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Local Purinergic Control of Arteriolar Reactivity in Pancreatic Islets and Renal Glomeruli

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Abstract
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Local control of regional blood flow is exerted mainly through the arterioles. An adequate minute-to-minute regulation of blood perfusion of the kidney and the pancreas is obtained by the modulation of arteriolar reactivity, which will influence the organ function. The importance of purinergic signaling in this concept has been addressed, with special emphasis on the role of the adenosine A_1 receptor. The effects of adenosine on two specialized vascular beds, namely the renal glomerulus and the pancreatic islets, have been examined. Characteristic for these regional circulations is their very high basal blood flow, but with somewhat different responses to vasoconstrictor and vasodilator stimuli. By adapting a unique microperfusion technique it was possible to separately perfuse isolated single mouse arterioles with attached glomeruli or pancreatic islets *ex vivo*. Microvascular responses were investigated following different additions to the perfusion fluid to directly examine the degree of dilation or constriction of the arterioles. This has been performed on transgenic animals in this thesis, *e.g*. A_1 receptor knockout mice. Also effects of P2Y receptors on islet arterioles were examined in both normoglycemic and type 2 diabetic rats. Furthermore, interference with adenosine transport in glomerular arterioles were examined.. Our studies demonstrate important, yet complex, effects of adenosine and nucleotide signaling on renal and islet microvascular function, which in turn may influence both cardiovascular and metabolic regulations. They highlight the need for further studies of other purinergic receptors in this context, studies that are at currently being investigated.

Keywords: afferent arteriole, islet arteriole, adenosine, A1 receptor, ATP, P2Y receptor, microperfusion, angiotensin II, type 2 diabetes, hypertension, oxidative stress, nitric oxide, tubuloglomerular feedback

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List of Papers

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals

I. Gao, X., Patzak, A., Sendeski, M., Scheffer, P.G., Teerlink, T., Sällström, J., Fredholm, B.B., Persson, A.E.G., Carlström, M. (2011) Adenosine A_1-receptor deficiency diminishes afferent arteriolar and blood pressure responses during nitric oxide inhibition and angiotensin II treatment. *Am J Physiol Regul Integr Comp Physiol 301:R1669-1681*

II. Gao, X.*, Peleli, M.*, Zollbrecht, C., Patzak, A., Persson, A.E.G., Carlström, M. Role of adenosine A_1 receptor dependent and independent pathways in modulating renal vascular responses to angiotensin II. *Submitted*.

III. Gao, X.*, Yang, T.*, Sandberg, M., Zollbrecht, C., Zhang, X.M., Hezel, M., Liu, M., Peleli, M., Lai, E.Y., Harris, R.A., Persson. A.E.G., Fredholm, B.B., Jansson, L., Carlström, M. Abrogation of adenosine A_1 receptor signaling improves metabolic regulation in mice by modulating oxidative stress and inflammatory responses. *Submitted.*

IV. Gao, X., Sandberg, M., Bodin, B., Persson, A.E.G., Jansson, L. Important role of P2Y receptors for islet blood flow regulation in anesthetized rats during acute and chronic hyperglycemia. *Manuscript.*

*These authors contributed equally to the work.

Contents

Abbreviations

$A_1^{-/-}$	Adenosine A_1 receptor knockout mouse
$A_1^{+/+}$	Adenosine A_1 receptor wildtype mouse
ACE	Angiotensin-converting enzyme
ADP	Adenosine biphosphate
AMP	Adenosine monophosphate
Ang II	Angiotensin II
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
DEXA	Dual energy X-ray absorptiometry
DMEM	Dulbecco's modified essential medium
EDTA	Ethylene-diamine tetra-acetic acid
GFR	Glomerular filtration rate
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide 1
HIF-1α	Hypoxia-inducible factor-1α
HPLC	High-pressure liquid chromatography
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12p70	Interleukin-12p70
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
JGA	Juxtaglomerular apparatus
KC/GRO	Keratinocyte chemoattractant or growth regulated oncogene-α
Kf	Filtration coefficient
KRBH	Krebs-Ringer bicarbonate buffer with Hepes
L-NAME	L-nitro-arginine methylester
LPA	Lysophosphatidic acid
MAP kinase	Mitogen-activated protein kinase
MDA	Malondialdehyde
MLC	Myosin light chain
MSD	Meso-Scale Discovery
NADPH	Nicotinamide adenine dinucleotide phosphate
NBTI	S-(4-nitrobenzyl)-6-theoinosine

NF-kB	Nuclear factor k-light-chain-enhancer of activated B cells
NKCC	Na/K/Cl co-transporter
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG-VSMC	Preglomerular vascular smooth muscle cells
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SD	Sprague-Dawley
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
T2D	Type 2 diabetes
TGF	Tubuloglomerular feedback
TNF-α	Tumor necrosis factor α
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VSMC	Vascular smooth muscle cells

Introduction

PURINES

Adenosine is ubiquitously present in intra- and extracellular compartments in concentrations in the nanomolar range under physiological conditions. It is mainly formed by metabolism of ATP via intra- or extracellular adenine nucleotidases [1], but some contribution occurs from hydrolysis of *S*-adenosyl homocysteine [2]. Intracellular ATP concentrations are high, *i.e.* in the millimolar range). Thus, transient or permanent traumatic damage of cell membranes markedly increases extracellular ATP, and the subsequent rapid formation of adenosine through nucleotidases.

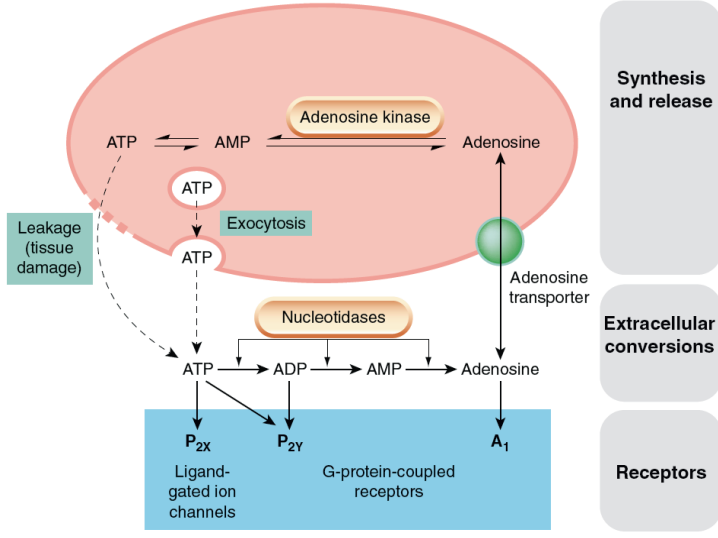


Figure 1: Schematic drawing of purinergic signaling (1).

The nucleotides ATP, ADP and AMP act on either ligand-gated ion channels, *i.e.* P2X receptors of which 7 subtypes ($P2X_1$ to $P2X_7$) are described, or G-protein-coupled receptors (GPCR) out of which 12 differencs can be found in humans [3, 4]. A brief summary of P2Y receptors are given in Table 1, and they are further discussed in relation to their expression in the pancreas, since Study IV deals with these receptors.

Table 1: P2Y receptors and their presence in the pancreas

RECEPTOR NAME	NUCLEOTIDE BINDING	PRESENT IN PANCREAS
$P2Y_1$	ADP (ATP)	VSMC in all size vessels [5] Duct cells [5] Islet capillaries [5] β-cells (mouse [6], but not rat [5]
$P2Y_2$	ATP, UTP	VSMC in large vessels Duct cells
$P2Y_4$	UTP	α- and β-cells
$P2Y_5$/LPA6	Lysophospatidic acid	Not described
$P2Y_6$	UDP	β-cells [7]
$P2Y_8$	Orphan receptor	Not described
$P2Y_9$/LPAR4/GPR23	Lysophospatidic acid	Not described
$P2Y_{10}$	Orphan receptor	Not described
$P2Y_{11}$	ATP	Islet endocrine cells (human) [8]
$P2Y_{12}$	ADP	Islet endocrine cells (human) [8] β-cells (rat; own unpublished observation)
$P2Y_{13}$	ADP	β-cells (mouse) [9]
$P2Y_{14}$	UDP-glucose	VSMC in intestines [10] Islet endocrine cells [10]

The gaps in the list are due to previously falsely assigned P2Y receptors. Lysophosptafic acid (LPA) referred to in the table is a phospholipid derivative that can act as a signaling molecule. LPA acts as a potent mitogen due to its activation of high-affinity GPCR, LPA receptors.

ADENOSINE

Adenosine acts on four GPCR referred to as A_1, A_{2A}, A_{2B} and A_3. It should be noted that the first three of these receptors are the major target for caffeine. Already the concentrations achieved following a single cup of coffee or tea suffices to cause significant inhibition of these adenosine receptors, with associated biological effects [11]. Mice with targeted deletions of each of the receptors exist and have been very important in the characterization of their physiological and pathophysiological roles, and some of these mice are used in the work included in this thesis.

Physiological adenosine concentrations are sufficient to activate A_1, A_{2A} and A_3 receptors, at least if they are abundantly expressed [12]. By contrast, adenosine A_{2B} receptors require higher concentrations that are believed to be

present only during pathological conditions [12]. It should be noted that the potency of adenosine as an agonist is very dependent on the density of receptors. The immune system, especially during stress, can influence the expression of adenosine receptors. Furthermore, expression of adenosine receptors in nerve cells is also regulated by nerve activity. Hypoxia can regulate adenosine concentrations by many means and can also stimulate the expression of adenosine receptors, besides A_{2A} receptors.

Since virtually all cells express one or more adenosine receptor subtypes the substance produces many pharmacological effects, both in the periphery and in the central nervous system [4, 13]. Based on its ability to inhibit cell function and thus minimize the metabolic requirements of cells, one of its functions may be as a protective agent that is rapidly released when tissue integrity is threatened [11]. Variations in adenosine release may play a role in controlling blood flow and (through effects on the carotid bodies) respiration, matching them to the metabolic needs of the tissues, as further outlined below in our studies [14]. Furthermore, adenosine is an_inhibitor cardiac conduction probably through all four of the receptors.

Besides these actions, adenosine receptors are found on all the cell types involved in asthma and causes enhanced mucus secretion, bronchial constriction and leukocyte activation [13]. Acting through A_1 and A_{2A} receptors, adenosine inhibits on many neurons, and the stimulation experienced after consumption of methylxanthines such as caffeine occurs partly as a result of block of these receptors [13].

NBTI (S-(4-nitrobenzyl)-6-theoinosine) is the nucleoside transport blocker, which affects the regulation of extracellular adenosine levels and the cardiac system signaling associated with adenosine.

ADENOSINE IN RENAL FUNCTION

Glomerular filtration rate

Glomerular filtration rate (GFR) is the formation of primary urine that is subsequently modified by tubular reabsorption and secretion to form secondary urine. GFR and tubular reabsorption are to a large extent matched to maintain fluid and electrolyte homeostasis. Autoregulation of renal blood flow and GFR is performed by both the myogenic response and tubuloglomerular feedback (TGF). The filtration *per se* depends on the net filtration pressure and the filtration coefficient (Kf) as defined by the Starling equation. The filtration pressure, which is the sum of the factors favoring filtration and those opposing filtration, can be influenced by several factors.

Glomerular filtration pressure is directly influenced by the renal plasma flow, the tone of the afferent and efferent arterioles, as well as the rate of

reabsorption in the proximal tubulus. An increase in renal plasma flow will reduce oncotic pressure in capillaries, which thereby will increase filtration pressure and GFR. An increased contraction of the afferent arteriole will reduce GFR by decreasing plasma flow and hydrostatic capillary pressure. The effects of the changing efferent arteriolar tone are more difficult to predict. An increased contraction will reduce glomerular plasma flow, which reduces GFR, but will also increase hydrostatic capillary pressure, which will counteract a reduction in GFR. An increased rate of reabsorption in the proximal tubulus affects filtration pressure by reducing hydrostatic pressure in Bowman's space, thus favoring filtration.

The renin-angiotensin-aldosterone (RAAS) system

The RAAS system is important for the normal regulation of blood pressure and electrolyte homeostasis, and is stimulated by a reduction in blood pressure or low sodium intake. Renin is an enzyme that is produced from the granulated juxtaglomerular cells in the wall of the afferent arteriole and converts the inactive protein angiotensinogen to angiotensin I, which is rapidly converted into the active peptide angiotensin II (Ang II) by the action of angiotensin-converting enzyme (ACE). Angiotensinogen is continuously released from both the liver and the kidney, and ACE is mainly localized to the vascular endothelium. The classical physiological effects of Ang II include general vasoconstriction, increased renal electrolyte reabsorption, and thirst. Ang II will also increase aldosterone release from the adrenal gland, thereby promoting sodium retention. All these effects are mediated through AT_1 receptors and act to prevent a fall in blood pressure. Ang II also acts on AT_2 receptors, which appear to[15] have opposite effects to those of AT_1 receptors and have sex differences. Moreover, increased activity of the RAAS and activation of AT_1 receptor signaling have been associated with oxidative stress and progressive inflammation in various pathological conditions including renal disease, hypertension and diabetes [15].

