receptor ligands on the MAP kinases, Akt/PKB and CREB phosphorylation status of MIN6c4 cells. (a-b) Akt/PKB and (c-d) ser-133-CREB. Membranes were reprobbed with the anti-Akt and CREB antibody. Each bar represents the fold increase of phospho-Akt or phospho-CREB relative to control after normalizing against total non-phosphorylated Akt or CREB.

curves in the presence and the absence of MRS2211 we were able to verify the MTT experiments as MRS2211 significantly increased the proliferation. Both of these studies showed that blockade of P2Y₁₃ receptor by using MRS2211 promotes growth and proliferation of MIN6c4 cells.

Taken together, we have found a new pro-apoptotic role of extracellular ADP where ADP acts on the P2Y₁₃ receptor through inhibition of the cAMP/PKA/ERK/CREB/Akt transcriptional pathway in β -cells (Figure 12). And finally, by means of a P2Y₁₃ antagonist MRS2211 it is possible to protect β -cell from apoptosis thereby improves β -cell survival and insulin secretion. Similar P2Y₁₃-antagonists might be the basis of future diabetes therapies.

Signaling Pathway of P2Y₁₃ Receptor in Beta-cell

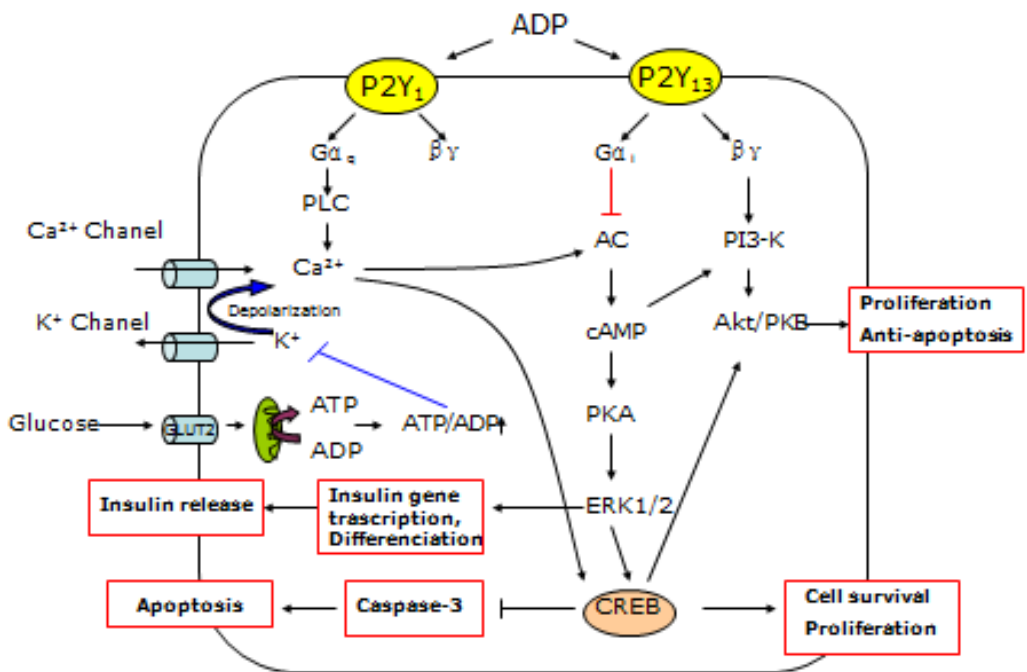


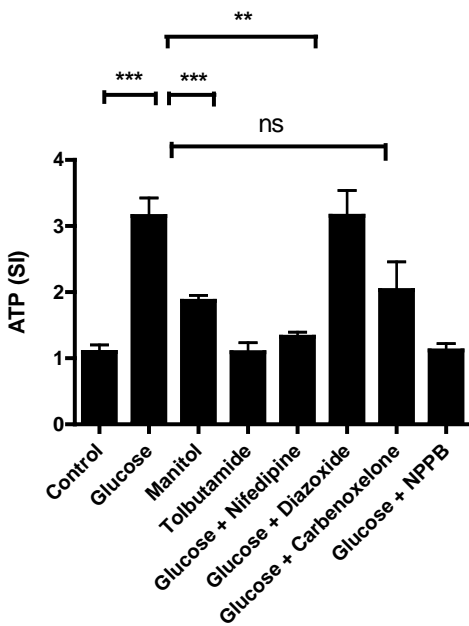
Figure 12. Model shows signalling pathway of P2Y₁₃ receptor in β -cell.

Paper III: High glucose and free fatty acids induce beta-cell apoptosis via autocrine effects of ADP acting on the P2Y₁₃ receptor

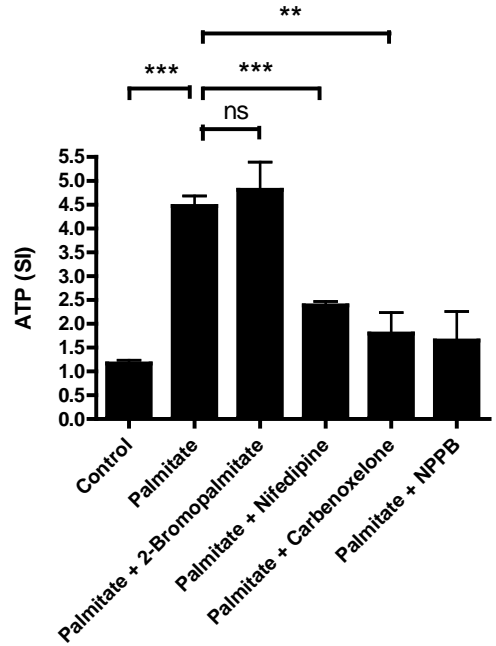
High glucose and palmitate regulate ATP release in β -cells

The high levels of glucose and saturated fatty acids, like palmitate, contribute to the increasing prevalence of obesity and have detrimental effects on β -cell function and survival⁹⁷. However, the signaling pathways mediating these effects are not entirely known. Based on our study of Paper I and II, we next wanted to investigate if autocrine/paracrine purinergic signaling, related to ADP and purinergic receptors, is involved in the proapoptotic effects of high glucose and FFAs in β -cell.

