Potential influence of the pericardial space on myocardial function: Cardiac interactions of adenosine and endothelin-1

Ph.D. Doctoral Dissertation

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1 LIST OF ABREVATIONS

5'N	5' nucleotidase
AA	arachidonic acid
AC	adenylate cyclase
ACE	angiotensin convertase enzyme
ACS	acute coronary syndrome
ADA	adenosine deaminase
ADH	antidiuretic hormone
ADO	adenosine
ADP	adenosine diphosphate
Adr	adrenaline
AK	adenosine kinase
AM	adrenomedullin
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
ANP	atrial natriuretic peptide
ATP	adenosine-triphosphate
Ang II	angiotensin II
AV	atrioventricular
bFGF-2	basic fibroblast growth factor-2
big ET-1	big endothelin-1
BNP	brain natriuretic peptide
CAD	coronary artery disease
CBF	coronary blood flow
CNP	C type natriuretic peptide
COX	cyclooxigenase
DA	dopamine
DAG	1,2 diacyl-glicerole
DHA	docosahexaenoic acid
dP/dt_{max}	left ventricular contractility
ECE	endothelin converting enzyme
EDHF	endothelium-derived hyperpolarizing factor
EF	ejection fraction
EHNA	eritro-4-(2-hidroxi-3-nonil) adenine-hidro-chloride
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
GMP	guanosine-monophosphate
cGMP	cyclic-guanosine-monophosphate
G-protein	guanosine-nucleotide binding protein
HCYS	homocystein
H-FABP	heart-type cytoplasmatic fatty acid-binding protein
HPLC	high performance liquid chromatography
HR	heart rate
HT	hypertension
HXA	hypoxanthine
IGF-1	insulin-like growth factor-1
IL-1	interleukine-1

IMP	inosine monophosphate
INO	inosine
ip.	intrapericardial
IP_3	inozitol-(1,4,5) triphosphate
ISF	interstitial fluid
iv.	intravenous
LVendo	left ventricular endocardial monophasic action potential
LVepi	left ventricular epicardial monophasic action potential
MABP	mean arterial blood pressure
MAP	monophasic action potential
MAPD	duration of monophasic action potential
$MAPD_{90}$	duration of MAP at 90% of repolarization
MAPK	mitogen-activated protein kinase
MET	metionin
mRNA	messenger ribonucleic acid
N	native pericardial fluid
NE	norepinephrine
NEP	neutral endopeptidase
NO	nitrogen monoxide
NOS	nitrogen monoxide synthase
o.d.	outer diameter
PARP	poly(ADP-ribose) polymerase
PBSA	phosphate buffered saline containing 0.5% bovine serum albumin
PF	pericardial fluid
PGI	prostacyclin
PI	pericardial infusate
PLC	phospholipase C
PKC	protein kinase C
PUFA	omega 3 (n-3) poly-unsaturated fatty acids
RAS	renin-angiotensin system
ROS	reactive oxygen species
SA	sinoatrial
SAN	stable angina
SAH	S-adenozil-homocystein
SAHH	S-adenozil-homocystein hydrolase
SAM	S-adenozil-metionin
TNFα	tumor necrosis factor-α
TGFβ	transforming growth factor-β
UV	upstroke velocity
USAN	unstable angina
VHD	valvular heart disease

2 INTRODUCTION

2.1 PERICARDIAL SPACE

In the past decades, the general consensus on the physiological role of the pericardial fluid assigned the function of providing continuous lubrication for the beating heart. Regarding the composition of pericardial fluid, with the exception of potassium it seems to be a passive plasma ultrafiltrate containing between one quarter and one third of the protein of plasma with a far higher proportion of albumin to other proteins [1].

Now, it is widely accepted that the pericardial fluid possesses an essential storage function as another fluid compartment of the heart and contains a large number of endogenous regulatory agents, which function locally in either an autocrine or paracrine manner in the heart, such as adenine nucleosides [2, 3], endothelin-1 [4], natriuretic peptides [5-8], catecholamines [9], angiotensin II [10], adrenomedullin [11], cytokines and growth factors [12-14]. Under physiological conditions, the concentrations of these regulators were found to be several magnitudes higher in the pericardial fluid than in the peripheral and coronary venous blood (Table 1.). The high pericardial concentrations of these cardioactive regulators may reflect their myocardial derivation and suggest a teleological role for the pericardial space as a functionally distinct compartment with potential (patho)physiological function in cardiac regulation. Moreover, the pericardial space offers an exceptional opportunity to examine the effects and interactions among the aforementioned agents, whose pericardial concentrations may be substantially elevated in certain circumstances like cardiovascular pathologies (Table 2.).