The tubuloglomerular feedback (TGF) mechanism and adenosine

The TGF mechanism is a negative feedback loop controlling GFR, and this feedback system operates within the juxtaglomerular apparatus (JGA) of each nephron. When the macula densa detects an increased tubular NaCl load TGF is activated and a paracrine signal is generated leading to constriction of the parent afferent arteriole to match tubular sodium load to its reabsorption capacity. More than 30 years ago it was suggested that adenosine was the mediator of the TGF response, thereby providing a link between the metabolic demand from the Na/K/Cl co-transporter (NKCC) and isoenzyme 2 transporters in the macula densa cells and vasoconstriction. Since then many studies have been performed to address this question.

Moreover, the afferent and efferent arterioles are innervated by sympathetic nerves. Contractile effects of norepinephrine are mediated by adrenergic α_1 receptors, whereas β_1 receptors diminish this contraction. Glomerular filtration is also modulated by various hormones and endocrine substances. Ang II acts on AT_1 receptors in both afferent and efferent arterioles to reduce renal blood flow. Adenosine is formed in the kidney by ATP metabolism, as outlined above, which can constrict afferent arterioles via activation of A_1 receptors or dilate via A_2 receptors [16]. Adenosine plays an important role in the TGF mechanism as outlined further below.

The afferent arterioles mainly express adenosine A_1 receptors, which mediates vasoconstriction through GPCR increasing intracellular calcium $[Ca^{2+}]_i$ in the vascular smooth muscle cells (VSMC). Further studies in $A_1^{-/-}$ confirmed that the TGF mechanism is dependent on A_1 receptors, since these mice completely lack the TGF response [17, 18]. The key role of adenosine is further emphasized by the fact that it also may, by activation of A_2-receptors (A_{2A} or A_{2B}) and release of nitric oxide (NO), attenuate TGF [19]. Furthermore, elevated levels of Ang II and reactive oxygen species (ROS) may enhance TGF and preglomerular vascular reactivity [20, 21], and have indeed been associated with hypertension in several experimental models [21, 22]. The balance between NO and ROS levels seem to determine glomerular hemodynamics. If NO is dominating there will be vasodilation and increased urine output while if ROS is dominant vasoconstriction prevails and an increase in blood pressure will occur. Since increased TGF sensitivity has been linked with hypertension in several experimental models, studies are warranted that investigate afferent arteriolar and blood pressure responses in animals that lack TGF. In the first study of this thesis we further investigated the hypertensive response to chronic inhibition of nitric oxide synthase (NOS) or infusion with Ang II in adenosine A_1 receptor knockouts. Both treatments are generally known to enhance TGF and increase blood pressure.

Ang II has been shown to elevate intrarenal adenosine concentrations, either by increased release [23] or decreased metabolism of adenosine [24]. As yet the role of A_1-receptors in development of hypertension is unclear.

We and others have found that there is a synergistic interaction between Ang II and adenosine in the kidney [25]. This condition have turned out to have a major influence on renal microcirculation [26]. The mechanisms of synergism between Ang II and adenosine and its role in blood pressure regulation remains incompletely understood. Studies in isolated and perfused afferent arterioles have shown that interaction between adenosine and Ang II potentiates the contractile response [26]. The interaction depend partly on what adenosine receptor that is activated. In the low concentration range of adenosine, A_1 receptors are activated and found to sensitize the contractile response to Ang II. With higher concentrations of adenosine the vasodilatory A_2 receptors are activated which can counterbalance or desensitize the con-

tractile response to Ang II. We have also demonstrated that during certain conditions [27] the sensitization effect is not caused by receptor activation, but rather through the entry of adenosine into the cell, thereby activating p38 MAP kinase signaling. Thus, blocking the entry of adenosine by inhibiting the adenosine transporters by NBTI blocks this sensitizing effect. This indicates that the sensitizing effect of adenosine on Ang II-mediated vasoconstriction can be mediated by both receptor activation and via receptor-independent mechanism via entry of adenosine into cells using adenosine transporters. In the second study of this thesis we further investigated this interaction between adenosine and Ang II by using adenosine A_1 knockout mice.

PANCREATIC ISLETS AND PURINES IN GLUCOSE HOMEOSTASISDiabetes mellitus

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Insulin is a hormone that regulates blood sugar. Hyperglycaemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, the heart, eyes, kidneys, especially the nerves and blood vessels (120).

In type 1 diabetes (or insulin-dependent diabetes mellitus) pancreatic b-cells are destroyed/defective and treatment with exogenous insulin is essential. In type 2 diabetes b-cells are unresponsive to glucose, insulin secretion is decreased and/or target tissues are resistant to action of insulin, and one or more metabolic abnormalities develop. Pancreatic diseases that destroy islets can also lead to diabetes, sometimes referred to as type 3 diabetes (7). The metabolic syndrome has pronounced effects on small blood vessels, e.g. islet arteriole, and this leads to many chronic complications in other organ systems.

Type 2 diabetes (T2D) is characterized by beta-cell dysfunction and insulin resistance, shows increased incidence with age and obesity [28-31] and leads i.a. to endothelial dysfunction with devastating long-term consequences on the vasculature [32-34]. Sex influences the incidence [35], and the emergence of cardiovascular disease, metabolic syndrome and T2D increase following menopause [36, 37]. Considering the growing incidence of T2D, and reduced quality of life associated with the disease, the demand for new mechanistic insights and therapeutic approaches are warranted. Several clinical and epidemiological studies have demonstrated that coffee consumption is associated with reduced risk of developing T2D [38]. It has been suggested that caffeine accounts for this protection, but also other substances in

coffee are important [39]. Caffeine acts by blocking the receptor-mediated actions of adenosine [40].

Pancreas

The pancreas performs both exocrine and endocrine functions. The bulk of the pancreas is exocrine, comprising 70–90% acinar cells and 5–25% duct cells, depending on the species. Endocrine cells in the islets of Langerhans contribute only 1-2% of the pancreas. The pancreas is an organ exhibiting several serious diseases – cystic fibrosis, pancreatitis, pancreatic cancer and diabetes (7).

Islet anatomy, especially the vasculature

Pancreatic islets consist of 5 different types of endocrine cells located in aggregates with a diameter up to 400 μm, usually containing 1000-2000 cells. In the human pancreas there is 1-2 x 10^6 islets, whereas the mouse has approximately 500 islets. Each islet contains mainly insulin-producing β-cells and to a lesser extent glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP-cells and ghrelin-producing ε-cells [41]. The frequence of the cells varies between species, and e.g. β-cells comprise 55-60% of a human islets but 70-75% of a mouse islet [41]. All islets possess a dense vascular network with fenestrated capillaries [42] and most, if not all, endocrine cells have contact with at least one capillary [43].

The islets are supplied by separate arterioles implying that the islets can regulate their blood perfusion separately from that of the exocrine parenchyma [44]. Normally islet blood flow is 5-10 times higher than that in the exocrine parts [45]. Besides mediating transport of nutrients and hormones, the islet endothelium also affects a number of processes including the differentiation of endocrine islet cells during development [46, 47], and in adults the regulation of β-cell proliferation [48]. Both islet vasculature and blood flow are affected in animal models of T2D. It has been suggested that islet vascular defects, including impaired signalling between β- and islet endothelial cells, adversely affect the endocrine function in human diabetes [49, 50].

Despite species differences most islets are usually supplied with arterioles separate from those to the exocrine pancreas (Figure 2). Each islet receives 1-5 arterioles, which branch into fenestrated capillaries. An important issue is how the arterioles enter the islets. A summary of this debate has been provided [45]. Briefly, it was claimed that the arterioles enter through discontinuities in the islet periphery lacking β-cells and branch into capillaries [51]. However, other morphological data indicate that the arterioles branch into capillaries before the entry into the islets [45]. A third theory is that the cel-

lular order of blood perfusion varies between the species, depending upon islet cytoarchitecture [45, 52].

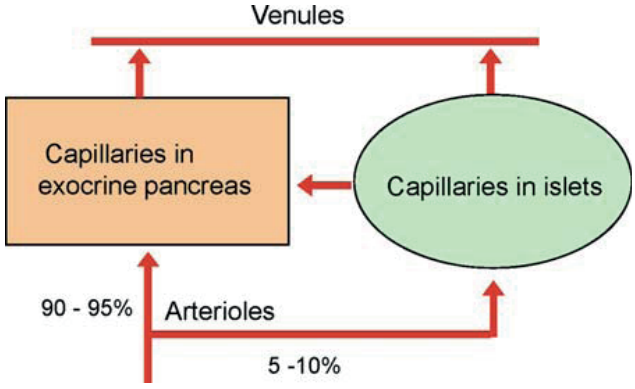


Figure 2: Schematic drawing of the vasculature in the pancreas. Arterial blood flow is separate for the endocrine and exocrine parenchyma, and 5–10% is diverted to the islets.

We recently developed a technique to perfuse large single rodent islets, and found that the branching occurs in the periphery (Figure 2). However, it cannot be excluded that arterioles in some large islets can enter the central parts of the islets [53]. In a recent set of experiments a real-time, multidimensional imaging technique allowed the study of the islet blood flow and its direction in vivo [53]. A flow from pole-to-pole was found in some islets, and centripetal flow in others. These observations support the idea of a difference in blood flow pattern between islets in the same animal.

The drainage of the islets is accomplished directly through veins, as seen in large islets, but also by an insulo-acinar portal system [54]. Especially the latter is the subject to large species variations, and the functional importance is unclear. Evidence has been provided that the portal system allows exposure of peri-insular acini to high concentrations of islet hormones [55, 56], and hereby resulting in an increase in their protein synthesis. Taken together available data suggest that precapillary VSMC in the arterioles is the most important site for islet blood flow regulation, and that postcapillary venulae contribute little, or not.

Adenosine and islet function

When islet cells are metabolically activated they increase their production of ATP [57]. ATP-consuming processes during insulin secretion are then likely to increase the formation and concentration of adenosine within the pancreatic islets, where it may affect not only insulin release in itself, but also mediate metabolically induced vasodilatation [58, 59]. It should be noted that an uptake of interstitial adenosine into the b-cells affecting insulin secretion may occur [60]. We have, indeed, previously demonstrated, as mentioned above, that adenosine mediates parts of the glucose-induced islet blood flow increase in rats through actions on A_1-receptors [61]. Thus, adenosine may

affect glucose homeostasis in multiple ways, and it has been suggested that interference with this substance may provide a novel way of modulating diabetes.

Previous studies have shown that adenosine is an important regulator of adipose tissue physiology, as mentioned above, mainly by actions of A_1 receptors decreasing lipolysis and increasing lipogenesis [62]. Studies utilizing gene-modified mice suggested that activation of adenosine A_1 receptors interact also with insulin and glucagon signalling [63, 64]. Thus, Johansson and co-workers demonstrated that administration of glucose was associated with elevated insulin and glucagon concentrations in $A_1^{-/-}$ compared with $A_1^{+/+}$ mice. Moreover, abrogation of A_1 signalling improved blood glucose disposal and altered insulin secretion patterns in young $A_1^{-/-}$ mice [65]. However, other recent studies have revealed conflicting results regarding the role of A_1 receptors in metabolic regulation [66]. Some of the controversies regarding the role of A_1 receptors in metabolic regulation may account to different age intervals. In the third study of this thesis we investigated the role of A_1 receptor signalling in modulating the metabolic phenotype during aging and obesity.

ATP and islet function

ATP plays a key role in insulin secretion, since ATP formed from glucose metabolism within the β-cells affects, and closes, the ATP-dependent K^+-channels in the plasma membrane [67, 68]. This leads to plasma membrane depolarization and an activation of voltage-gated Ca^{2+} channels which stimulates insulin release. Also external ATP may affect the β-cells, and complementary to gap junctions, acts as a coordinator of oscillatory activity in the β-cells [67]. Furthermore, ATP induces $[Ca^{2+}]i$ transients in the β-cells which may activate a repolarizing K^+ current [69]. In this context it has been suggested that neural ATP released in pulses may adjust islets in different oscillatory phases into a common rhythm [70]. We have also studied if external ATP and UTP may influence mouse islet endothelial cells and found a more pronounced Ca^{2+} response than in b-cells, but no response to ADP or acetyl choline [71]. This was interpreted to suggest that islet endothelial cells may provide a tonic inhibition b-cell P2 receptors resulting in impaired synchronization of insulin release pulses [71].

In a ddition to neural release [72] ATP has been shown to be released together with insulin from pancreatic secretary granules by exocytosis and to stimulate glucagon and insulin secretion from isolated perfused rat pancreas [73]. Adenosine, also derived from ATP breakdown, inhibited insulin secretion stimulated by glucose [74]. On the other hand, adenosine, ADP released glucagon in isolated perfused rat pancreas [75].

Adenosine stimulated the secretion of glucagon, but not insulin, suggesting that α-cells are more sensitive to adenosine than the β-cells [76]. ATP

and acetylcholine have synergistic effects on insulin release [77], consistent with their roles as co-transmitters from parasympathetic nerves (7). A recent study shows that over-expression of the α_{2A} adrenoceptor contributes to development of type 2 diabetes [78].