a



b



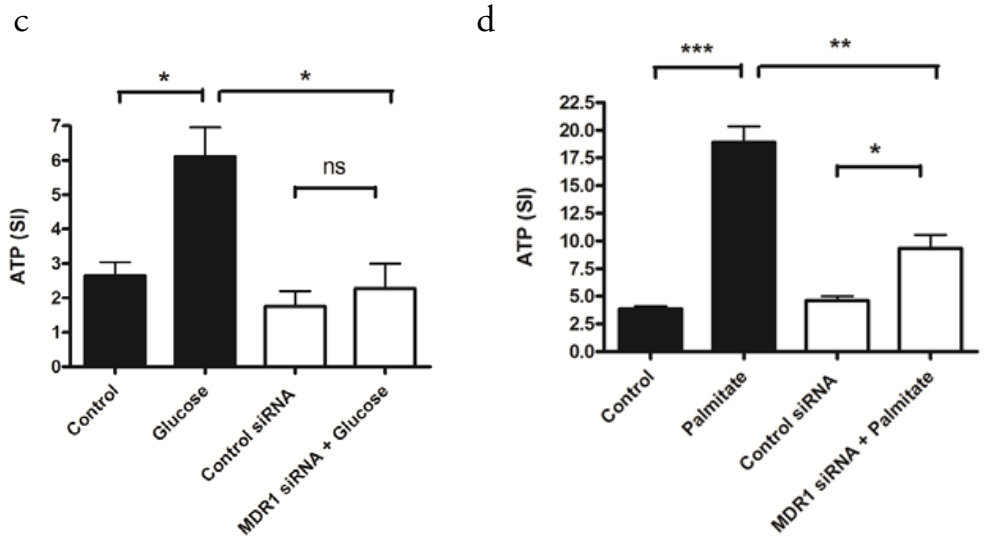


Figure 13. ATP release from MIN6c4 cells. ATP release stimulated by 16.7 mM glucose (a) or 100 μ M palmitate (b) in the present or absent of a series of inhibitor. Silencing of MDR1 gene substantially reduced 16.7 mM glucose (c) and 100 μ M palmitate (d) induced ATP release in MIN6c4 cells.

We first tested if high glucose and palmitate causes ATP release from β -cells. Mouse insulinoma cell line MIN6c4 was used as our primary model system and isolated mouse pancreatic islets as our secondary model. As shown in Figure 13 a, b, after stimulating the cells for 60 seconds in the presence of high glucose or in the presence of palmitate, the release of ATP were both significantly elevated, while equimolar concentration of mannitol was without effect. The calcium-channel blocker nifedipine, the pannexin inhibitor carbenoxelone, and the VRAC-channels inhibitor NPPB could inhibit these effects.

These results indicate that, glucose and palmitate do induce ATP release. Calcium-channels and VRAC-channels might be involved in the ATP release mechanism of β -cells. The multidrug resistance protein MDR1, also known as p-glycoprotein, has previously been shown to be important for activation of VRAC-channels.

Indeed, silencing the MDR1 gene also blocked nutrient generated ATP release (Figure 13c, d). We found that both glucose and palmitate stimulated ATP release were dependent on the MDR1 protein and activation of VRAC-channels. Also, even though the ATP release is related to insulin release, they are not completely co-released, the glucose and palmitate induced ATP release is quicker than the insulin release (Figure 14). The ATP release mechanism is described as Figure 15.

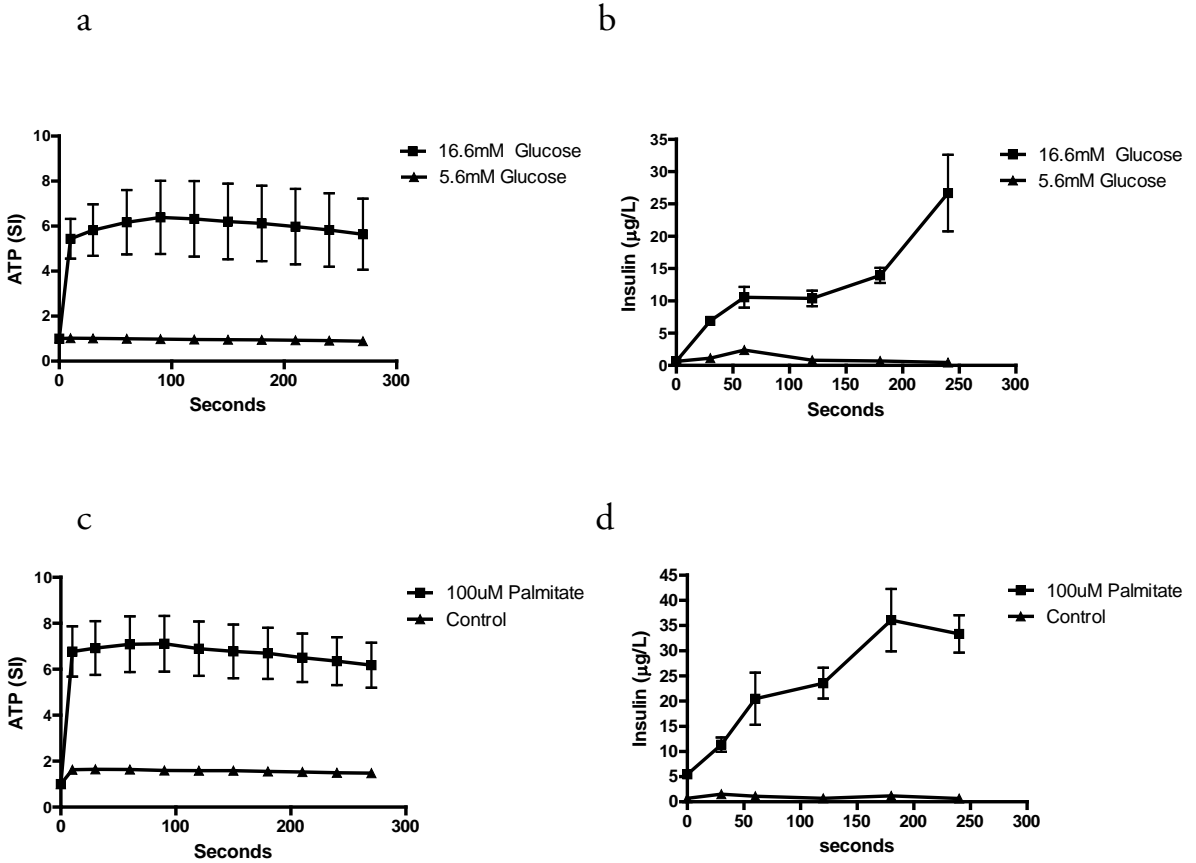


Figure 14. ATP release and insulin secretion from MIN6c4 cells. ATP release stimulated by 16.7 mM glucose (a) or 100 µmol/l palmitate (c) was faster than the 16.7 M glucose or 100 µM palmitate induced insulin secretion (b, d) under same conditions.