Previously it was found that in patients with coronary artery disease the pericardial concentrations of adenosine and inosine were significantly higher than in patients with valvular heart disease [3]. In both subject groups, pericardial levels of adenine nucleosides exceeded by an order of magnitude the plasma adenine nucleoside levels. The enhanced release of adenine nucleosides by the ischemic myocardium, as a result of supply/demand imbalance of the heart, has an important role in metabolic autoregulation of coronary arteries and these alterations of their concentrations in the interstitial fluid can be reflected by the alterations of their pericardial concentrations.

Table 1. Different values of pericardial and plasma concentrations of some endogenous regulators

	Pericardial concentrations	Plasma concentrations	References
ADO	738 nmol/l	63 nmol/l	[3]
INO	347 nmol/l	52 nmol/l	[3]
ET-1	73 pg/ml	3,3 pg/ml	[4]
ANP	258 pg/ml	28 pg/ml	[15]
BNP	144 pg/ml	5 pg/ml	[16]
H-FABP	16.3 μg/ml	8.5 μg/ml	[17]
AM (active)	9 pmol/l	1 pmol/l	[11]

(ADO: adenosine, INO: inosine, ET-1: endothelin-1, ANP: atrial natriuretic peptide, BNP: brain type natriuretic peptide, H-FABP: heart-type cytoplasmic fatty acid-binding protein, AM: adrenomedullin)

Atrial (ANP) and brain type (BNP) natriuretic peptides are also the members of a family of vasoactive substances that are produced and released by the heart during increased wall tension and stretch of heart chambers. Apart from acting as systemic hormones, ANP and BNP may have local actions at the site of their synthesis, namely they have an important role in hampering cardiac fibroblast growth and structural remodeling of the heart. Furthermore, recent studies demonstrated that natriuretic peptides are more concentrated in the pericardial fluid than in blood, and pericardial fluid BNP levels provide more sensitive, stable and accurate information than do plasma BNP levels on left ventricular dysfunction due to ischemic heart disease [16]. There is also increasing evidence that adrenomedullin secreted from cardiac myocytes and fibroblasts may exert its local pathophysiological actions against cardiac remodeling in the failing heart. In patients underwent cardiac surgery significantly higher pericardial concentrations of total and active adrenomedullin were found compared to the peripheral plasma and

Table 2. High pericardial concentrations of cardiac regulatory agents in different cardiovascular pathologies.

Pericardial fluid	Control concentrations	Pathological concentrations	References
ADO	738 nmol/l (VHD)	1545 nmol/l (CAD)	[3]
INO	347 nmol/l (VHD)	658 nmol/l (CAD)	[3]
ANP	15 pg/ml	177 pg/ml	[16]
	23 ng/ml (SAN)	66 ng/ml (ACS)	[11]
BNP	144 pg/ml	1720 pg/ml	[16]
	354 ng/ml (SAN)	1022 ng/ml (ACS)	[11]
H-FABP	9.6 ng/ml	16.3 ng/ml (ACS)	[17]
AM (active)	7 pmol/l (SAN)	12.5 pmol/l (ACS)	[11]
NE	20 nmol/l	70 nmol/l (HT)	[9]
bFGF	289 pg/ml	2036 pg/ml (USAN)	[12]
IGF-1	20 ng/ml (EF>50%)	45 ng/ml (EF<30%)	[13]

(ADO: adenosine, INO: inosine, ANP: atrial natriuretic peptide, BNP: brain type natriuretic peptide, H-FABP: heart-type cytoplasmic fatty acid-binding protein, AM: adrenomedullin, NE: norepinephrine, bFGF: basic fibroblast growth factor, IGF-1: insulin-like growth factor-1, VHD: valvular heart disease, CAD: coronary artery disease, SAN: stable angina, ACS: acute coronary syndrome, HT: hypertension, USAN: unstable angina, EF: ejection fraction)