With regard to P2X and P2Y receptors in the pancreas it is known that P2X receptors mediate vasoconstriction of the rat pancreatic vascular bed [79], while P2Y receptors mediate vasodilation [80], probably via endothelium-derived relaxing factor affecting VSMC (7). CD39, and $P2X_7$, $P2Y_2$ and $P2Y_6$ receptors are significantly increased in biopsies of pancreatic cancer [81] and intestinal adenocarcinomas [5], but the relevance of this iss till obscure.

Islet blood flow

We also wanted to further elucidate the mechanisms responsible for the coupling between islet β-cell metabolism and islet blood flow regulation. We have previously found complex interactions between nervous and metabolic factors ([82], which can be modulated by the release of endothelium-derived mediators such as nitric oxide (NO) [83]. We have previously focused on the effects of glucose, since its metabolism is the major regulator of insulin secretion and we noted that acute hyperglycemia increases islet blood flow time- and concentration-dependently (Jansson 1997). In contrast chronic hyperglycemia in rodent models of type 2 diabetes results in a elevated islet blood flow, which is not possible to further stimulate by an acute glucose challenge [84-86]. In view of this, we chose to use both these models in the present study, *i.e*. normal rats stimulated by acute glucose administration as well as GK rats, a type 2 diabetes model [87]. An unexpected finding was that the Goto-Kakizaki (GK) rats were hypertensive, with a 30% increase in mean arterial blood pressure. We have not seen this in our previous experiments, but we have used GK rats from other suppliers earlier. This led us to also calculate vascular conductance, not only blood flow, to compensate for these differences in pressure.

The metabolic component of glucose-induced islet blood flow increase begins approximately 5 min after acute hyperglycemia, and then continues until blood glucose concentrations are normalized [82]. As mentioned we have previously shown that adenosine, working through A_1 receptors are involved in this response, and we have also shown that such receptors are present in islet arterioles and react to exogenous adenosine (Study III). To summarize our previous experiments there seems to be a redundancy in mechanisms for islet blood flow regulation [88]. It is therefore empting to speculate that this occurs also for purinergic receptors, and we decided to further examine the role of not only adenosine, but also ATP, ADP and AMP.

We therefore focused on P2Y receptors, which are GPCR that bind differentially to ATP, ADP, AMP or the uracil nucleotides UTP and UDP, depending on receptor subtype. Many purinergic receptor isoforms have been identified in both islets [4, 89, 90](and vascular cells [91]. It has been shown that binding of ATP to $P2X_1$ receptors localized on VSMC causes constriction of the blood vessel [92]. ATP in the blood vessel lumen, on the other hand, can bind to $P2Y_1$ and $P2Y_2$ purinergic receptors localized to endothelial cells, which release Ca^{2+} from the endoplasmic reticulum via activation of inositol trisphosphate (IP_3) receptors. This activates endothelial cell nitric oxide synthase, inducing vasodilation [93].

Furthermore, it is known that luminal perfusion of ATP also elicits vasodilation via activation of endothelial cell P2X receptors [94], whereas ATP activation of VSMC P2Y receptors causes vasoconstriction in different vascular beds [95]. If this reasoning is applied to islets, most islet vasculature consists of fenestrated capillaries that are in close contact with the β-cells. Both ATP and ADP are known to be present in the insulin secretory granules [96], and can be released thereby inducing paracrine effects [97]. Also ATP formed in the cytoplasm by the direct metabolism is likely to be released from the β -cells [4]. The released ADP and ATP could then affect endothelial NO production, and due to the high sensitivity of islet vasculature to NO [84] this may help to increase blood flow. It also fits well with our previous data that glucose-induced islet blood flow increase can be prevented by inhibition of nitric oxide synthase [84]. However, it is unlikely that the islet capillaries are important regulators of blood flow *per se*, since they lack VSMC. Pericytes are present [98], but their role in islet blood flow regulation, if any, is still unclear. Islet arterioles are, however, often penetrating into the islets, meaning that their media with VSMC can be exposed to ATP, ADP or adenosine released from metabolically stimulated β-cells. Another possible, but more speculative, mechanisms would be retrograde transmission of signals from intra-islet endothelium, stimulated by purinergic receptors, in the direction towards the afferent arteriole. This could thereby signal upstream to affect blood flow in the distal capillaries, analogous to what has been suggested to occur in other vascular beds [99]. In the fourth study we examined the role of P2Y receptors in the regulation of arteriolar reactivity and islet blood flow.

Aim

The overall aim of this thesis was to further evaluate the role of especially adenosine A_1 receptors in glomerular and islet function with focus on their effects in the arterioles in these organs. Since an endothelial dysfunction is a key feature in diabetes, we deemed it important to further explore if adenosine and ATP/ADP could be involved in the vascular abnormalities seen in the kidney and pancreas. To achieve this we have used an A_1 receptor knockout model ($A_1^{-/-}$ mice). In view of the so far unknown roles of ATP in regulation of islet blood flow we have also evaluated if metaboropic P2Y receptors in general and P2Y13 receptors in particular.

In Study I, we tested the hypothesis that the absence of adenosine A_1 receptors that abolishes TGF could prevent the development of hypertension and oxidative stress in models with reduced NO or elevated Ang II levels. We therefore studied renal afferent arteriolar contractile responses and blood pressure responses during chronic treatment with either L-NAME or low dose of Ang II infusion in $A_1^{-/-}$ and $A_1^{+/+}$ mice.

In Study II, we used $A_1^{-/-}$ and $A_1^{+/+}$ mice to investigate the contribution of A_1 receptor-dependent and –independent signalling pathways, responsible for the important synergism between Ang II and adenosine on the contractile response of the afferent arteriole in the kidney.

In Study III, we investigated young and aged $A_1^{-/-}$ and $A_1^{+/+}$ mice to further elucidate the role of A_1 receptors in metabolic regulation and islet endocrine as well as arteriolar function during aging. Furthermore, the role of A_1 receptors in modulation of oxidative stress and inflammatory responses was assessed.

In Study IV, the aim was to further elucidate the mechanisms responsible for the coupling between islet β-cell metabolism and islet blood flow regulation. in control rats and in GK rats, a type 2 diabetes model. In view of the recent findings on the importance of $P2Y_{13}$ on insulin secretion, we also investigated if a selective inhibitor of this receptor, MRS2211, affected islet blood flow regulation.

Methods

Animals (Study I-IV)

In Study I-III experiments were conducted on adenosine A_1-receptor knockouts ($A_1^{-/-}$) and corresponding wild-type mice ($A_1^{+/+}$) from heterozygous breeding pairs. The strain was developed by Johansson et al. (2o4) and backcrossed by the Jackson Laboratory (Bar Harbor, ME) to a C57BL/6J background. Genotyping of the offspring was performed by PCR. Both genders of $A_1^{-/-}$ and corresponding $A_1^{+/+}$ mice were used, with equal distribution for young (3-5 months, study I+II) and aged (14-16 months, study III) mice. A subset of $A_1^{+/+}$ mice was given a high fat diet (34.9% fat) for 12 months and the effects of acute A_1 receptor inhibition were studied in these aged and obese mice (study III). The animals had free access to tap water and pelleted food throughout the course of the studies.

In Study IV we used 10-14 week old, male Sprague-Dawley (SD) rats weighing 300-350 g, as well as adult male GK rats (Jackson Laboratories, Bar Harbor, ME, USA), a T2D model [87].

When anesthesia was needed referred it was induced by spontaneous inhalation of isoflurane (2.2%; Forene®; Abbott Scandinavia, Solna, Sweden) in air in mice, and with thiobutabarbital sodium (120 mg/kg body weight intraperitoneally; Inactin; Sigma-Aldrich; St. Louis, MO, USA) in rats.

The animals were then placed on a servo-controlled heated operating table to maintain body temperature at 37.5°C and breathed spontaneously through a tracheostomy.

All studies were approved by the Animal Ethical Committees at Uppsala University and Karolinska Institutet (Stockholm, Sweden), and were performed according to the National Institutes of Health guidelines for the conduct of experiments in animals.

Vascular reactivity of isolated and perfused renal afferent arterioles (Study I + II)

This technique has previously been described in detail [100]. Briefly, the outer cortical afferent arterioles were dissected at 4°C in Dulbecco's Mini-

mal Essential Medium (DMEM; Life Technologies Europe, Stockholm, Sweden) with 0.1% bovine serum albumin (BSA; ICN Biomedicals, Aurora, OH, USA) added. Arterioles with their glomeruli were perfused in a thermoregulated chamber (37°C) by a perfusion system, which allowed adjustment of outer holding and inner perfusion pipettes (Vestavia Scientific, Vestavia Hills, AL, USA). The chamber and the perfusion system were fixed to the stage of an inverted microscope (Nikon, Badhoevedorp, the Netherlands). A 5-µm diameter perfusion pipette, inserted into the holding pipette, was connected to a reservoir containing the perfusion solution to provide a pressure of 100 mmHg in the pressure head, producing a flow of 50 nl/min and a pressure of approx. 60 mmHg. The criteria to use an arteriole were a satisfactory, remaining basal tone and an intact myogenic response. Rapidly increasing perfusion pressure and assessing the change in the luminal diameter, confirming a constriction, verified both criteria. All experiments were ended by ascertaining a fast and complete constriction in response to KCl (100 mmol/L) solution.

The setup for the renal afferent arteriolar experiments is demonstrated in Fig. 3. The experiments were recorded by a video system, digitalized off-line, and analyzed as described previously [100]. Changes in luminal diameters were measured to estimate the effect of vasoactive substances. In all series, the last 10 s of a control or treatment period were used for statistical analysis of steady-state responses. The experimental period (15 min) with L-nitroarginine methylester (L-NAME, Sigma-Aldrich) and/or tempol (a superoxide dismutase mimetic; Sigma-Aldrich) were analyzed every 5th min. For a detailed protocol with the used doses, see Methods section in Study I.

To test the arteriolar contractility during conditions with low NO generation or ROS bioavailability we pretreated the arterioles for 15 minutes with either L-NAME to inhibit endogenous NOS system or Tempol to scavenge superoxide (O_2^-) before we started to do our dose-response curve for Ang II. Arterioles used to study Ang II or norepinephrine dose responses alone had not been exposed to any other drug prior to the actual experiment. Each experiment used a separate dissected afferent arteriole. As previously described [101], inner luminal diameter and media thickness were measured during baseline (before application of any substances), and the areas were calculated to compute the media-to-lumen ratios to assess vascular remodeling.

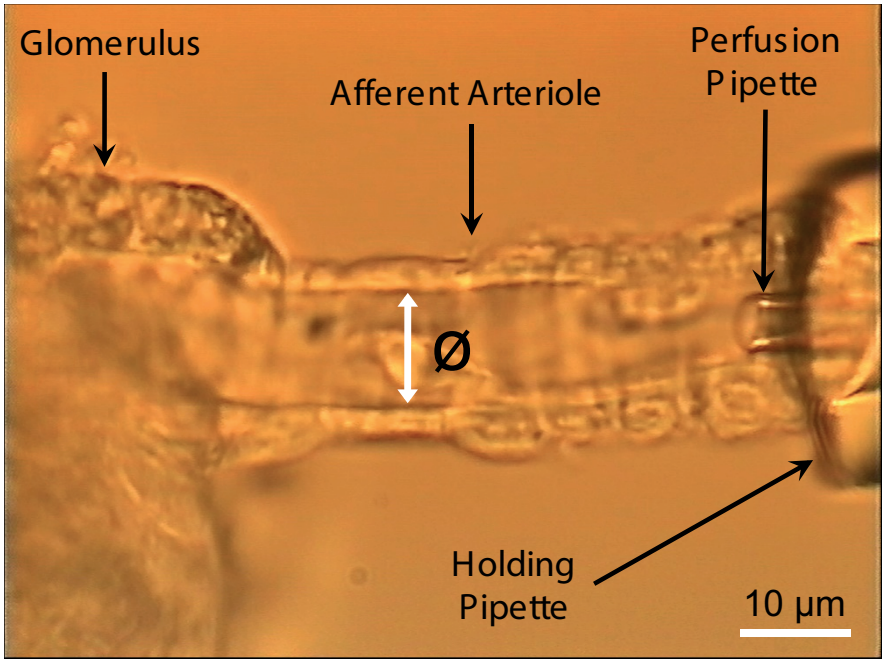


Figure 3: Microphotograph shows a glomerulus and its afferent arteriole held by one pipette and perfused with another. Also the glomerulus is attached with a holding pipette (not seen). The inner luminal diameter (Ø) of the arteriole was measured at the active site (indicated by white arrow) to estimate effects of vasoactive substances.