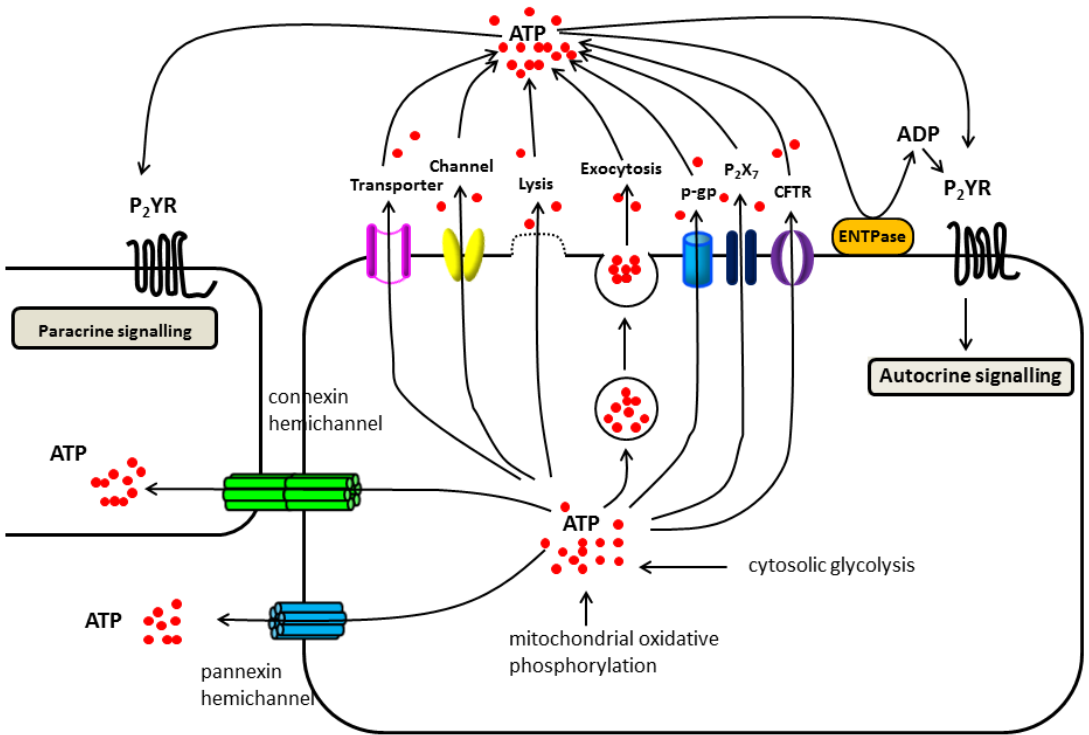


Figure 15. ATP released signal efflux pathways, and purine effects on P₂Y ADP receptors.

ATP release triggered by high glucose or palmitate inhibits cAMP production

As G α i linked P₂Y₁₃ receptor is known to decrease cAMP production. To confirm that P₂Y₁₃ is involved in the autocrine inhibition of adenylate cyclase, cAMP content was also measured in MIN6c4 cells which were treated with either high glucose or palmitate in the presence or absence of MRS2211. We found that in a high glucose medium containing 10 μ M of the P₂Y₁₃ antagonist MRS2211, the cAMP accumulation significantly increased, and blocking the P₂Y₁₃ receptor by MRS2211 during palmitate stimulation resulted in a similar increase in cAMP levels.

Autocrine effect of glucose and palmitate on β -cell apoptosis

Caspase-3 is a key mediator of apoptotic pathways in mammalian cells, then we next investigated if high glucose or palmitate could influence Caspase-3 level in MIN6 cells and to what extent this effect could be inhibited by MRS2211. The Caspase-3 activity induced by high glucose (25 mM) was suppressed by 10 μ M of MRS2211 (Figure 16 a). Similarly, 100 μ M of palmitate induced a significant increase in Caspase-3 activity in MIN6c4 cells and this effect was markedly inhibited by 10 μ M of MRS2211 (Figure 16 b). This result indicates that high glucose and palmitate stimulation causes autocrine activation of Caspase-3 through P2Y₁₃ receptor.

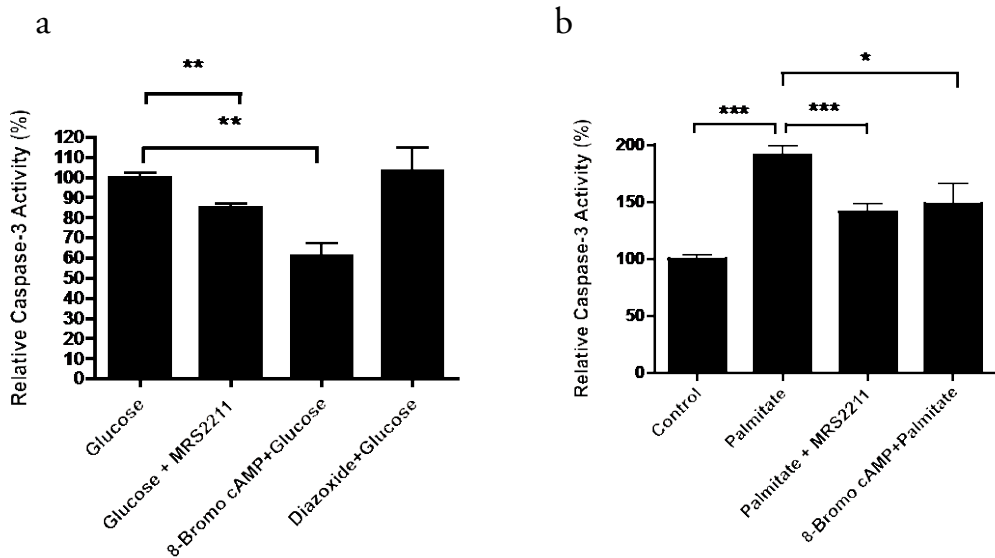
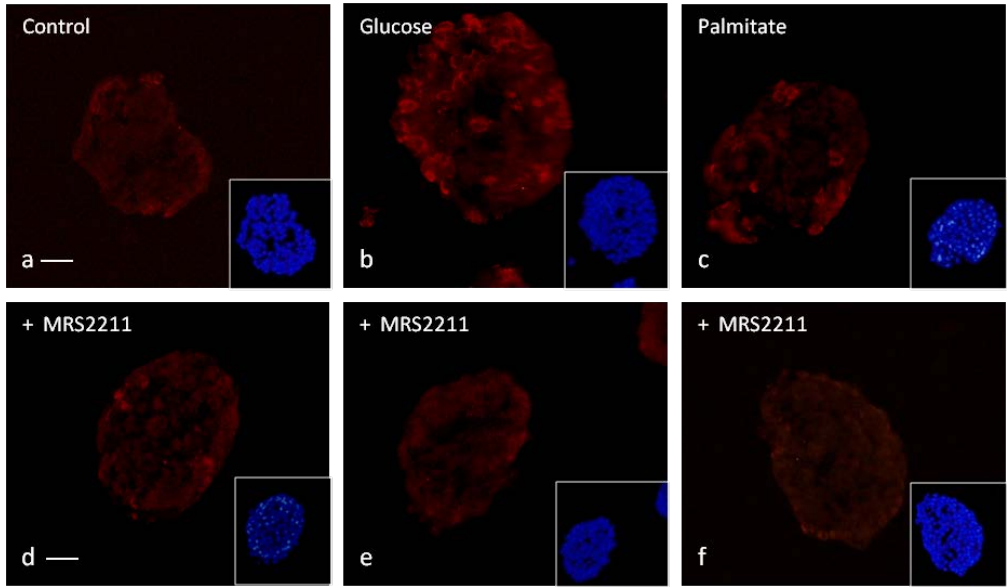


Figure 16. Effects of the P2Y₁₃ antagonist MRS2211 on high glucose (a) and palmitate (b) induced Caspase-3 activity in MIN6c4 cells. MIN6c4 cells growing in 6-well dishes were treated for 36 h with high glucose (25 mM) or palmitate (100 μ M) in the presence or absence of MRS2211 (10 μ M) or 8-Bromo cAMP.