they were correlated with left ventricular hemodynamic variables, while plasma concentrations failed to do so. [11]. Previous experimental studies have reported several roles of insulin-like growth factor-1 (IGF-1) in the heart and similar to renin-angiotensin system (RAS), there is a local (tissue) IGF-1 system regulated differently from the circulating IGF-1 system. Moreover, experimental and clinical studies suggested that local IGF-1 system is up-regulated with the progression of left ventricular dysfunction. In a human study of 87 coronary artery disease patients pericardial IGF-1 levels and IGF-1 and its receptor expression in myocardial tissue were enhanced and inversely correlated with left ventricular ejection fraction [13]. Angiogenic growth factors, such as basic fibroblast growth factor (bFGF) produced and released by cardiac tissue, are also concentrated in pericardial fluid and its pericardial concentration was found to be significantly higher in patients with ischemic heart disease than nonischemic patients [12].

Pharmacological actions evoked from the pericardial space

In the pharmacological treatment of heart diseases one of the problems to overcome is to achieve satisfactory concentrations of therapeutic agents at the target site. When agents are applied systemically, the potential risk of side effects exists and therapeutic efficacy may be low, due to metabolism and tissue binding of the agent. Higher therapeutic efficiencies and smaller risks of peripheral side effects are anticipated if agents are administered locally. An alternative approach is administration of agents into the pericardial space. The local advantages of intrapericardially applied agents can be mainly explained by their low turnover in pericardial space compared to the relatively high plasma clearance [8]. Furthermore, the myocardial cells, coronary arteries, autonomic nerves, and conducting tissue can be directly affected from the pericardial space. In certain cardiovascular pathologies or in case of specific agents with extremely short plasma half-life intrapericardial application might have great significance. Previous studies demonstrated that certain regulatory agents applied into the pericardial space may exert characteristic cardiovascular effects (Table 3.), which suggested the possibility of pharmaco-therapeutic intervention via the pericardium. In experimental studies, marked characteristic actions of intrapericardially applied sotalol, atenolol and esmolol were found to be developed compared to intravenous application [18, 19].

During another experimental studies of myocardial ischemia intrapericardial administration of the nitroglycerin and docosahexaenoic acid (DHA) exerted a pronounced antiarrhythmic effect and avoided malignant arrhythmias [20, 21].

Table 3. Summary of different actions of regulatory agents applied into the pericardial space

	Actions from the pericardial space	References
Sotalol, Atenolol	antitachycardiac effect negative chronotropic and inotropic effect	[18]
Esmolol	antitachycardiac effect	[19]
Nitroglycerine	coronary vasodilatation	[22]
	antifibrillatory effect during AMI	[20]
L-arginine	antiarrhythmic effect, reduction of NE release during sympathetic stimulation	[23]
Digoxin	reduction of QTc interval	[24]
Amiodarone	antiarrhythmic effect	[25]
ET-1	inducing ventricular arrhythmias prolongation of QT and MAPD	[26]
DA	positive chronotropic effect	[27]
DHA	reduction of myocardial infarct size and malignant arrhythmias	[21]
bFGF	neovascularization in chronic myocardial ischemia	[28]
Arachidonic acid	Increase of pericardial prostaglandins electrophysiological effect	[29]

(ET-1: endothelin-1, DA: dopamine, DHA: docosahexaenoic acid, bFGF: basic fibroblast growth factor, MAPD: monophasic action potential duration, AMI: acute myocardial infarction, NE: norepinephrine)

Pharmaco-therapeutic intervention has become a matter of intense debate with recent attempts to induce myocardial angiogenesis by targeting genes or gene-derived products

to the heart. Basic fibroblast growth factor (bFGF-2) has been intensely studied for therapeutic myocardial angiogenesis in different experimental models of chronic myocardial ischemia. It has been demonstrated that intrapericardially injected bFGF-2 significantly increased the number of angiographic collaterals and ameliorated the perfusion of the ischemic myocardium [28]. Gene carriers applied into the pericardial space could also be used for gene delivery to the heart [30].

2.2 ADENINE NUCLEOSIDES

The adenine nucleosides, adenosine (ADO) and its first metabolite inosine (INO) are ubiquitous compounds found in every cell of the human body. The coronary vasodilator and negative chronotropic effects of adenosine were first described 80 years ago and in 1963 Berne presented experimental evidence for the "adenosine hypothesis", which suggested the major role of adenosine in the adaptation of coronary blood flow to metabolic demand [31-33]. Adenosine is formed in the myocardial cells as a result of reduced myocardial O_2 supply (hypoxia, ischemia) or increased myocardial workload by the breakdown of high energy phosphate compounds. Many of the cardiovascular actions of ADO are homeostatic and protective in nature and equalize local energy requirements with energy supply [34].