Vascular reactivity of isolated and perfused islet arterioles (Study III+IV)

Pancreas was quickly removed and placed in cold (4ºC) albumin-enriched (1%) DMEM (Life Technologies). Single islets with attached afferent arterioles were dissected and perfused as previously described [102]. The time for dissection was limited to 60 min and the obtained islets had diameters of approximately 200-300 µm. The experimentalset-up allowed us to measure the diameter of the afferent islet arteriole continuously and to record changes at a resolution of < 0.2 µm similar to the technique used for glomeruli described above. Each experiment began with a 15-min equilibrium period with buffer containing 5.5 mmol/L glucose in both bath and perfusion solutions. Thereafter the arteriolar responses to Ang II (10^{-6} to 10^{-12} mol/L, each dose applied for 2 min) alone, or with simultaneous treatment with apocynin (10^{-4} mol/L) were investigated during low (2.8 mmol/L) and high (16.7 mmol/L) glucose concentrations, or to adenosine (10^{-4} to 10^{-11} mol/L, each dose applied for 2 min) alone. Each perfusion was terminated by administration of KCl (100 mmol/L) to ascertain that the arterioles were viable and able to contract.

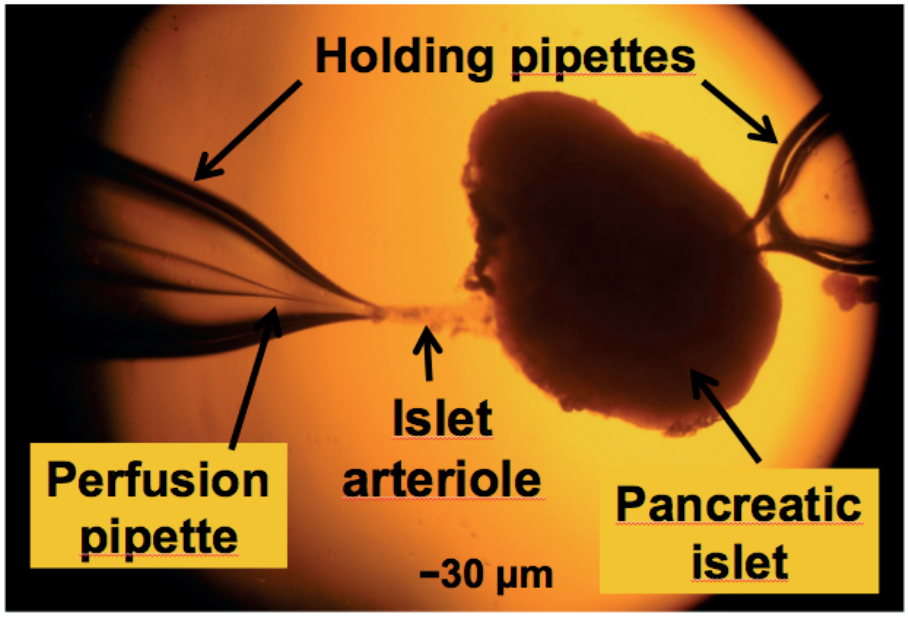


Figure 4: Experimental setup for ex vivo perfusion of a pancreatic islet. Islet diameter 250 µm.

Studies of islet arteriolar reactivity (Study IV)

Each experiment began with a 15-min equilibrium period with buffer containing 2.8 mmol/L D-glucose in both bath and perfusion solution. Thereafter either suramin (3, 6, 15, 30, 60, 150 or 300 µg/mL) or MRS2211 (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/L) was added to the buffer and changed with 5-min intervals. After this, the glucose concentrations was changed to 16.7 mmol/L for 15 min and then suramine or MRS2211 was once again added together with the high glucose concentrations at doses given above in 5-min intervals. Note that each islet preparation was treated with either MRS2211 or suramine, not combinations of them. Each perfusion was terminated by evaluating the arteriolar reactivity by increasing the perfusion pressure. This has proven to be as effective as the previously used aminostration of KCl to acieve the same result.

Plasma analysis (Study I+II+III)

Study I: To determine the regulation of major NO producing system the concentrations of the NOS substrate arginine, and the endogenous inhibitors of NOS asymmetric and symmetrical dimethylarginine in plasma were measured with high-pressure liquid chromatography (HPLC) as described previously [103], using modified chromatographic separation conditions [104]. In brief, solid-phase extraction on polymeric cation-exchange columns was

performed after addition of mono-methylarginine as the internal standard. After derivatization with orthophtal-dialdehyde reagent containing 3-mercaptopropionic acid, analytes were separated by isocratic reversed-phase HPLC with fluorescence detection. For all analytes the intra- and interassay coefficients of variation were <1.5% and <3.5%, respectively.

Study I: To determine the degree of oxidative stress the total, *i.e.*, free and protein-bound malondialdehyde (MDA) concentration in plasma was measured in duplicate by HPLC and fluorescence detection after alkaline hydrolysis and reaction with thiobarbituric acid, as previously described [105]. The intrarun and interrun variations were 3.5% and 8.7%, respectively.

Study III: Meso-Scale Discovery (MSD) Multi Array Technology was used to analyse metabolic and inflammatory markers in plasma. The MULTI-SPOT® 96well, 4-Spot Prototype Mouse Metabolic Kit (Cat No. N45ZA-1) was used to detect total glucagon-like peptide-1 (GLP-1), glucagon, insulin and leptin. The mouse ProInflammatory 7-Plex Ultra-Sensitive Kit (Cat No. K15012C-2) was used to detect interferon-γ (IFN-γ), interleukin 10 (IL-10), interleukin 12 (IL-12p70), interleukin-1β (IL-1β), interleukin-6 (IL-6), keratinocyte chemoattractant or growth regulated oncogene-α (KC/GRO), and tumor necrosis factor-α (TNF-α). For detailed method description, please visit http://www.meso-scale.com/.

Blood pressure in response to prolonged L-NAME or Ang II treatment (Study I)

Telemetric devices were implanted in young $A_1^{-/-}$ and $A_1^{+/+}$ mice as previously described [106] and blood pressure was initially measured continuously for 72 hours to determine basal levels. Blood pressure in $A_1^{-/-}$ and $A_1^{+/+}$ mice was then measured continuously during a 10-day period of treatment with L-NAME (10^{-4} mol/L; drinking water) or Ang II treatment (400 ng/kg/min; Alzet osmotic minipumps) [107].

Renal cortical mRNA expression (Study I)

The mice were killed by cervical dislocation followed by infusion of cold PBS to remove the blood. The kidneys were explanted, blotted, and weighed. The renal cortex was separated and homogenized in lysis buffer (1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L NaF, 80 mmol/L Tris, pH 7.5) containing enzyme inhibitors (Complete Mini®; 1 tablet/1.5 ml; Roche Diagnostics, Mannheim, Germany). RNA was isolated with RNA-Bee-reagent® (Biozol, Eching, Germany) and reverse transcribed with random hexamers (High Capacity cDNA RT-Kit®, cat. no. 4374966; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Quantitative polymerase chain reaction (PCR) analysis was performed

with a StepOnePlus device (Applied Biosystems). SYBR Green® was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 12.5 _µl with 0.4 pmol/µL of each primer (for primer sequences see Study I), and ImmoMix® (Bioline, Luckenwalde, Germany): 2 µl cDNA corresponding to 20 ng RNA was used as a template. Experiments were performed in triplicate with similar results. The expression levels of mRNA were normalized to β-actin by the ΔCt-method. Parallelism of amplification curves of the test and control was confirmed.

Cell culture of VSMC (Study II)

Aortic VSMC: Primary VSMC from $A_1^{+/+}$ and $A_1^{-/-}$ mice were isolated and cultured by a modified method of that originally described by Kobayashi and colleagues [108]. In isoflurane-anesthetized mice, the abdominal aorta was cut at the middle to release blood, and then perfused with 1 ml of PBS containing 1000 U/ml of heparin (Hospira, Inc. Lake Forest, IL. USA). The aorta was dissected out from the aortic arch to the abdominal aorta, and immersed in 20% fetal bovine serum (FBS, from ATCC, Manassas, VA. USA) and DMEM; Sigma-Aldrich containing 1000 U/ml of heparin. The fat or connecting tissue was rapidly removed with fine forceps under a microscope. The inside of the lumen of aorta was briefly washed with serum-free DMEM. From the other side it was washed with collagenase type II solution (2 mg/ml, dissolved in serum-free DMEM) (Sigma-Aldrich). After incubation for 45 minutes at 37 °C, endothelial cells were removed from the aorta by flushing with DMEM containing 10% FBS. The aorta was cut lengthwise, and put onto a 60 mm dish. With a scalpel blade the aorta was cut into almost square pieces (approximately and the culture period was 10-14 days 2 mm each) and allowed to dry briefly. DMEM with 20% FBS and penicillin/streptomycin was added gently and the cells were transferred to 48-well culture dishes and placed in an incubator, and left undisturbed for a week. All the solutions at every isolation step had antibiotic/antimycotic mix added (Gibco, Carlsbad, CA. USA). After one week, the culture plates were examined under the microscope, to observe the presence of VSMC in the medium. The cells were rinsed two times with 20% FBS containing DMEM, and replaced with fresh medium with antimycotic/antibiotic added. The cells were cultured for 10-14 days and medium was replaced two times a week.

Preglomerular VSMC (PG-VSMC): Isolation and culturing of primary PG-VSMC from rats were performed as previously described [109], and the phenotype was confirmed as described by Dubey and colleagues [110].

All experiments were carried out during early passage (between 3 to 9), when it is known that the cells express VSMC specific markers, such as antibodies against myosin light chain kinase and smooth muscle actin. Cells were seeded in 75cm^2 flasks andcultured in DMEM D0572 (Sigma-Aldrich)

supplemented with 10% FBS, 2mM L-Glutamine and 100 U/ml Penicillin-Streptomycin (Life Technologies, Grand Island, NY, USA). When used for the experiments, the cells were plated in 60 mm dishes until they reached 80% confluence and then serum deprived for 24 hours before the treatments.

Cellular protocol (Study II)

The cellular protocol was similar to that used for renal afferent arterioles. Briefly, the VSMC were divided in 3 groups: a) untreated, b) treated with adenosine (10^{-8} mol/L; 15 min) and then AngII (10^{-7} mol/L, 15min) and c) pretreated with NBTI ($3x10^{-7}$ mol/L; 5 min) and then treated with adenosine (10^{-8} mol/L; 15 min) and ANG II (10^{-7} mol/L; 15min). In experiments using the selective adenosine A_1 receptor antagonist CPX ($5x10^{-8}$ mol/L), the cells were pre-incubated with the antagonist for 1 hour before the same treatment protocol described above was started. In that case, the control cells were pretreated with CPX ($5x10^{-8}$ mol/L, 1 hour) and then with NBTI ($3x10^{-7}$ mol/L; 5 min). After the different treatments, VSMC were placed on ice immediately, washed twice with cold DPBS and lysed in 100ìl of lysis buffer supplemented with phosphatase and protease inhibitors (Sigma-Aldrich). Cell lysates were centrifuged (14000 g for 15 min) and the supernatant was collected and placed in -80°C until further analysis.

Western blot analysis of p38 MAPK and MLC phosphorylation (Study II)

Protein concentration in the supernatants was determined by Bradford protein assay (Bio-Rad). Equal protein amounts were separated by 4%–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad), followed by transfer to a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 5% nonfat dry milk in Tween-containing Tris-buffered saline, membranes were incubated with specific primary antibodies (phospho-p38 MAP Kinase (Thr180/Tyr182) rabbit polyclonal antibody-Cell Signaling #9211, MLC-2B (pSer19) rabbit polyclonal antibody-Acris Antibodies #R1535P or mouse monoclonal antibody, Cell Signaling #3675S, GAPDH mouse monoclonal antibody-Santa Cruz #sc-47724) and secondary antibodies (horseradish peroxidase-conjugated goat antibodies to rabbit or mouse IgG; DAKO). Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) was used to remove bound antibodies from the membranes, followed by blocking and re-probing the membranes with primary and secondary antibodies. Bands were detected by a SuperSignal West Femto chemiluminescence substrate (Thermo Scientific), and results were normalized with GAPDH. Images were analyzed by a luminescent image analysis system

LAS 1000 + (Fujifilm). The results were quantified by densitometry (Image J) and reported as relative optical density of the specific proteins.

Glucose tolerance test (Study III)

Intraperitoneal glucose tolerance tests (IPGTT) were performed sometimes between 8 a.m. and 2 p.m. following 6 hours of withdrawal of food [111]. Before starting the experiments, the animals were weighed to determine the amount of glucose to inject. The IPGTT was performed in a quiet room and handling was kept at a minimum to reduce stress during the procedure. A bolus of glucose was injected (2 g/kg body weight; 30% D-glucose) into the intraperitoneal cavity and blood was sampled from the tail tip at 0, 15, 30, 60, and 120 min. Plasma glucose levels were determined with a portable glucose meter (FreeStyle Lite®; Abbot Diabetes Care Inc, Stockholm, Sweden).

The effect of acute adenosine A_1 receptor inhibition on IPGTT was assessed in a subset of aged wild-type mice chronically fed a high fat diet. Paired measurements were conducted in mice given saline (control) or DPCPX (A_1 antagonist, 0.2 mg/kg body weight; Sigma-Aldrich) 45 min prior to the IPGTT.