To confirm that the P2Y₁₃ mediated Caspase-3 activation, which is observed in the MIN6c4 cells, also takes place in natural cells, we cultured isolated mouse islets

with either high glucose or palmitate in the presence or absence of MRS2211. Staining the mouse islets for activated Caspase-3 clearly confirmed our previous results as both glucose and palmitate mediated activation of Caspase-3 was suppressed in the presence of MRS2211 (Figure 17).



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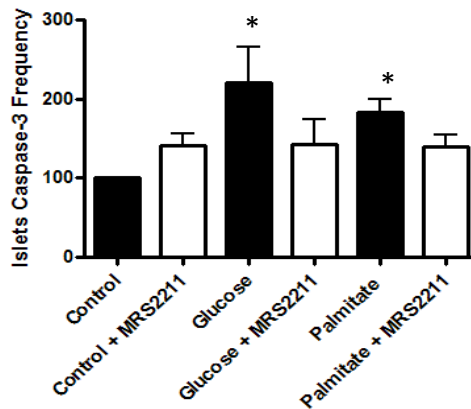
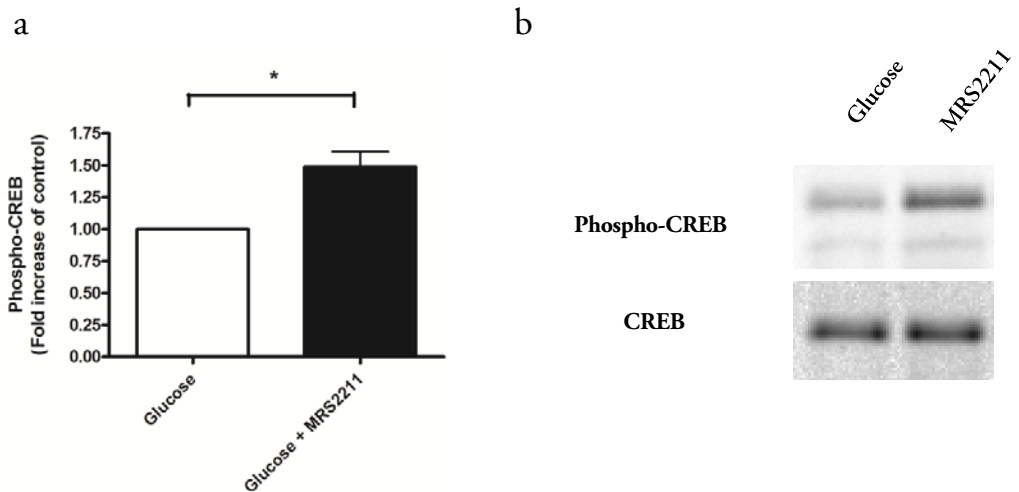


Figure 17. Effect of the P2Y₁₃ antagonist MRS2211 on activated Caspase-3 frequency in isolated mouse islets. Images shown are representative for their treatments; inserts show Hoechst staining for the represented islet. Bar equals 20 μm.

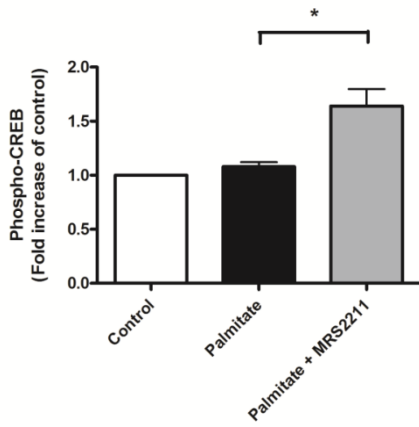
Apoptotic signal-transduction pathway of autocrine P2Y₁₃ activation

CREB is a transcription factor that plays a role in β -cell survival and differentiation, Bad is a proapoptotic member of the Bcl-2 that is inactivated upon phosphorylation at Ser112, while IRS-1 plays important biological function for both metabolic and mitogenic (growth promoting) pathways like intracellular pathways PI3K / Akt and ERK, MAP kinase pathways.

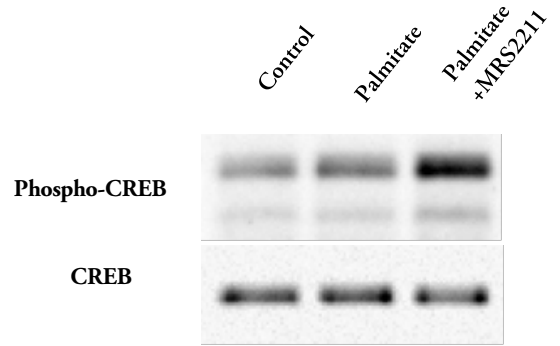
We next determined if the important for cellular survival signal pathways are activated by autocrine/paracrine activation of the P2Y₁₃ receptor by using western blot analysis. MIN6c4 cells incubated in high glucose medium in the presence of MRS2211, displayed an enhanced CREB activation (Figure 18 a, b). Blocking P2Y₁₃ receptor by MRS2211, in the presence of palmitate (100 μ M), also elevated the CREB activation (Figure 18 c, d). These results were paralleled by the results of the analysis of Bad, since P2Y₁₃ inhibition also produced a strong phosphorylation of Bad (Figure 18 e, f). The effect on the IRS-1 phosphorylation state was significant although less pronounced (Figure 18 g, h). Protein studies showed that blocking the P2Y₁₃ receptor resulted in enhanced CREB, Bad and IRS-1 phosphorylation, which are known to be involved in β -cell survival and insulin secretion.