Synthesis, release and metabolism of adenosine

As it is illustrated in Figure 1, adenosine may be formed in cardiac tissues by two major pathways: the dephosphorylation of AMP to ADO and phosphate, and the hydrolysis of S-adenosyl-homocysteine (SAH) to ADO and homocysteine [35, 36]. In the heart, the relative importance of the two pathways varies under different circumstances, such as normoxia or hypoxia. Thus, during normoxia, ADO is formed in the cardiac vascular endothelium and in the cardiomyocytes intracellularly via the transmethylation pathway. SAH is hydrolyzed to ADO and homocysteine, which is catalyzed by the enzyme SAH-hydrolase [37]. The rate of ADO release via this pathway is approximately equal to the rate of total ADO release from the heart during normoxia

and does not appear to increase significantly during hypoxia [38]. Therefore, the dephosphorylation of intracellular AMP by the enzyme 5'-nucleotidase is the main mechanism responsible for the increased formation of ADO during hypoxia. The concentration of intracellular AMP depends on the energy charge of the cell, so an increase in the hydrolysis of ATP or a decrease in the rate of ATP synthesis, leads to a drop in energy charge and increase AMP concentration. The enzyme 5'-nucleotidase activity is negative feedback regulated by ADO and ATP, but stimulated by low energy levels (hypoxia, ischemia), increased concentration of AMP and NO, and α_1-adrenergic receptor activation [39-41]. The enzyme 5'-nucleotidase is localized both in the cytosol (cytosolic 5'-nucleotidase) and at the surface membrane (ecto-5'-nucleotidase) of cardiomyocytes. ADO formed by cytosolic 5'-nucleotidase released by a nucleoside carrier into the extracellular space, where it performs its actions. The down-regulation of both endo- and ecto-enzymes after 30 minutes exposure to ADO may serve to protect the heart from excessive ADO accumulation.

Two enzymes play a key role in catabolizing ADO: adenosine deaminase (ADA) and adenosine kinase (AK) [36]. The former is a high capacity and high Km enzyme, degrades adenosine into inosine. The latter is a low capacity and low Km enzyme catalyzing the phosphorylation of ADO to AMP, which represents the preferential pathway of ADO inactivation by re-incorporation of the ATP pool [35]. At physiological concentrations ADO is predominantly metabolized into AMP and AK is critically important in maintaining the physiological levels of ADO low [42]. While at higher ADO concentrations, e.g. following exogenous administration, ADO is deaminated into inosine. ADO, before being inactivated intracellularly by these two cytosolic enzymes, must first be taken up by endothelial cells and cardiomyocytes via the nucleoside transporters. There are inhibitors for ADO transporters, including the drug dipyridamole, which inhibits the cellular reuptake of ADO and however it also inhibits ADA enzyme, thus increases extracellular ADO concentrations by two different ways. Following the degradation of ADO to inosine (INO), inosine is further degraded to hypoxanthine, which is finally metabolized through xanthine to urate (Figure 2.).