Insulin tolerance test (Study III)

Intraperitoneal insulin tolerance tests (IPITT) were performed similarly to the IPGTT, but in non-fasting mice as previously described [112]. A bolus of insulin (0.75 IE/kg body weight; Actrapid 100 IE/ml®; Novo Nordisk A/S, Glostrup, Denmark) was injected (0.2 IE/ml in saline) into the intraperitoneal cavity and blood was sampled from the tail tip at 0, 15, 30, 60, and 120 min. Plasma glucose levels were determined with a portable glucose meter (FreeStyle Lite®).

Body composition analysis (Study III)

Dual-emission X-ray absorptiometry (DEXA) studies were performed in the anesthetized mice to determine their total fat and lean mass, and the abdominal fat fraction [113]. In short, anaesthesia was induced and continued by inhalation of isoflurane in air as given above. Mice were scanned using a Lunar PIXImus® densitometer (GE Medical-Lunar, Madison, WI, USA). These bone, fat and lean measurements exhibit excellent correlation to their total ashed or chemical extraction weights (r=0.99, 0.93, 0.99 respectively) [113]. The PIXImus® employs a cone beam X-ray source generating energies at 35 and 80 keV. The detector is flat (80 × 65 mm), comprised of individual pixels of 0.18 × 0.18 mm. Because of the limited imaging area, heads were excluded from all analyses, thus all data presented are for subcranial

body composition. The instrument was calibrated according to the manufacturer's instructions, using an aluminium/lucite phantom (corresponding to bone mineral density of 0.0639 g/cm^2, and 8.8% fat). The phantom was analysed daily before animal testing for quality control purposes. All scans were analysed using the software provided by the manufacturer.

Islet insulin release and insulin contents measurement (Study III)

Pancreatic islets were isolated from mice by collagenase digestion [114], and cultured in groups of 150 islets for 3 days in 5 ml of culture medium consisting of RPMI 1640 (Life Technologies) supplemented with L-glutamine (Sigma-Aldrich), benzylpenicillin (100 U/ml; Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (0.1 mg/ml; Sigma-Aldrich) and 10 % (vol/vol) fetal calf serum (Sigma-Aldrich). After the initial islet culture the islets were submitted to insulin release experiments and islet insulin content was measured.

Groups of 10 islets from each animal were transferred to vials containing KRBH with 2 mg/ml BSA (ICN Biomedicals). The KRBH buffer contained 1.67 mmol/L D-glucose during the first hour of incubation at 37°C (O_2/CO_2, 95:5). The medium was then removed and replaced by KRBH supplemented with 16.7 mmol/L glucose and the islets were then incubated for a second hour. The islets from each vial were harvested, and the incubation medium was retrieved. The islets were homogenized by sonication in 200 µl redistilled water. A fraction of the homogenate was mixed with acid-ethanol (0.18 M HCl in 95% (vol/vol) ethanol) from which insulin was extracted overnight. Insulin contents in incubation media and homogenates were determined by a mouse insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

Histology (Study III)

Pancreatic tissue was weighed, rinsed and placed in 4% paraformaldehyde fixative for 24 hours, and then dehydrated in 70% ethanol and embedded in paraffin. Sections (5 µm thick) were stained with haematoxylin-eosin. An observer unaware of the origin of the sections investigated the morphology of the sections. Randomly chosen sections from the different groups were photographed and the volume of the islets was determined with a point-sampling technique [115].

Blood flow measurements with microspheres (Study IV)

The rats were anaesthetized with Inactin™, Sigma-Aldrich. The animals were then placed on a servo-controlled heated operating table to maintain body temperature at 38°C and breathed spontaneously through a tracheostomy. Heparinized catheters were inserted into the right carotid artery and

left femoral artery and vein. The tip of the former catheter was positioned in the ascending aorta 1-2 mm above the aortic valves. The catheter in the femoral artery was used to enable continuous measurement of mean arterial blood pressure, whereas the venous catheter was used to continuously infuse Ringer solution (4 ml/kg body weight/h).

When mean arterial blood pressure had remained stable for 20 min the rats were injected intravenously with 1 ml/kg body weight of either saline, MRS2211 (1 mg/ kg body weight; Tocris, Bioscience, Bristol, UK) or suramine (30 mg/kg body weight; Tocris, UK). Five minutes after this injection 1 ml of saline or 30% /wt/vol) D-glucose was injected intravenously. All measurements were then made 5 min after this, *i.e.* 15 min after the first injection, and both SD and GK rats were subjected to this protocol.

Blood flow measurements were then performed with a microsphere technique as previously described [116, 117] 10 min after administration of the test substances as given above. Briefly, a total of 2.5×10^5 black non-radioactive microspheres (EZ-Trac™; Triton Microspheres, San Diego, CA, USA), with a diameter of 10 μm were injected via the catheter with its tip in the ascending aorta during 10 sec. Starting 5 sec before the microsphere injection, and continuing for a total of 60 sec, an arterial blood sample was collected by free flow from the catheter in the femoral artery at a rate of approximately 0.6 mL/min. The exact withdrawal rate was confirmed in each experiment by weighing the sample. Finally, arterial blood was collected from the carotid catheter for determination of hematocrit and blood glucose and serum insulin concentrations. Blood glucose was determined with test reagent strips (MediSense) and insulin with ELISA (Rat Insulin ELISA™, Mercodia AB, Uppsala, Sweden).

The animals were then killed and the pancreas and adrenal glands were removed *in toto*, blotted and weighed. Samples (approximately 100 mg) from the mid-regions of the duodenum, colon descendens and left kidney were also removed, blotted and weighed. The number of microspheres in the samples referred to above, including the pancreatic islets, was counted in a microscope equipped with both bright and dark field illumination, after treating the organs with a freeze-thawing technique [118]. The number of microspheres in the arterial reference sample was determined by transfer of samples to glass microfiber filters (pore size <0.2 μm), and then counted under a microscope.

The organ blood flow values were calculated according to the formula $Q_{org} = Q_{ref} \times N_{org}/N_{ref}$ where Q_{org} is organ blood flow (ml/min), Q_{ref} is withdrawal rate of the reference sample, N_{org} is number of microspheres present in the organ and N_{ref} is number of microspheres in the reference sample. With regard to islet blood perfusion it was expressed per gram wet weight of the whole pancreas. Since there were differences in mean arterial blood pressure between the groups, we also calculated vascular conductance in the

different organs by dividing the blood flow per gram tissue with the value for mean arterial blood pressure at the time of measurement.

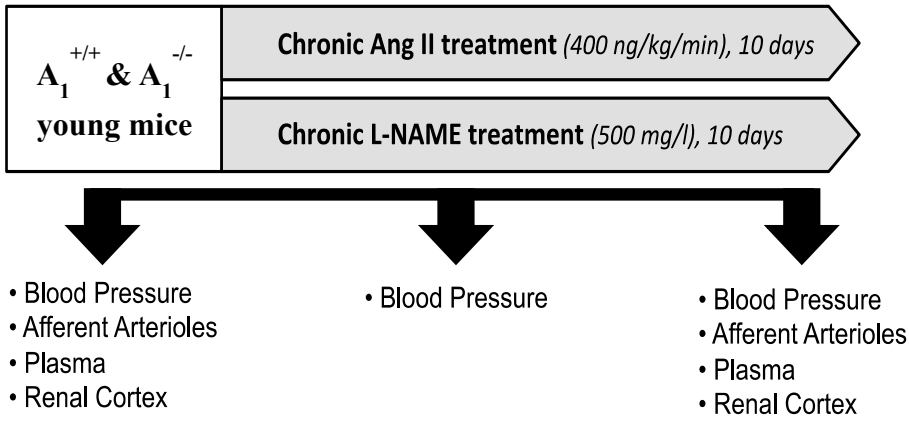
Blood flow values based on the microsphere contents of the adrenal glands were used to confirm that the microspheres were adequately mixed in the circulation. A difference <10% in the blood flow values was taken to indicate sufficient mixing, and this occurred in all animals in the present study (data not shown).

Statistical analysis (Study I + II+III+IV)

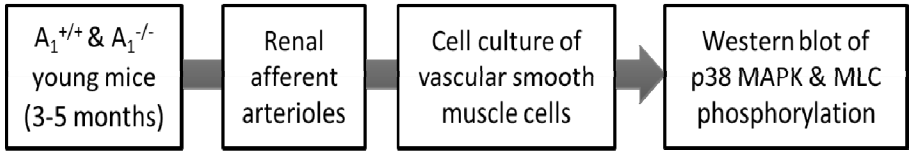
Values are presented as mean ± SEM. Single comparisons between normally distributed parameters were tested for significance with Student's paired or unpaired t-test as appropriate. For multiple group comparisons, one-way ANOVA followed by Bonferroni's or Tukey's *post-hoc* test for normally distributed values and Mann-Whitney's rank-sum test for non-parametric values. Statistical significance was defined as $P<0.05$ and we used SigmaStat™ (SSSP; Erfart, Germany) for all calculations

Study design

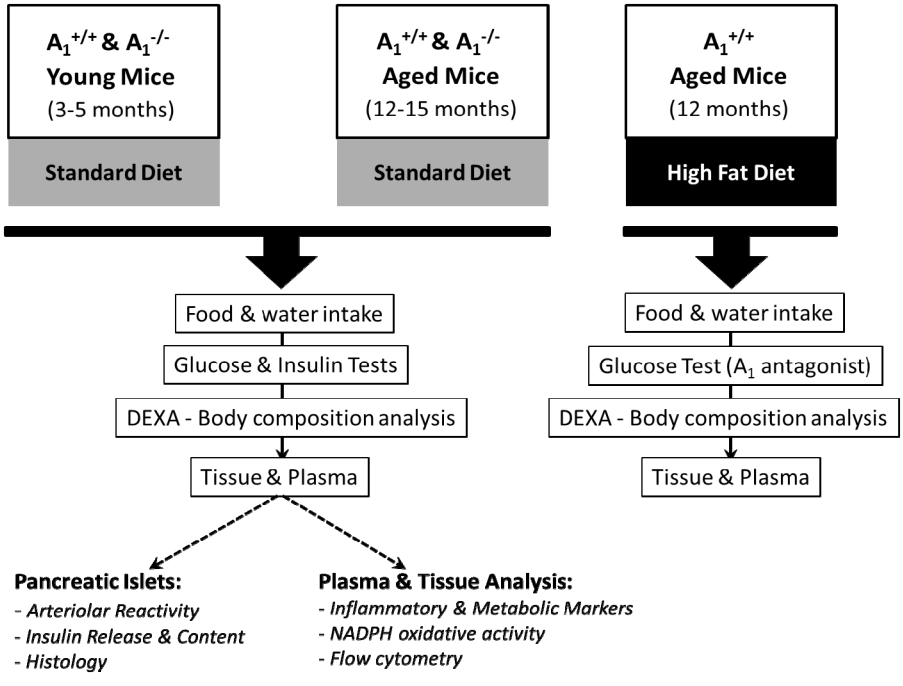
Study I



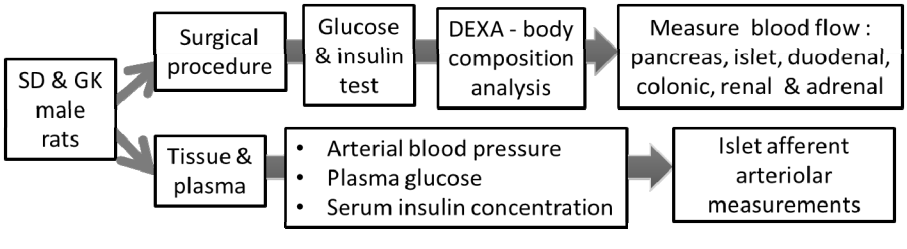
Study II



Study III



Study IV



Results and Discussion

Adenosine A_1 receptor deficiency and glomerular function (Study I)

To summarize, our findings show that A_1-receptor deficiency was associated with blunted arteriolar and blood pressure responses to both L-NAME and Ang II. The decreased vascular reactivity in $A_1^{-/-}$ mice was not a general phenomenon, as arteriolar responses to both KCl and norepinephrine were similar to that seen in wild-types. Although further mechanistic studies are required, our results suggest that different regulation of NO and ROS signaling may contribute to the different responses between genotypes.

Blood pressure in mice

Basal blood pressure was slightly higher in $A_1^{-/-}$ than in the $A_1^{+/+}$ mice. The mean blood pressure elevation during L-NAME treatment was 14 mmHg in $A_1^{+/+}$ mice, but only 4 mmHg in $A_1^{-/-}$. Prolonged Ang II infusion increased blood pressure with 13 mmHg in $A_1^{+/+}$ mice, whereas progressive blood pressure elevation was not seen in $A_1^{-/-}$ mice. These findings are showed as below. These findings have been confirmed in a recent study [119].