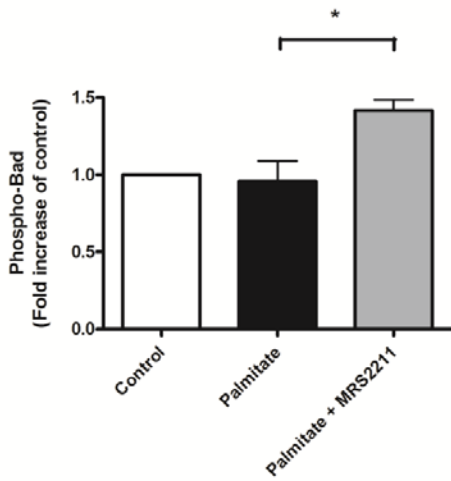
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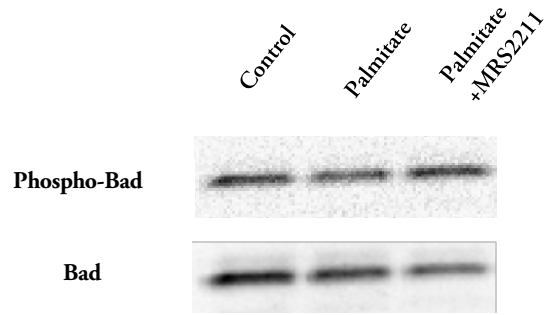
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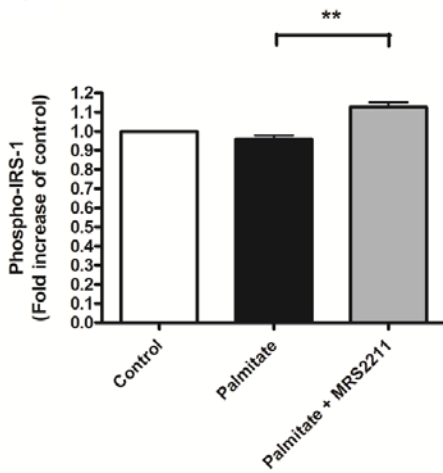
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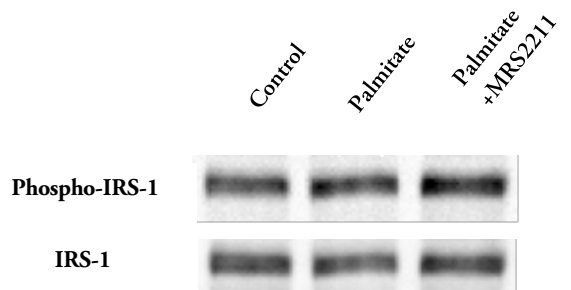
f



g



h



o

Figure 18. *Effects of high glucose and palmitate on the phosphorylation status of CREB, IRS-1 and Bad in MIN6c4 cells. MIN6c4 cells were incubated for 30 min in medium containing: control, high glucose (25 mM) or palmitate (100 μ M) with or without MRS2211 (10 μ M).*

Autocrine activation of P2Y₁₃ receptor mediates β -cells survival and proliferation

To investigate the functional consequences of autocrine P2Y₁₃ activation, the changes in the cell viability and proliferation were determined by means of MTT assay and by cell growth. As measured by the MTT assay 3 days after treatment, the growth of MIN6c4 cells was inhibited by treatment with 100 μ M palmitate, an effect that was reversed by MRS2211. Similar results were obtained when MIN6c4 cells were incubated in 25 mM glucose. These results were confirmed by cell growth that showed an increased proliferation in the presence of MRS2211. Both the results of the MIN6c4 cell MTT assay and the cell proliferation study show that blocking the P2Y₁₃ receptor promotes both the cell viability and proliferation.

In conclusion, these findings provide the important concept that P2Y₁₃ plays an important role in β -cell apoptosis and suggest that autocrine/paracrine mechanisms, related to ADP and P2Y₁₃ receptors, are involved in the proapoptotic effects of high glucose and free fatty acids.

Paper IV: High Glucose Induces ATP Release and Alters Ectonucleotidase Expression in Vascular Smooth Muscle Cells

Identify VSMC by immunofluorescence

How high glucose stimulation modulates extracellular nucleotide release and degradation is unclear in VSMCs. To confirm the identity and purity of primary rat VSMCs we used in our study, immunofluorescent staining of the smooth muscle specific marker α -actin was applied. As shown in Figure 19, primary rat VSMCs are stained for smooth muscle alpha-actin (red; nuclei are pseudocolored

green). It confirmed the rat VSMCs we used and the purity of these cells is more than 80 % in the cell population.

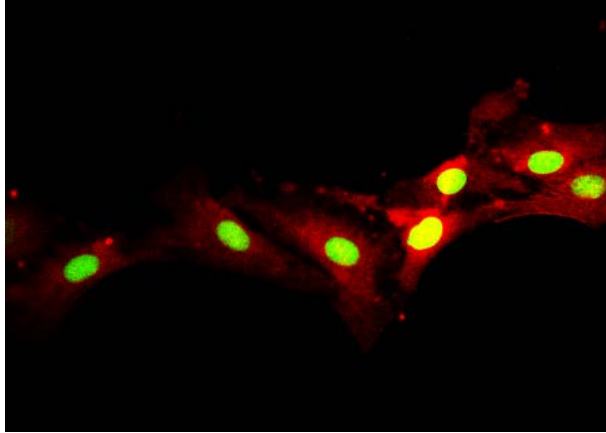


Figure 19. Confocal image of primary rat VSMCs with immunofluorescent labeling of smooth muscle alpha-actin (pseudocolored red, nuclei pseudocolored green) to confirm the identity of the VSMCs.

High glucose regulates gene expression of ectonucleotidases in primary rat VSMCs

Diabetes-induced vascular cell dysfunction has been linked to chronic inflammation and VSMC proliferation, and there are several studies regarding the role of ectonucleotidases and purine signalling in diabetes and vascular disease^{40; 98; 99; 100; 101}. However, little is known about the impact on the vascular wall itself. CD39 is a scavenger of ATP/ADP and UTP/UDP, while CD39L1 activity results in accumulation of UDP and ADP, in turn potentially stimulating proliferation and inflammation. Our hypothesis is that metabolic stress tilts the balance of the extracellular nucleotide cascade towards accumulation of the pro-inflammatory and VSMC stimulating nucleotides ADP and UDP by modulating the expression of ectonucleotidases.

We first determined the gene expression of ectonucleotidases with or without high glucose stimulation by using quantitative real-time PCR. In the primary rat VSMCs, gene transcription of CD39L1 was significantly increased two more times

after 48 hours culture in high glucose (25.5 mM) compared to low glucose control (5.5 mM). Meanwhile, CD39 gene expression was significantly declined to around 30% after exposure to high glucose. High glucose did not have effect on CD39L3, NTPDase8 and CD73 (Figure 20 a). Time cross mRNA analysis further reveals that gene transcription of CD39L1 was significantly increased in rat VSMCs after 48 hours treatment of high glucose (25.5 mM) compared to control (5.5 mM) (Figure 20 b).