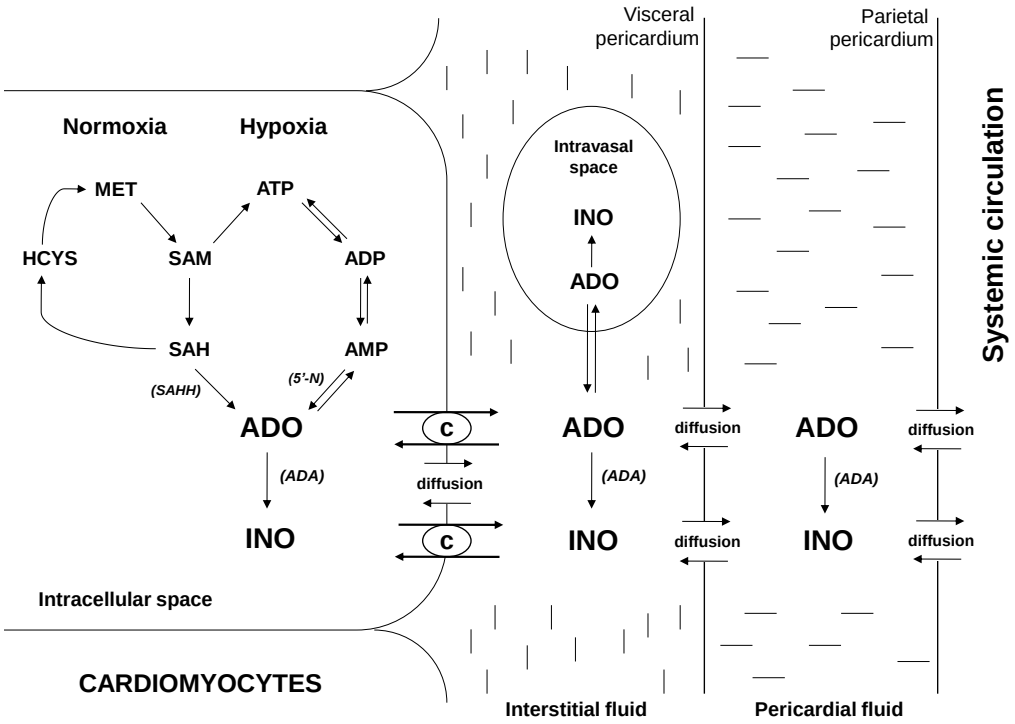

Figure 1. Schematic illustration of metabolism and dynamic transport of the adenine nucleosides among different fluid compartments of the heart.

(ADO: adenosine, INO: inosine, AMP: adenosine monophosphate, ADP: adenosine diphosphate, ATP: adenosine triphosphate, MET: metionine, HCYS: homocysteine, SAH: S-adenosyl-homocysteine, SAHH: SAH hydrolase, SAM: S-adenosyl-metionine, ADA: adenosine deaminase, AK: adenosine kinase, 5'-N: 5'-nucleotidase)

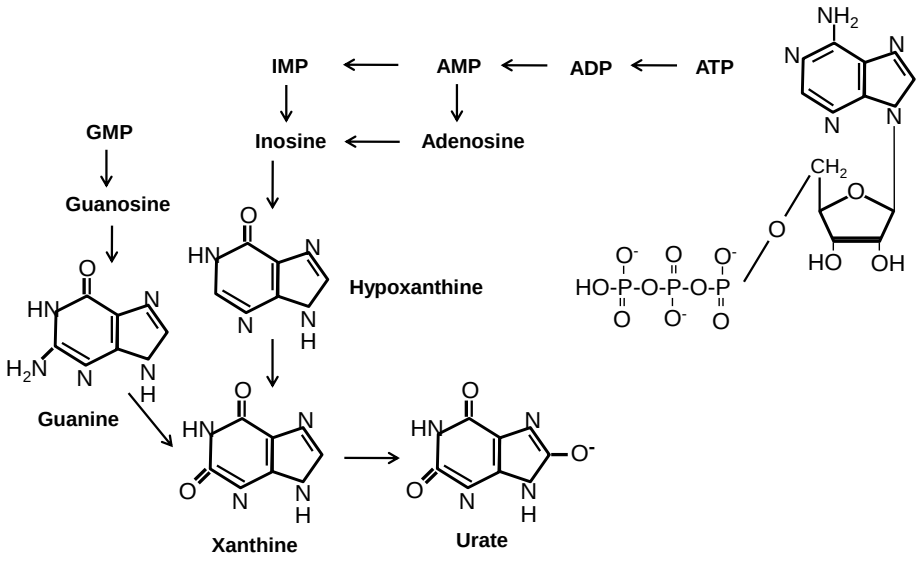

Figure 2. Biochemical summary of ATP breakdown

(ATP: adenosine triphosphate, ADP: adenosine diphosphate, AMP: adenosine monophosphate, IMP: inosine monophosphate, GMP: guanosine monophosphate)

Adenosine receptors

Adenosine acts on four, G-protein coupled purinergic receptors: A_1, A_{2a}, A_{2b}, A_3 receptors [43, 44]. Adenosine in concentrations present under basal conditions is sufficient to activate A_1, A_{2a} and A_3 receptors. However, adenosine A_{2b} receptors require higher concentrations of ADO to be significantly activated, as high concentrations that are believed to be present during more extreme or pathological conditions.