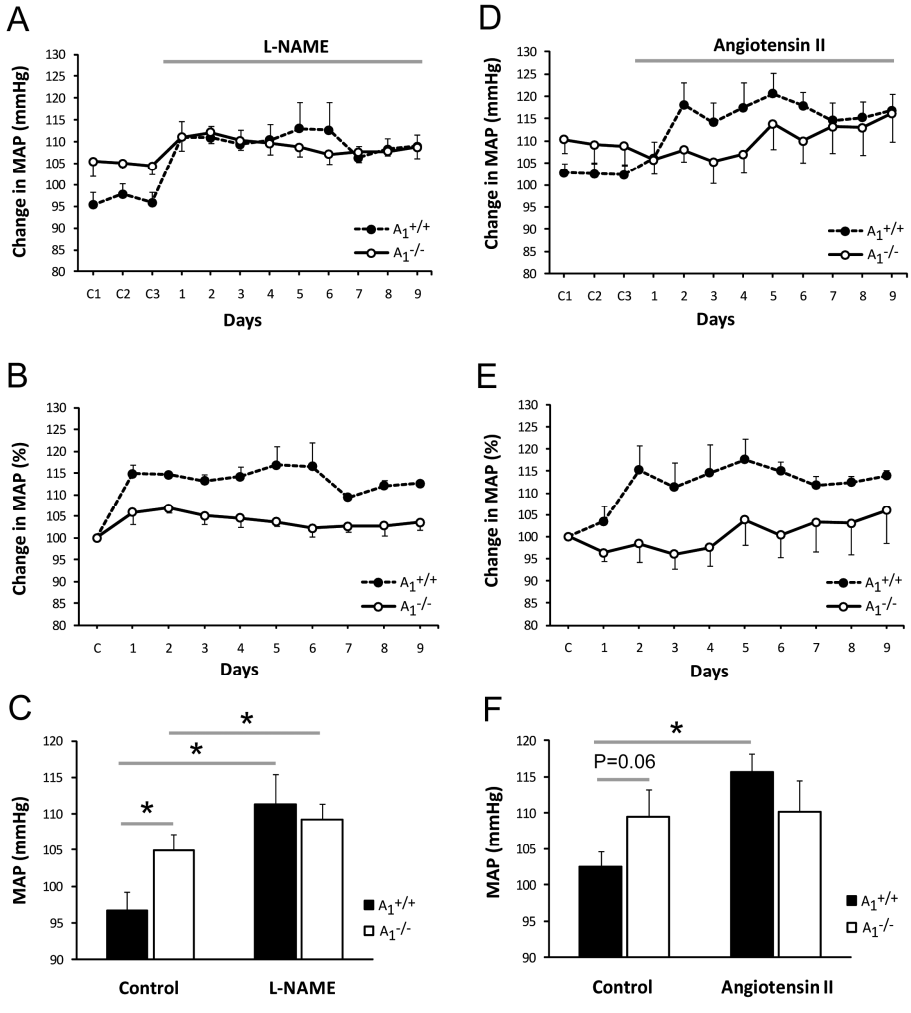


Figure 5: A1-receptor deficiency attenuates hypertensive responses to L-NAME and ANG II.

Renal afferent arteriolar response

Ang II constricted renal afferent arterioles with a maximum response of approximately 40% in $A_1^{+/+}$ mice and 18% in $A_1^{-/-}$. The kidney arteriolar contraction to L-NAME (10^{-4} mol/l; 1 5 min) alone was significantly lower in $A_1^{-/-}$ than in $A_1^{+/+}$ mice. Simultaneous L-NAME treatment enhanced the contractile response for both $A_1^{-/-}$ mice and $A_1^{+/+}$ mice, but preferentially in the latter. In wild type mice, addition of tempol (superoxide dismutase mimetic) attenuated the contractile response to L-NAME as well as to the following

maximal response to Ang II. In the $A_1^{-/-}$ mice, simultaneous application of tempol did not change this response.

Chronic L-NAME treatment for 10 days did not significantly change arteriole responses to L-NAME or Ang II, compared with all non-treated mice. Chronic Ang II treatment for 10 days attenuated maximal response to L-NAME (15 min) in $A_1^{+/+}$ compared with non-treated mice, but had no effect in $A_1^{-/-}$ mice. The following maximal response to Ang II was not changed in $A_1^{+/+}$ mice, but was attenuated in $A_1^{-/-}$ mice compared with non-treated mice.

Real-time PCR did not reveal any significant differences in renal cortical mRNA expression for NOS-, NADPH oxidase or SOD2/SOD3-isoforms, or in Ang II and adenosine A_2 receptors between non-treated $A_1^{-/-}$ and $A_1^{+/+}$ mice.

In the kidney, both A_1- and A_2-receptors are widely expressed in preglomerular vessels [120-123]. In renal afferent arterioles A_1 mediates vasoconstriction and A_2 vasodilatation [121, 124]. In afferent arterioles from A_1-deficient mice, or during pharmacological inhibition of A_1-receptors in $A_1^{+/+}$ mice, adenosine only produces vasodilatation [121]. Recent studies have demonstrated that A_2-receptors attenuate TGF responses by counteracting the effects of A_1-receptors [19, 125]. Together these findings show that A_2-receptors buffer A_1-induced vasoconstriction in preglomerular vessels and thus contribute to regulation of renal hemodynamics and blood pressure. There is considerable evidence for a synergism between Ang II and adenosine in regulation of preglomerular resistance [126]. In isolated and perfused afferent arterioles, a low concentration of adenosine significantly enhanced Ang II-mediated contraction, which was blocked by A_1-receptor inhibition [121]. Interstitial tissue concentrations of adenosine in the kidney may be increased by Ang II through either increased release, formation or reduced metabolism of adenosine [127, 128]. Our findings suggest that Ang II-mediated adenosine release is associated with opposite effects between genotypes. In $A_1^{+/+}$ mice, elevated adenosine levels may enhance arteriolar contraction, but in A_1-deficient mice this would only activate A_2-receptors, and thus attenuate the contractile response to Ang II. Moreover, NO produced in the JGA may attenuate the contractile responses through direct actions on VSMC [107, 125] or indirectly by scavenging ROS [129, 130].

Oxidative stress implies imbalance between ROS and NO bioavailability and has been linked to development of hypertension and cardiovascular disease [131]. Both Ang II treatment and NOS inhibition increase oxidative stress in the kidney, which may cause hypertension by increasing preglomerular vascular responses [132, 133]. During chronic L-NAME or Ang II treatments, the arteriolar contraction to Ang II and L-NAME was enhanced in $A_1^{+/+}$ mice, but not changed in $A_1^{-/-}$ mice. It was previously shown that pretreatment with Ang II sensitized arteriolar contraction in wild types, but did not alter Ang II responses in NADPH oxidases 2 deficient mice [107]. Activation of GPCR, including AT_1 receptors, may stimulate NADPH oxi-

dases and thereby increase ROS formation. *In vitro* and *in vivo* studies have shown that oxidative stress may increase renal adenosine production by activating ecto-5′-nucleotidase [134], whereas NO may inhibit these enzymes and thus reduce A_1 activation [135]. This suggests that both ROS and NO may reciprocally influence adenosine production and hence renal hemodynamics.

In addition to a blunted arteriolar response to Ang II, $A_1^{-/-}$ mice displayed reduced contraction to L-NAME compared with $A_1^{+/+}$ mice. Simultaneous treatment with tempol attenuated L-NAME-induced contractions in $A_1^{+/+}$ but did not influence arteriolar responses in $A_1^{-/-}$ mice. Thus, during basal conditions, preglomerular tone of A_1-deficient mice is less dependent on NO production. Also compatible with this hypothesis was the finding that arteriolar diameter in nontreated $A_1^{-/-}$ mice was somewhat larger despite lower expression of both neuronal NOS and endothelial NOS. Chronic Ang II treatment increased expression of constitutive NOS isoforms only in $A_1^{-/-}$ mice, which may balance any elevation in ROS formation and hence maintain low arteriolar reactivity.

In VSMC, oxidative stress increased A_1-receptor expression by activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [136]. AT_1-receptors may stimulate NADPH oxidase activity and ROS production via protein kinase C, phospholipase C and phospholipase D dependent mechanisms [137]. Similar to Ang II, adenosine may stimulate these signaling pathways via A_1-receptor activation [138]. In addition, A_1-receptors may act synergistically with other receptors to increase intracellular calcium concentrations [139, 140]. It was recently shown that pro-oxidant stressor-induced increases in ROS and intracellular calcium levels were more pronounced in primary cells from $A_1^{+/+}$ compared with $A_1^{-/-}$ mice [141].

Together these findings indicate a positive feedback between A_1-receptors and NADPH oxidase signaling. Firstly, the SOD-mimetic tempol attenuated arteriolar contractions to Ang II and l-NAME in $A_1^{+/+}$ mice, but had no effect in A_1-deficient mice. Secondly, chronic treatment with L-NAME or Ang II increased plasma levels of oxidative stress marker in $A_1^{+/+}$ but not in $A_1^{-/-}$ mice. Thirdly, elevated levels of dimethylarginines have been associated with Ang II infusion and oxidative stress and may contribute to renal and cardiovascular disease [142, 143]. In our study, both ADMA and SDMA levels were higher in Ang II-treated $A_1^{+/+}$ than in $A_1^{-/-}$ mice. Finally, elevation in several NADPH oxidase isoforms/subunits was observed in chronically treated $A_1^{+/+}$ but not in $A_1^{-/-}$ mice. However, the functional consequences are still yet not elucidated.

Adenosine A_1 receptor deficiency and afferent arteriolar function (Study II)

Synergistic interaction between Ang II and adenosine has been demonstrated, but the mechanisms are not clear. The present study investigates the afferent arteriolar contractile response and the important interaction between adenosine and Ang II in regulation of vascular resistance, which may alter both renal hemodynamics and filtration properties [122, 144-146]. We and other groups have demonstrated that the adenosine A_1 receptor is important for the sensitizing effects between adenosine and Ang II [121, 145, 147, 148], but receptor independent mechanisms may also contribute [27]. The present study aimed to further investigate the contribution of receptor dependent and independent effects of adenosine on Ang II responses in renal afferent arterioles. Contractility studies were performed in isolated and perfused arterioles, together with more mechanistic studies using cultured primary VSMC.

Renal afferent arteriolar response

Similar to that described in Study 1, Ang II-mediated arteriolar contraction is attenuated in A_1 receptor knockout mice, however simultaneous administration of adenosine surprisingly sensitizes the contractile response in both wild type and knockout mice (Figure 6; Panel A). This finding suggested involvement of receptor independent mechanism. To further investigate this hypothesis we studied the vascular effects during simultaneous inhibition of cellular adenosine transporters using NBTI. In A_1 knockouts, NBTI inhibited the synergistic effect of adenosine on Ang II responses in the renal microvasculature. However, NBTI had no significant effect on arteriolar contraction in wild type mice.

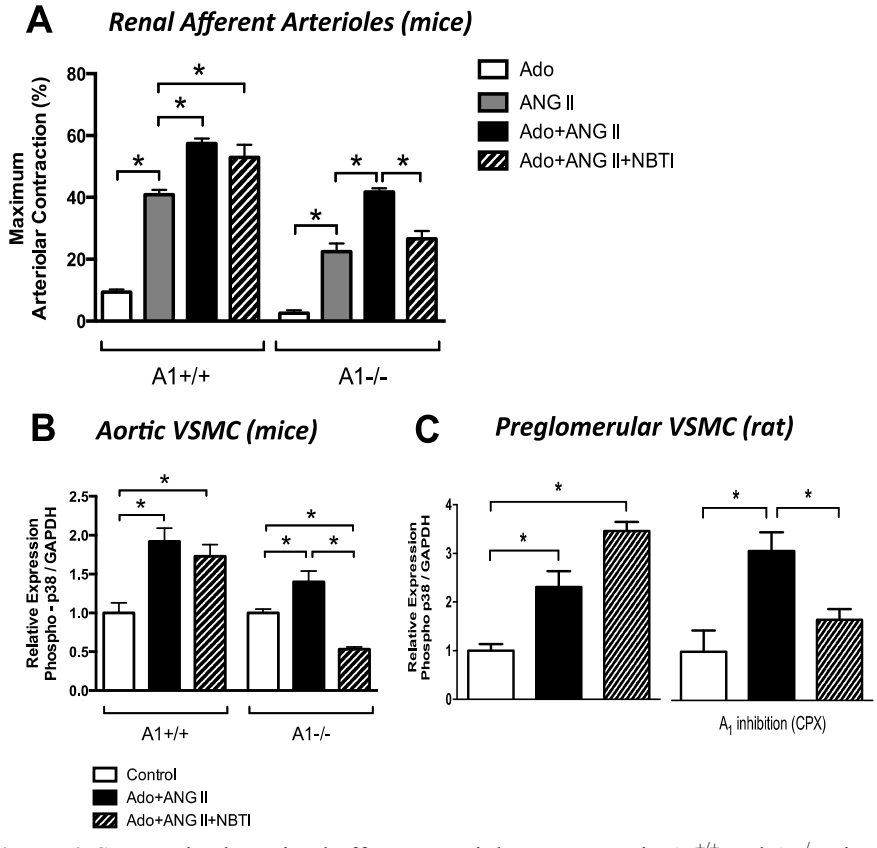


Figure 6: Summarized maximal afferent arteriolar responses in $A_1^{+/+}$ and $A_1^{-/-}$ mice (A). Regulation of p38 MAPK and MLC phosphorylation in aortic VSMC from $A_1^{+/+}$ and $A_1^{-/-}$ mice (B) and regulation of MLC phosphorylation in preglomerular VSMC during pharmacological inhibition of A_1 receptor signaling (C)

Regulation of p38 MAPK and MLC phosphorylation in VSMC

Simultaneous treatment with adenosine and Ang II increased phosphorylation of important contractile proteins (p38 MAPK and MLC) in aortic VSMC from wild-type mice. Similar changes were also observed in the $A_1^{-/-}$ VSMC, but treatment with NBTI only blocked phosphorylation in the knockouts (Figure 6, Panel B). Since aortic VSMC not necessarily reflects the phenotype of VSMC in the afferent arterioles we also investigated preglomerular VSMC (PG-VSMC) from the rat. The rationale for using aortic VSMC is due to the technical issues and absence of standardized protocol for isolating and culturing VSMC from mouse afferent arterioles. In agreement with that observed in aortic cells, incubating PG-VSMC with

Ado+ANG II enhanced phosphorylation of p38 and MLC, but this was not affected by NBTI. In contrast, if the A_1 receptors had been blocked pharmacologically, simultaneous treatment with NBTI reduced phosphorylation of both p38 and MLC to control levels (similar to that observed in the $A_1^{-/-}$ VSMC) (Figure 6, Panel C).