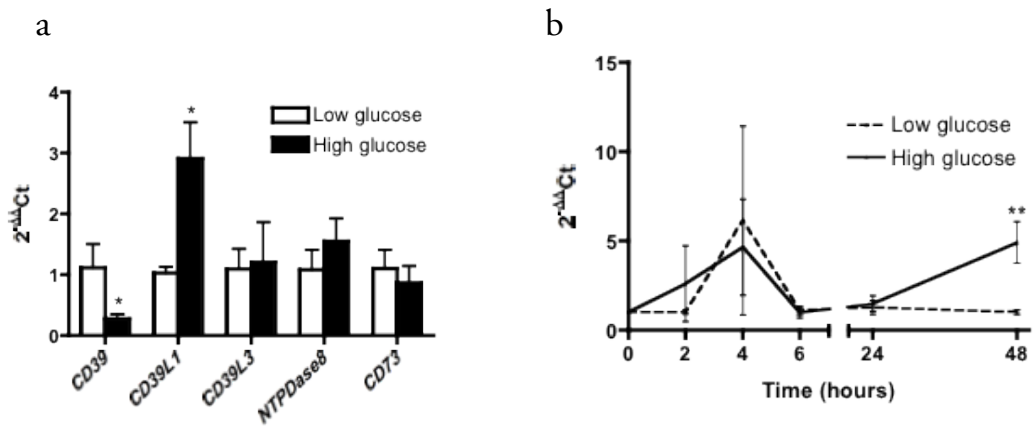


Figure 20. (a) Gene expression of extracellular ectonucleotidases in primary rat VSMCs compared to the housekeeping gene β -actin after 48 hours of culture in low (5.5 mM) or high (25.5 mM) glucose using quantitative PCR. (b) Time curve for CD39L1 mRNA expression in primary rat VSMCs during culture in low (5.5 mM) or high glucose (25.5 mM).

High glucose stimulates ATP release from primary rat VSMCs

Another hypothesis is that hyperglycemia modulates extracellular nucleotide signaling in VSMCs by increasing the rate of nucleotide release. High glucose (25.5 mM) caused a 1.3-fold transient increase in extracellular ATP levels within seconds of the exposure compared to control (5.5 mM glucose + 20 mM mannitol). Blocking of ectonucleotidases using the inhibitor ARC67156 (100 μ M) led to sustained release of ATP demonstrating functional presence of ectonucleotidases on the surface of the VSMCs (Figure 21).

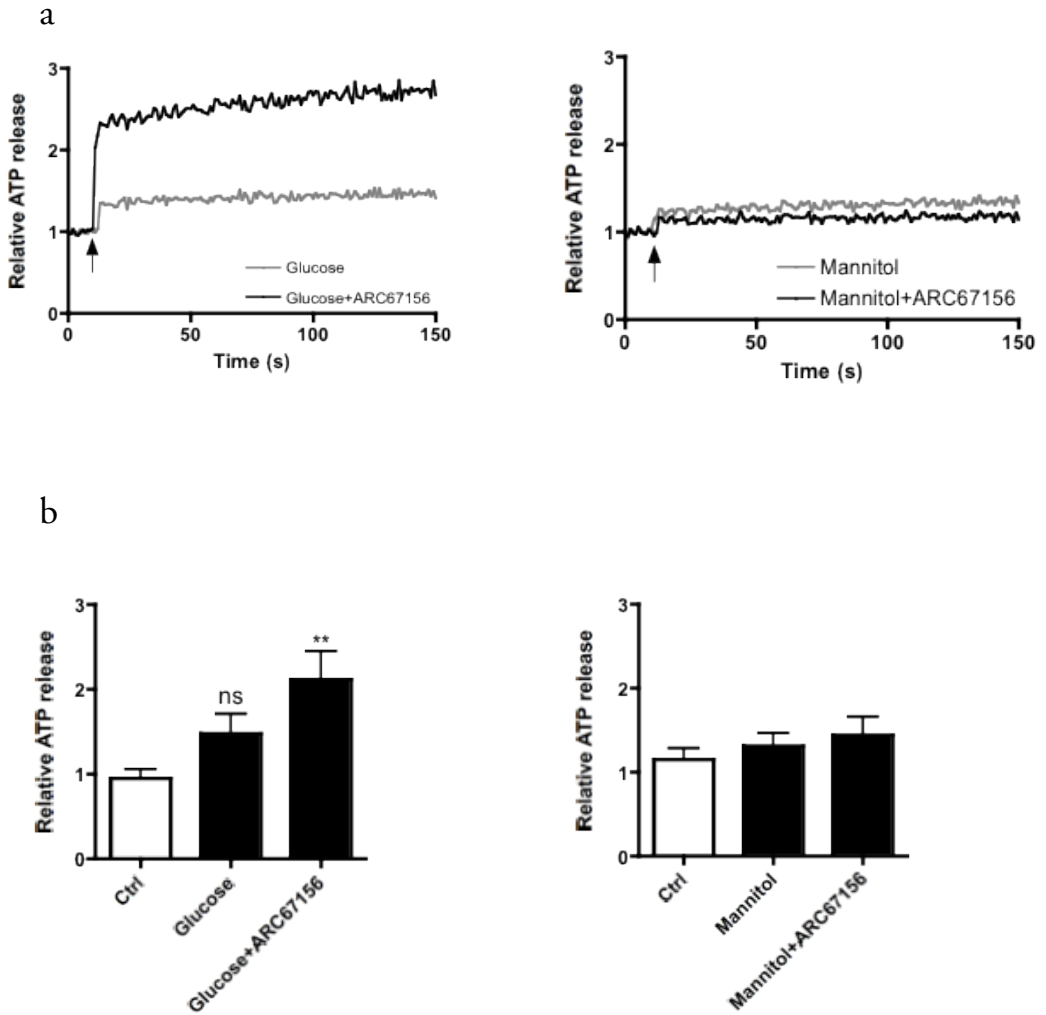


Figure 21. (a) ATP release from primary rat VSMCs in response to high glucose (25.5 mM) or the mannitol control (5.5 mM glucose + 20 mM mannitol) with or without pre-incubation with the ectonucleotidase inhibitor ARC67156 (100 μ M). (b) Statistical analysis of ATP release from primary rat VSMCs.

Taken together, the main finding of the Paper IV was that high glucose increases ATP release from VSMCs and up-regulates the expression of CD39L1 in primary rat VSMCs, predicting accumulation of ADP and UDP that could result in increased vascular inflammation and VSMC proliferation. Figure 22 shows the function of nucleotidases in VSMCs.