A_1 receptors are located on supraventricular and ventricular cardiomyocytes (SA-nodal and AV-nodal cells, atrial and ventricular cardiomyocytes). A_1 receptors coupled via pertussis toxin-sensitive G-protein (G_i) activate K_{ATP} channels, protein kinase C (PKC) and inhibit intracellular cAMP production when they are stimulated [36]. Activation of A_1 receptor mediates the negative chronotropic, dromotropic and anti-adrenergic effects of ADO (Table 4.). Thus, ADO antagonizes the cAMP-dependent stimulation of L-type Ca^{2+} channels, delayed rectifier K^+ channels, Cl^- channels and pacemaker ion channels and the inotropic and arrhythmogenic effects of β-adrenergic agonists. There is clear evidence, that adenosine A_1 receptors are involved in mediating cardioprotection in the ischemic heart [45]. ADO induced activation of sarcolemmal and mitochondrial K_{ATP} channels of cardiomyocytes may play an important role, at least in part, in the protective effects of ADO during myocardial ischemia [46].

Most of the blood vessels dilate in response to adenosine, except afferent arterioles of the kidney, which as a part of the tubuloglomerular feed back mechanism contracts via adenosine A_1 receptors [47]. Adenosine A_{2a} and A_{2b} receptors mediate vasodilatation, partly by a direct action on the vascular smooth muscle cells, partly endothelium mediated. ADO induced A_2 receptor activation lead to a stimulation of adenylate cyclase enzyme activity via G_s proteins and a subsequent increase in intracellular cAMP levels. The increase of intracellular cAMP concentration activates the cAMP-dependent protein-kinases, which cause relaxation of smooth muscle in the vasculature. In addition, A_2 receptor activation reduces platelet aggregation and the adherence of leukocytes to endothelial cells, prevents the release of superoxide anions by activated neutrophils, inhibits monocyte and macrophage function, increases glucose uptake, stimulates gluconeogenesis, glycolytic flux and the release of nitrogen monoxide from the vascular endothelium [36].

Adenosine A_3 receptors of cardiomyocytes, similar to A_1 receptors, are coupled to G_i protein, activate PKC and may participate in the ischemic preconditioning and enhancement of mediator release from mast cells [48].

Table 4. Summary of the effects of adenosine.

Effects of adenosine	**Cell type**	**Receptor subtype**
vasodilatation	vascular smooth muscle	$A_{2a,}$ A_{2b}-receptor
negative chronotropic effect negative dromotropic effect	SA and AV node	A_1-receptor
anti-adrenergic effect	cardiomyocytes	A_1-receptor
cardioprotection	cardiomyocytes	A_1és A_3-receptor
anti-platelet aggregation	thrombocytes	A_{2a}-receptor
anti-inflammatory effect (inhibition of leukocyte, monocyte and macrophage function)	polymorphonuclear leukocytes, monocytes, macrophages, mast cells	A_{2a}-receptor
stimulation of glucose uptake, glycolysis and gluconeogenesis	cardiomyocytes	A_1-receptor
stimulation of NO release	endothelial cells	A_{2a}-receptor

(SA: sinoatrial, AV: atrioventricular, NO: nitrogen monoxide)

Role of adenosine in the regulation of cardiac function

ADO is continuously released into the interstitial fluid from cardiomyocytes and in small amounts from the vascular endothelium. ADO acts chiefly on small resistance coronary arterioles (diameters are less than 20μm) as an endothelium-independent vasodilator. Hypoxia, myocardial ischemia and exercise are the main triggers of increased myocardial ADO production. Berne suggested that an inadequate O_2 supply for the prevailing metabolic rate would increase the ADO level, resulting in vasodilatation, and thereby increase O_2 delivery. This would then feed back through the

system, increasing the tissue O_2 tension and subsequently lowering the ADO concentration. Local metabolic adaptation is one of the most essential mechanisms in the regulation of coronary circulation and adenosine possesses a key role in it.

Beside its vasodilator effects adenosine has negative chrono-, dromotropic effects and interacts with the adrenergic system in many ways. Adenosine inhibits the presynaptic norepinephrine release, antagonizes β-adrenergic effects on cardiomyocytes via A_1 receptor mediated decrease of cAMP levels. However, the activation of α_1 and β-adrenoceptors increases adenosine release from cardiomyocytes and the activation of α_2 receptors potentiate the vasodilatory effect of adenosine.