Taken together, these findings demonstrate that the synergistic effect between adenosine and Ang II usually depends on the existence of receptor dependent signaling that leads to phosphorylation of p38MAPK and MLC. However, during certain conditions this mechanism can be, at least partly, compensated by a receptor independent mechanism that involves transportation of adenosine into the cell. The intracellular mechanism warrants further investigations. Future *in vitro* studies are planned to investigate if, or how, adenosine sensitize the contractile machinery by enhancing calcium sensitivity in VSMC, and *in vivo* studies are aimed to determine the significance of this novel receptor independent pathway for adenosine in modulating renal hemodynamics and filtration properties both during health and disease.

Adenosine A_1 receptor deficiency and islet function (Study III)

In summary, A_1-receptors have age-specific effects on glucose metabolism, presumably by affecting insulin resistance. Furthermore, these receptors also directly modulates islet arteriolar responses and in response to Ang II. This shows complex effects of adenosine on islet function and highlights the need for studies of other adenosine receptors in this context.

Islet arteriolar response in old mice (Study III)

To provide a better possibility to study only the islet arteriole we used the recently developed technique with isolated and perfused single islets with attached arterioles modified from the technique used for glomeruli and used in Studies I and II [102]. Hyperglycaemia as well as stimulation with adenosine induced arteriolar dilatation, as previously described [102], and this response was similar in $A_1^{+/+}$ and $A_1^{-/-}$ mice. This is of considerable interest since it points towards the fact that hyperglycemia induces a metabolic vasodilatation which can be dissociated from A_1 receptors. If indeed adenosine is responsible for the metabolic component of islet blood hyperperfusion during hyperglycemia it must be through other adenosine receptors, a subject that warrants further investigations. As further studied in this thesis (Study IV) it may also be through the purines ATP/ADP/AMP.

Adenosine induced dose-dependent vasodilatation in both $A_1^{+/+}$ and $A_1^{-/-}$ mice of 5-10% at 10^{-4} mol/L. Also glucose in itself induced an arteriolar

vasodilatation, which was similar in $A_1^{+/+}$ and $A_1^{-/-}$ mice. This dilatation, also in the presence of apocynin to inhibit NADPH oxidase, did not differ between $A_1^{+/+}$ and $A_1^{-/-}$ mice.

Arteriolar contractile responses to increasing Ang II concentrations were attenuated in $A_1^{-/-}$ (18%) compared with $A_1^{+/+}$ mice (24%). The maximum contraction to Ang II was not significantly affected by glucose concentration. Simultaneous incubation with apocynin attenuated contractile responses to Ang II in $A_1^{+/+}$ mice, but had no effect on arterioles from $A_1^{-/-}$ mice, suggesting different regulation of NADPH oxidase function between genotypes. With apocynin, there were no longer any significant differences in arteriolar responses between the $A_1^{-/-}$ and $A_1^{+/+}$ mice.

Several studies have demonstrated that an increased islet blood flow is normally associated with islet insulin release [45, 49]. An effect of A_1 receptors in this context has been shown, since an acute A_1 receptor inhibition prevented glucose-induced islet blood flow increase (36). However, in these experiments there were confounding effects from systemic vascular responses and exocrine blood vessels. The conclusion from that previous paper was that adenosine participated in the metabolic coupling between insulin release and increased islet blood flow. We speculated that hyperglycemia would, through ATP generation, increase extracellular adenosine concentrations within the islets, which hypothetically could influence arteriolar VSMC. This could occur either directly on the part of the arteriole within the islet or through retrograde transmission of signals from islet capillaries to the arteriole, as suggested to occur in other vascular beds [99].

Similar to that described for renal afferent arterioles in Stucy I, islet arterioles from $A_1^{-/-}$ mice displayed reduced contractility to Ang II, both during normo- and hyperglycemia, although effects were more marked during the latter. Ang II is known to stimulate NADPH oxidase-mediated superoxide formation, which may be one reason for its pronounced vasoactive properties. Interestingly, in particular superoxide anion, has been shown to reduce islet blood flow [149]. Reduction of oxidative stress by incubation with apocynin, used as an inhibitor of NADPH oxidase, attenuated Ang II mediated contraction in $A_1^{+/+}$ mice, but had no effect on islet arterioles from $A_1^{-/-}$ mice. This suggests potential differences between $A_1^{+/+}$ and $A_1^{-/-}$ mice in the generation of reactive oxygen species, or in antioxidant capacity. This notion has also been described in the model with Ang II induced hypertension, where blood pressure elevation and the oxidative stress are markedly attenuated in A_1 knockout mice [119, 150]. It should, however, be noted in this context that apocynin has also been suggested to modulate redox status through NADPH oxidase independent processes [151] and may also directly affect voltage-gated potassium channels [152].

Body weight and body composition studies

There was no difference in body weight between young $A_1^{+/+}$ and $A_1^{-/-}$ mice (Study I and III). In the more thorough evaluation of body mass in Study III we found that there was no difference in body weight in aged male $A_1^{+/+}$ and $A_1^{-/-}$ mice, but in females body weights tended to be lower in $A_1^{-/-}$ than in $A_1^{+/+}$ mice. As expected, there was an obvious difference in body weight between male and females. Daily food intake was similar between genotypes, however, as previously reported $A_1^{-/-}$ displayed somewhat higher water consumption compared with $A_1^{+/+}$ mice [65, 153].

Accumulating evidence demonstrates that adenosine, through A_1 receptor signalling, influences adipocyte metabolism [154]. It was therefore expected that enhanced lipolysis and diminished lipogenesis in $A_1^{-/-}$ mice would lead to lower body weight or more specifically, to reduced fat mass. Previous studies on this issue have generated conflicting results [17, 63, 65, 66, 154, 155], which at least to some extent may be explained by sex differences and/or different ages of the studied animals. In the present study we addressed this, and found that young mice displayed similar food intake and body weights, with no differences between genotypes. Furthermore, DEXA analysis for fat and lean body mass revealed no significant differences between $A_1^{+/+}$ and $A_1^{-/-}$ mice, neither for females nor for males. These findings support those of a previous investigation using young $A_1^{+/+}$ and $A_1^{-/-}$ mice of the same genetic background [154].

In aged mice, food intake and body weight were not significantly different between genotypes, but female $A_1^{-/-}$ tended to be somewhat lighter than corresponding wild-type mice. DEXA analysis in wild-types showed an age-dependent increase in fat mass and reduction in lean body mass. Both total fat and abdominal fat fraction was lower in female $A_1^{-/-}$ compared with wild-type mice. For both genders, age-dependent reduction in lean mass was not observed in the A_1 knockout mice. Taken together, these observations support the notion that enhanced lipolysis and smaller lipogenesis in $A_1^{-/-}$ mice may influence fat mass with aging. However, the role for this on general metabolism and glucose homeostasis is still unknown, even though cytokine measurements referred to below may provide a link between these factors.

Glucose and insulin tolerance

In young mice there was no difference in basal glucose concentrations or in glucose tolerance between $A_1^{+/+}$ and $A_1^{-/-}$ mice irrespective of gender. However, in aged mice basal blood glucose was higher in $A1^{+/+}$ than in $A_1^{-/-}$ mice. Glucose tolerance tests revealed better overall glucose homeostasis, *i.e.* lower plasma glucose concentrations, in $A_1^{-/-}$ mice compared with $A_1^{+/+}$ mice.

Insulin tolerance tests in aged mice showed lower plasma glucose values in $A_1^{-/-}$ mice than in $A_1^{+/+}$ mice. In aged males, there was no difference in the early response (0-60 min), but the sustained glucose lowering effect (60-120 min) was more pronounced in $A_1^{-/-}$ mice. In aged females, both the early and the sustained responses were more pronounced in $A_1^{-/-}$ mice. In young mice there were no significant differences between genotypes.

To evaluate the role of acute A_1-receptor inactivation wild type mice fed a high fat diet for 12 months displayed increased body weight, total fat mass and abdominal fat fraction, and reduced lean mass (both total and fractional) compared with aged matched male $A_1^{+/+}$ mice fed regular chow. The more pronounced obesity in high fat treated mice was associated with worsening of glucose tolerance compared with the control $A_1^{+/+}$ mice.in the present study Administration of an A_1 receptor inhibitor, prior to glucose challenge, was associated with improved glucose tolerance. In high fat diet treated mice given an A_1 receptor inhibitor, total AUC was similar to that of $A_1^{+/+}$ mice given regular chow and not significantly different compared with $A_1^{-/-}$ mice.

Advanced age and increased body fat mass have been associated with a higher risk of T2D, as demonstrated by reduced glucose tolerance and insulin resistance [156-161]. The interest for adenosine receptors as potential regulators of glucose and insulin homeostasis has been boosted by large epidemiological studies suggesting that caffeine, in a dose dependent manner, reduces the risk for T2D [38]. Glucose and insulin tolerance tests *in vivo* in the present study showed more improvement in young females than in young males, but did not reveal any differences between $A_1^{+/+}$ and $A_1^{-/-}$ mice. As expected, advanced age was clearly associated with elevated plasma glucose levels, impaired glucose tolerance and insulin responses in female $A_1^{+/+}$ mice whereas but more surprisingly knockouts did not display this. In aged males these differences were not as obvious, but $A_1^{-/-}$ mice still demonstrated somewhat improved glucose handling and insulin responses when compared with $A_1^{+/+}$ mice. Taken together, these findings suggest that A_1 receptor signalling influences glucose homeostasis during aging, and may be of specific importance after menopause.

Adenosine is also known to affect the endocrine pancreas *per se* [4]. Previous studies have demonstrated an effect of A_1 receptors on insulin and glucagon plasma concentrations [63]. Thus, it was demonstrated that basal insulin and glucagon was similar in $A_1^{+/+}$ and $A_1^{-/-}$ mice, whereas plasma insulin were significantly higher in A_1 knockouts compared with wild types following glucose challenge. Simultaneous measurements of glucagon showed that plasma concentrations decreased in $A_1^{+/+}$, but increased in $A_1^{-/-}$ mice. Moreover, studies using *in situ* pancreatic perfusions demonstrated an effect on insulin dynamics upon glucose challenge, with higher insulin levels in A_1 knockout during the second secretory phase [63, 64]. Such an effect may have contributed to the improved glucose handling in A_1 knockouts in the present study.

One issue, which we did not address in the present study, was a direct assessment of peripheral insulin sensitivity by *e.g.* clamp studies. This was due to technical problems with isoflurane anaesthesia, which in our hands caused a slight but consistent hyperglycemia. This thereby precluded the use of this technique. Clamp studies were done by Faulhaber-Walter and co-workers who reported an impaired peripheral glucose handling and worsened glucose tolerance in $A_1^{-/-}$ mice [162], *i.e.* results opposite to ours. We cannot provide any convincing explanation for their different in results compared to those of us.

Insulin release and insulin content in isolated islets

High glucose stimulated islet insulin release in both young and aged $A_1^{+/+}$ and $A_1^{-/-}$ mice, but to a lower degree in aged mice. The relative change in insulin release in response to high glucose was reduced in aged $A_1^{+/+}$ compared with both young $A_1^{+/+}$mice and also compared with age-matched $A_1^{-/-}$. There were no differences in total insulin content between the groups of mice.

We observed also differences in plasma levels of insulin and glucagon between genotypes during aging, which will be discussed more in detail below.

Histological evaluation of pancreas

Pancreas weight, islet morphology and volume were not significantly different among groups. The number of islets was 0.58 ± 0.05 and 0.52 ± 0.07 islets per mg pancreas (n=5) in young $A_1^{+/+}$ and $A_1^{-/-}$ mice, respectively. The values for aged mice were 0.65 ± 0.11 and 0.70 ± 0.12 islets per mg pancreas (n=5). Thus, there seems to be no change in islet morphological composition induced by A_1 receptor deficiency to explain the functional differences.