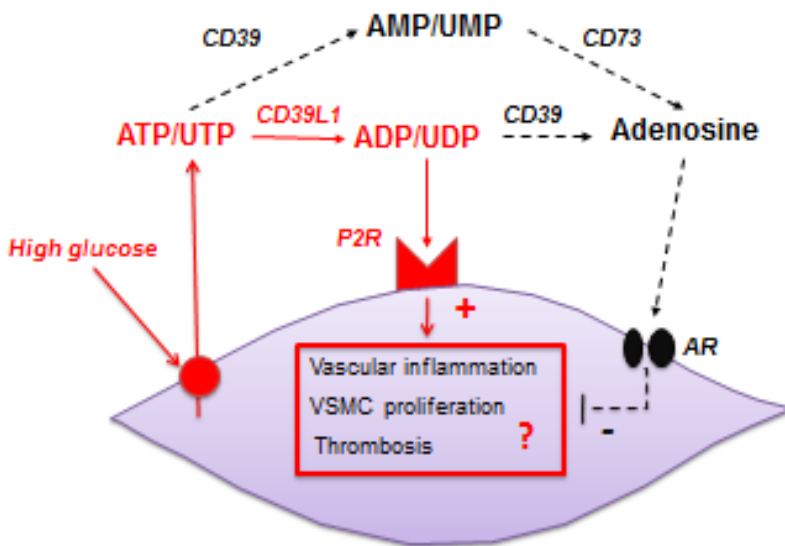


Figure 22. Sketch illustrating the function of nucleotidase in VSMCs. CD39 is a scavenger of ATP/ADP and UTP/UDP, CD39L1 activity results in accumulation of UDP and ADP, in turn stimulating proliferation and inflammation.

CONCLUSION

- ADP acting on the P2Y₁₃ receptors inhibits insulin release. (**Paper I**)
- Activation of the P2Y₁₃ receptor of mouse MIN6c4 cells has a pronounced proapoptotic effect and this effect is mediated by inhibiting the cAMP/ERK/CREB/Akt pathway. (**Paper II**)
- Nutrient-stimulated ATP release from MIN6 cells is dependent on VRAC and MDR1. (**Paper III**)
- High glucose and free fatty acids induce beta cell apoptosis via autocrine effects of ADP acting on the P2Y₁₃ receptor. (**Paper III**)
- High glucose increases ATP release from VSMCs and up-regulates the expression of CD39L1 in VSMCs. (**Paper IV**)

SAMMANFATTNING

Endokrina och metabola sjukdomstillstånd har många gemensamma komponenter. Att förstå de signalvägar som ligger till grund för dessa sjukdomar hjälper oss att utveckla bättre behandlingsmetoder. Det övergripande syftet med min forskning är att undersöka den roll s.k. G-protein kopplade receptorer spelar i endokrina metaboliska sjukdomar, mer specifikt de signalvägar som är kopplade till G α -subenheten. I förlängningen kan detta leda till nya behandlingsmetoder för sjukdomar som påverkar miljontals människor världen över.

Reglering av insulinutsöndring via ADP

Den första delen av mitt avhandlingsarbete fokuserade på att undersöka effekten av extracellulära puriner på insulinutsöndring från de langerhanska öarna i bukspottkörteln hos möss och i sådana fall huruvida de s.k. P2Y-receptorerna är inblandade. Höga glukoshalter stimulerar utsöndring av extracellulärt ATP från många typer av vävnader och celler. Många studier har visat att ATP spelar en viktig roll för insulinutsöndring, men huruvida ADP också är inblandat har varit en kontroversiell fråga. Anledningen till detta är förmodligen att flera olika typer av purinoreceptorer är inblandade. Vi har nu kunnat visa att glukos stimulerar utsöndring av insulin-reglerande puriner. ADP visade sig ha en dubbel roll i betacellerna, då vi fann att insulinutsöndring stimuleras via P2Y₁-receptorer och inhiberas via P2Y₁₃-receptorer. En antagonist till P2Y₁₃-receptorn, MRS2211, ökade glukos-stimulerad insulinutsöndring och minskade glukosnivåerna, vilket tyder på att P2Y₁₃-antagonism skulle kunna vara värdefullt för behandling av type 2 diabetes.

Reglering av β -cell apoptos via ADP

Avhandlingsarbetets andra del har fokuserat på vilken roll de P2-receptorer som binder ADP har för apoptos hos β -celler. Att förhindra celldöd hos β -cellerna är en nödvändig del av behandlingen av diabetes. Puriner och pyrimidiner är viktiga

signalmolekyler som påverkar många olika cellulära processer men vilken roll extracellulärt ATP och ADP spelar för apoptos hos β -celler har varit oklart. Vi upptäckte att ADP och P2Y₁₃ spelar en viktig roll för apoptos hos β -celler via aktivering av kaspas-3 och kartlade den signalväg som är inblandad. Genom farmakologiska interventionsstudier kunde vi se att aktivering av P2Y₁₃ orsakade inhibering av ERK1/2 medan aktivering av P2Y₁ stimulerade p38, vilket tillsammans leder till en pro-apoptotisk signal. Det är troligt att denna effekt uppkommer på grund av sänkta cAMP-nivåer till följd av P2Y₁₃-medierad G α i-aktivering. Vidare fann vi att inhibering av P2Y₁₃ ökade ser-133-CREB-fosforylering, vilket är en viktig överlevnadsfaktor hos β -celler. Aktivering av cAMP/PKA/CREB-signalvägen resulterar i en ökad insulin-medierad PI3K-fosforylering av Akt/PKB, vilket leder till ökad resistens mot apoptos. Den ökade viabilitet vi såg hos β -cellerna när vi blockerade P2Y₁₃ kan troligen förklaras delvis med aktivering av Akt. Sammanfattningsvis har vi visat att aktivering av P2Y₁₃-receptorn i en β -cellinje har en betydande pro-apoptotisk effekt via cAMP/ERK/CREB/Akt-signalvägen.

Höga nivåer av glukos/palmitat reglerar β -cell apoptos via autokrina effekter av ADP

I en studie tittade vi på autokrina effekter av ADP på β -cell apoptos via P2Y₁₃. Att glukolipotoxicitet påverkar β -cellfunktion och överlevnad negativt har visats tidigare, men vilka signalvägar som medierar dessa effekter är fortfarande okänt. Vi kunde visa att kalciumkanaler, VRAC-kanaler och proteinet MDR1 är involverade i glukos/palmitat-medierad ATP-frisättning. Höga nivåer av glukos och palmitat inhiberade produktion av cAMP och minskade cellproliferation i MIN6c4-celler och ökade kaspas-3 aktivitet i langerhanska öar från möss via P2Y₁₃. Vidare studier visade att blockering av P2Y₁₃ resulterade i ökad fosforylering av CREB, Bad och IRS-1, en signalväg som sedan tidigare visat sig vara viktig för överlevnad hos β -celler och insulinutsöndring. Dessa fynd visar att ADP spelar en viktig auto- och parakrin roll för β -cell apoptos medierad av höga nivåer av glukos och fria fettsyror via P2Y₁₃. Detta har betydelse för förståelsen av sjukdomsförloppet i diabetes.