Metabolic markers in plasma

In young mice there were no significant differences in GLP-1, glucagon, insulin or leptin concentrations between the genotypes. However, advanced age was associated with elevated levels of all these substances in $A_1^{+/+}$ mice. In aged $A_1^{-/-}$ mice however, levels were increased only for leptin. Moreover, aged $A_1^{+/+}$ mice had higher basal levels of GLP-1, insulin and leptin compared with age-matched $A_1^{-/-}$ mice.

Leptin has traditionally been linked with fat mass, and studies have also suggested leptin to be an inflammatory marker. Increased leptin concentrations, or leptin resistance, have been linked to aging-associated-disorders including obesity, cardiovascular diseases, metabolic syndrome and diabetes [163-167]. Many obese individuals are resistant to the anorectic effects of leptin [168, 169],

Influence of P2Y receptors on islet blood flow (Study IV)

The mean arterial blood pressure of GK rats, a type 2 diabetes model [170], was approximately 30% higher than in SD rats. Neither MRS2211 nor suramine affected the blood pressure in any of the groups. The plasma glucose concentrations in GK rats were, as expected, higher compared to SD rats, both during basal conditions and after glucose administration and unaffected by administration of MRS2211 or suramine in all groups. There were no differences in serum insulin concentrations between SD and GK rats, and the values were unaffected by MRS2211 and suramine.

Blood flow values

There were no differences in total pancreatic blood flow or pancreatic vascular conductance between any of the groups were seen (P=0.077 when ANOVA was used). In the control SD rats MRS2211 decreased basal islet blood flow, and suramine showed a trend towards this (P=0.07). Glucose administration increased islet blood flow in SD rats, and this increase was prevented by administration of both MRS2211 and suramine. Control GK rats showed higher basal islet blood flow than that of SD rats, and also this was decreased by MRS, and showed a tendency towards a decrease (P=0.11) after suramine injection. Glucose administration did not affect islet blood flow in GK rats and neither MRS2211 nor suramine changed islet blood flow in these animals (Figure 3C). When islet vascular conductance was calculated, to compensate for the differences in mean arterial blood pressure, a somewhat different picture emerged. Thus, no differences between SD and GK rats were seen in the saline-injected rats, but we still observed that MRS2211 reduced islet vascular conductance in saline-injected SD rats. Glucose markedly increased islet vascular conductance in SD rats, whilst no effect was seen in GK rats. Both MRS2211 and suramine decreased the vascular conductance in glucose-injected SD rats, but had no such effects in GK rats.

There were no differences in duodenal blood flow or duodenal vascular conductance between any of the experimental groups (Figures 4A and 4B), besides a decrease in MRS2211 treated saline-injected GK rats (P=.049). However, colonic blood flow and vascular conductance showed marked differences between the groups. Since the changes (and significant differences) in blood flow and vascular conductance mirrored one another, we only discuss the blood flow values further. There were no differences during basal conditions, *i.e.* after saline-administration, but glucose administration increased colonic blood flow in SD rats and decreased it in GK rats. Surprisingly especially suramine, but also MRS2211 markedly increased colonic

blood flow in SD rats, but had no significant effects in GK rats. This flow increase was unaffected by glucose administration.

There were no significant differences in renal blood flow or vascular conductance between the experimental groups. Neither suramine nor MRS2211 had any effect on adrenal blood flow in SD rats. However, glucose administration decreased adrenal blood flow in GK rats. MRS2211 decreased adrenal blood flow in control GK rats but MRS2211, but not suramine. None of these substances had any effects in SD rats. The changes in adrenal blood flow were mirrored by those in vascular conductance.

Islet arteriolar reactivity

The islets included in this study had a diameter of approximately 300 μm and the average arteriolar diameter was 44.9 ± 13.0 and 35.3 ± 7.8 μm for 12 SD rats and 17 GK rats, respectively. A consistent finding in both SD and GK rats was an approximately 10% dilatation caused by hyperglycemia, which peaked 15 min after start. When examining the effects of suramine in SD rats a dose-dependent reduction on arteriolar diameter was seen with a maximum of approximately 20% during both low and high glucose conditions. When MRS2211 was administered a similar, but much more pronounced response, amounting to an approximately 40-50% reduction in arteriolar diameter, was seen. Also this response was similar during low or high glucose concentrations.

GK rats reacted similarly but the constrictor responses were much less pronounced than those seen in SD rats. Thus, suramine caused a 5% and MRS2211a 10% vasoconstriction. Also this response was unaffected by the glucose concentrations in the medium.

Our results with suramine and MRS2211 clearly show that especially the ADP receptor $P2Y_{13}$ is of importance for islet blood flow. The differences in reaction between the two drugs are likely to reflect the fact that suramine affects all P2Y receptors, and may thereby directly affect both vasoconstrictor and vasodilator responses. In addition to $P2Y_{13}$, also $P2Y_{12}$ is a receptor which is preferentially activated by ADP. The latter receptor is present on thrombocytes and is a target for e.g. clopidogrel, which inhibits its activation and thereby diminishes thrombocyte activation [171]. In ongoing experiments we have seen that also clopidogrel inhibits islet blood flow in both SD and GK rats, thereby highlighting the importance of ADP for normal islet blood flow regulation.

The importance of extracellular purine nucleotide concentrations is likely to vary not only between vascular beds, but also during different functional and pathological conditions [91]. Adenosine was the first to be studied, and suggested to be of prime importance in the heart [172]. Later measurement of ATP in the venous effluent from the isolated rat hindlimb in response to hyperemia supporting a role for ATP acting on P2 receptors on endothelial

cells elicits the observed vasodilatory response. Furthermore, in the heart the vasodilation observed in response to luminal ATP has been shown to be more potent than that to adenosine [173].

In the blood vessel lumen, ATP concentration increases during periods of hypoxia and ischemia. Since the chronic islet blood flow increase seen in GK rats is dependent on NO, and also the nervous system [84, 174] it is tempting to speculate that this may be a mechanism behind the islet blood flow increase seen in this model. Indeed, MRS2211 decreased the basal blood flow *in vivo* in GK rats, as well as in SD rats, suggesting that this may be the case. It should be noted that the response to especially suramine, but also MRS2211, was much less in isolated perfused islets from GK rats when compared to SD rats. The reasons are not known but may reflect some additional sources of ATP and ADP *in vivo* as well as the presence of P2Y receptors in other organ causing secondary changes in islet blood flow. Alternatively the P2Y receptors may be down-regulated in GK rats, and this may be partially compensated for *in vivo*. The expression of P2Y receptors in islets from GK rats is presently being investigated. However, it is unlikely that only one purinergic receptor is involved in this response, so there is clearly a need for further investigations.

Other possible sources of luminal ATP in blood vessels is erythrocytes [175] as well as capillary endothelial cells [91] . ATP is released from erythrocytes in response to low oxygen tension [176] , presumably acting on endothelial cell purinergic receptors to induce vasodilation and increase blood flow and oxygen delivery to tissues. Increased shear stress on endothelial cells has also been shown to induce ATP release into the vessel lumen [177]. From sympathetic nerves ending on VSMC, ATP can be released as a co-transmitter along with noradrenaline from sympathetic nerves, which may be an additional way of exerting nervous effects on islet vasculature, since these nerves are known to stimulate islet blood flow [178]. Thus, the number of possible sources for ATP and ADP in islets is multiple, and would therefore provide numerous possibilities for blood flow control, which we have so far only begun to map. It should be noted that when perfusing isolated islets with ba buffer, *i.e*. without any erythrocytes, or innervations, we noted that both suramine and MRS2211 induced a pronounced vasoconstriction suggesting that is local production of purines which is important.

It has been suggested that chronically elevated ATP in blood vessels could potentiate pathological conditions such as hypertension and atherosclerosis [91]. ; It is therefore, essential that the extracellular concentration of this nucleotide is tightly regulated by ecto-nucleotidases. Such enzymes are present in pancreatic endothelial cells and VSMC [9], and in the human pancreas [179]. Furthermore, they can be upregulated in primary rat VSMC by hyperglycemia [9]. This is another important area to investigate in more detail and such studies are presently being performed.

An interesting finding when islet vascular conductance was calculated, to compensate for the differences in mean arterial blood pressure, was that there were no differences between saline-injected SD and GK rats. This would suggest that the changes we saw in islet blood flow were secondary to changes in blood pressure. This is in contrast to several previous studies [178, 180]. As mentioned above, we used GK rats from a different commercial supplier in this study, which may explain our findings. It should be noted, however, that MRS2211 induced the same effects on islet vascular conductance as on blood flow. None of the other organ blood flow values showed any discrepancy between blood perfusion and vascular conductance.

Colonic blood flow responded strongly to especially suramine administration. The blood flow was markedly increased in both normo- and hyperglycemic SD rats, whereas an opposite effect was seen in GK rats. The reasons for this unexpected, and to our knowledge previously unnoticed, finding are unknown. Several subtypes of P2 receptors are known to dilate the circular smooth muscle in the colon muscular layer, presumably by direct binding [181]. If this may occur also on VSMC in the colon, or if the effects are secondary to changes in motor activity is unknown. Binding to P2Y receptors in colon cancer cells can activate cyclo-oxygenase-2 [182], and if this occurs also in normal colon it could explain the flow increase in SD rats, but this is conjectural. The reasons for the opposite effects in GK rat are unknown. .

Summary and Conclusion

The hypothesis and aims behind this study are that a variant of the well-known endothelial dysfunction seen in diabetes and hypertension can be manifested also in the islet and glomerular vascular system, where it may modulate and ultimately impair specific organ function. Such a vascular dysfunction may be amenable to therapy, and thereby help to normalize islet insulin secretion early during the course of impaired glucose tolerance and diabetes, as well as restoring autoregulatory mechanisms in the kidney. This may provide new treatment modalities for diabetes and hypertension, which will supplement other therapies, and the likelihood of adverse reactions is small. Also the renal vasculature is a target for diabetes, and possibilities to interfere with and normalize an endothelial dysfunction would be advantageous also to prevent long-term renal complications.

Study I

Adenosine A_1 receptor knockouts have no TGF and we found in the present study that lack of adenosine A_1 receptors reduces preglomerular contractile responses. Lack of A_1 receptor sign aling also attenuates hypertension, induced by prolonged NOS-inhibition or Ang II infusion.

We conclude that abrogation of A_1 receptor signaling:

1) Reduces the effectiveness of L-NAME and Ang II to constrict renal afferent arterioles.

2) Reduces blood pressure responses to prolonged L-NAME or Ang II treatment.

We suggest that a functional TGF might be necessary to evoke sustained blood pressure elevation in these models of renal hypertension, and that A_1 receptor signaling may modulate the oxidative stress response in these models.

Study II

The interaction between Ang II and adenosine in the contractile response of the afferent glomerular arteriole usually involves A_1 receptor signaling, but this can be compensated for by receptor independent actions that act to phosphorylate p38 MAPK and MLC via adenosine entry into the VSMC.

Study III

A_1-receptors have age-specific effects on glucose metabolism, presumably by affecting insulin resistance. It also affects islet arteriolar responses in itself and to Ang II. This shows complex effects of adenosine on islet function and highlights the need for studies of other adenosine receptors in this context. These studies emphasize that A_1 receptors regulate metabolism and islet endocrine and vascular functions during aging by modulating oxidative stress and inflammatory responses.

Study IV

P2Y receptors are involved in islet blood flow regulation, and affect the hyperglycemia-induced islet blood flow changes. Furthermore, $P2Y_{13}$ antagonism elicited almost the same response as a total P2Y receptor inhibition, highlighting the importance of ADP for purinergic responses in islet blood flow.

Future perspectives

Our studies demonstrate important, albeit complex, effects of adenosine on both renal and islet function and ATP/ADP on islet vascular function. They highlight the need for studies of other adenosine receptors in this context, studies that are at present being performed.

Gene-modified mice with targeted deletions of the adenosine receptor subtypes will be used, together acute pharmacological modulation of the same receptors. We aim to study downstream signaling proteins that regulate the contractility of arteriolar VSMC *e.g.* MLC phosphorylation, and evaluate if there are differences between islets and glomeruli afferent arterioles. Moreover, the regulation of NADPH oxidase in both vascular beds will be further investigated.

Through collaborative efforts we are currently having access to adenosine A_{2a}, A_{2b} and A_3 receptor knockout mice, and studies on both glomerular and islet function in these animals are ongoing. In these studies we also intend to address the question on the importance of adenosine transporters and adenosine uptake for the effects on arteriolar function; a notion so far mostly ignored.

Our present findings in Study IV have demonstrated hitherto unknown effects of especially ADP on islet blood flow regulation, and we need to further examine the role of not only P2Y, but also P2X receptors in this context.

Within the next few years we also hope to be able to use our techniques on human kidneys and islets material to further evaluate the therapeutic potential of modulating specific purinergic receptor signaling pathways.

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