Höga glukosnivåer inducerar ATP-frisättning och påverkar uttrycket av ektonukleotidaser i vaskulära glattmuskelceller

Vaskulär dysfunktion är vanligt hos patienter med diabetes och har kopplats till kronisk inflammation och proliferation hos vaskulära glattmuskelceller (VSMC). I nuläget studerar vi hur höga glukosnivåer påverkar ATP-frisättning och ektonukleotidas-uttryck i VSMC. Våra resultat indikerar att inhibering av CD39L1 vid höga glukosnivåer blockerar nedbrytningen av ATP till ADP vilket resulterar i ökade ATP-nivåer. Dessutom orsakar höga glukosnivåer minskat uttryck av CD39 och ökat uttryck av CD39L1. CD39 verkar genom att bryta ned ATP/ADP samt UTP/UDP medan CD39L1-aktivering leder till ackumulering av ADP och UDP. Effekterna av höga glukosnivåer leder därför till en ackumulering av ADP vilket stimulerar proliferation och inflammation hos VSMC.

论文概要

许多不同的内分泌和代谢疾病实际上有着很多共同的内在特征。认识这些信号转导能帮助我们在更深层次上探究问题的本身，通过研究一系列的细胞膜受体能够让我们寻找到更好的治疗疾病的方法。我们研究的根本目标是认识 G 蛋白偶联受体(GPCRs) 对内分泌代谢疾病的作用和影响，研究 $G\alpha$ 亚基介导下的 G 蛋白偶联受体信号转导，并藉此开发治疗这些疾病的新型治疗方法以改善提高世界上数以百万计的人的健康水平和生活质量。

ADP 对胰岛素分泌的调控

我们第一部分的研究目标是探讨细胞外嘌呤类化合物对小鼠胰岛分泌胰岛素的影响，并且检验 P2Y 受体的参与。高血糖水平刺激 ATP 从多种组织和细胞中释放到细胞外间质中。大量的研究表明，细胞外 ATP 和 ADP 对胰岛素的释放承担着重要的作用，但是关于 ADP 如何影响胰岛素的释放还存在争议。这些不同的意见的原因很可能是由于这个过程涉及多种不同的嘌呤受体。在本研究中，我们为血糖能刺激胰岛细胞分泌具有胰岛素调控能力的嘌呤提供了新的证据。我们发现 ADP 在 β 细胞中具有双向的调控的能力，ADP 既能通过激活 P2Y₁ 受体刺激胰岛素分泌，也能通过作用于 P2Y₁₃ 受体抑制胰岛素的分泌。P2Y₁₃ 受体抑制剂 MRS2211 能够提高血糖诱导的胰岛素分泌，以此降低血糖水平。这些结果揭示了 P2Y₁₃ 的抑制剂对治疗二型糖尿病具有潜在的临床药物价值。

ADP 对 β 细胞凋亡的调控

我们第二个部分的研究内容主要关注 ADP 特异性嘌呤 P2Y 受体在 β 细胞凋亡中所扮演的角色。在糖尿病病理学过程中抑制 β 细胞的凋亡对阻碍糖尿病的发生是十分必要的。嘌呤和嘧啶是十分重要的信号分子，他们影响着一系列细胞生理过程。然而，细胞外 ADP 和 ATP 对 β 细胞凋亡的作用和影响还很少被研究。我们首次揭示了在 ADP 激活下嘌呤受体 P2Y₁₃ 能引起 β 细胞凋亡。并证实了 ADP 激活 Caspase-3 通路是通过其特异性激活 P2Y₁₃ 受体来实现的。药理学水平上的研究进一步展示了 P2Y₁₃ 能抑制 ERK1/2，同时 P2Y₁ 能激活 p38，从而共同整合发出引起细胞凋亡的信号。引起此细胞凋亡的一个主要因素是构成 P2Y₁₃ 受体的 $G\alpha_i$ 亚基的激活导致细胞中 cAMP 水平的降低。此外我们还发现了阻断 P2Y₁₃ 通路能显著提高 ser-133-CREB 磷酸化水平，而 ser-133-CREB 磷酸化是 β 细胞的一个重要存活因子。cAMP/PKA/CREB 细胞信号转导途径的激活提高了 PI3K 激酶对 Akt/PKB 的磷酸化水平，从而抑制了细胞凋亡。阻断 P2Y₁₃ 受体所引起的 Akt

的激活也反映在了其对 β 细胞的存活率有所提高上。简而言之, 我们揭示了 β 细胞中 $P2Y_{13}$ 受体的激活是通过 cAMP/ERKs/CREB/Akt 信号传导途径对细胞凋亡具有重要的介导调控作用的。

高血糖/脂 通过 ADP 的自分泌作用对 β 细胞凋亡的调控

我们还研究了 ADP 自分泌作用对 $P2Y_{13}$ 受体介导 β 细胞凋亡的影响。虽然糖毒性对 β 细胞功能和存活具有不利影响是广为人知的, 但是介导这些作用的细胞信号通路并不完全为人们所了解。我们发现, 钙离子通道, VRAC 通道和多药耐药蛋白 MDR1 是参与高血糖/脂引导的 ATP 释放机制的。高血糖/脂通过激活 $P2Y_{13}$ 受体抑制了 cAMP 的生成和细胞增值, 并使小鼠胰岛中具有 Caspase-3 活性的细胞数目有所提高。进一步研究显示, $P2Y_{13}$ 受体的阻断会提高 CREB, Bad 和 IRS-1 的磷酸化, 从而改善 β 细胞的存活和胰岛素的分泌。这些结果为高血糖/脂引起的自分泌和共分泌 ADP 作用于 $P2Y_{13}$ 受体后在 β 细胞凋亡中所展现的重要作用提供了重要的理论根据。综上所述, 我们的研究对认识内分泌科学具有重要意义, 并有可能提高和改善众多糖尿病病人的生活质量。

高血糖引起 ATP 释放并影响和改变血管平滑肌细胞中外核苷酸水解酶的表达

糖尿病引起的血管细胞功能障碍是与慢性炎症以及血管平滑肌细胞增值相关联的。目前, 我们在研究高血糖如何作用于血管平滑肌细胞中, 以及高血糖如何影响 ATP 的释放和外核苷酸水解酶的表达。我们的结果指出, 在高血糖的刺激下抑制 CD39L1 将阻断 ATP 降解成 ADP, 从而增加 ATP 的释放。此外, 我们的基因转录的研究表明, 高血糖会导致 CD39 的表达降低和 CD39L1 的表达增加。外核苷酸水解酶 CD39 能降解 ATP/ADP 和 UTP/UDP, 而 CD39L1 的激活却能促使 ATP 降解成 ADP, 从而导致 ADP 和 UDP 的积累, 藉此调控血管平滑肌细胞外核苷酸的功能和刺激细胞增值, 导致血管平滑肌细胞将更多 ATP 转换成 ADP 和促进炎症反应。总而言之, 代谢压力使血管平滑肌细胞中外核苷酸级联的平衡导向积累更多的促炎症因子, 并在通过增加核苷酸释放的方式和调控外核苷酸酶表达的方式刺激了更多 ADP 和 UDP 的生成。

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