The modulation of the ATP degradation pathway at different levels is an important strategy to reduce ischemia-reperfusion injury. The beneficial effects of adenosine [49], adenosine analogues [50], adenosine uptake and deaminase inhibitors [51, 52] have been described in the past decades. Adenosine exerts cardioprotective effects directly on cardiomyocytes during myocardial ischemia and reperfusion. The beneficial actions of ADO experimentally manifesting in the reduction of infarct size and in the improved recovery of ventricular function, are based on multiple underlying protective mechanisms [53].

The beneficial effects of adenosine could be partly attributed to its ability to reduce ATP depletion during ischemia and improve repletion of ATP during reperfusion. It is known that during ischemia-reperfusion an overload of Ca^{2+} exists that could cause cellular damage by activating phospholipases and proteases accelerating ATP degradation. The activation of A_1 receptors during reperfusion could modify the inflow of Ca^{2+} through the sarcolemma by inhibiting the adenyl cyclase enzyme, phosphorylating and opening ATP sensitive K^+ channels and by inhibiting the slow Ca^{2+} channels. The modulation of sarcoplasmatic reticulum Ca^{2+} handling may be involved in both A_1 and A_3 adenosine receptor mediated protection. This also may limit the development of Ca^{2+} overload [54]. Cardioprotection afforded by adenosine A_3 receptor stimulation is mediated, in part, via the activation of K^+_{ATP} channels [55]. The opening of mitochondrial K^+_{ATP} channels plays a crucial role in preservation of mitochondrial function and cell viability during ischemia. There is some evidence about the potential involvement of mitochondrial K^+_{ATP} channel activation in adenosine A_1 receptor mediated cardioprotection [56]. Moreover, adenosine could attenuate the activation of

neutrophils, which are proposed as crucial determinants of injury during ischemia-reperfusion, contributing to endothelial dysfunction, ROS generation and myocardial injury. A_1 receptor activation protects via enhancing antioxidant defense by reducing mitochondrial free radical formation and oxidative stress and by increasing cellular antioxidant capacity [57]. Besides, the activation of A_3 receptors also can decrease the degree of lipid peroxidation [58].

Adenosine also affects neurotransmission and interferes with the release of several hormones and endogenous active compounds, including renin, endothelin-1, prostacyclin and NO.

Inosine

Inosine in the form of its nucleotide, inosine 5'-monophosphate, is the precursor of AMP and GMP in the de novo biosynthesis of purine nucleotides. It is also an intermediate in the salvage pathway of purine nucleotide synthesis, as well as an intermediate in the degradation of purines to the purine end-product, uric acid.

Inosine (INO) is also the first metabolite of adenosine, formed by the enzyme adenosine deaminase. This pathway works mainly in case of increased release of ADO. ADO is rapidly taken up from the interstitial fluid by cardiomyocytes, vascular smooth muscle cells and endothelial cells or from the intravascular space by erythrocytes. Inosine was thought for many years to be an inert metabolite without biological effects. However, INO elicits marked coronary vasodilatation. It is comparable in magnitude to the adenosine-induced response, although, the molar potency of adenosine is much greater, than that of inosine. Inosine relaxes the coronary artery by stimulation of a site distinct from the vascular adenosine receptor [59]. While adenosine mainly dilates small resistance coronary arterioles (o.d. <20 μm), inosine primarily acts on epicardial coronary arteries (o.d. 1-2 mm). It has been also demonstrated that inosine can enhance the actions of adenosine and almost the complete blockade by aminophylline of adenosine-induced coronary vasodilatation was significantly reduced with inosine [60].

As an own characteristic effect, inosine increases myocardial contractile force [61]. The positive inotropic action of inosine has been demonstrated in both healthy and pathological myocardium. This effect is not due to stimulation of the cardiac β-adrenergic receptors, as beta-blockers do not antagonize the positive inotropic effect of

inosine. Administration of inosine has been shown to improve myocardial function during acute left ventricular failure and decrease infarct size after coronary artery occlusion [62, 63]. The inotropic and coronary vasodilator effects of inosine are not associated with any modification of the chronotropic function and inosine does not affect atrioventricular conduction.

In contrast to adenosine which acts mainly via surface purine receptors, inosine might have more direct effect on intracellular signaling. It has been shown recently, that inosine may also exert cardioprotective effects during ischemia-reperfusion and may modulate oxidant-induced cell death by interfering with the poly(ADP-ribose)polymerase (PARP) activation pathway. In a rat heart transplantation model inosine improved myocardial and endothelial functions during early reperfusion, restored ATP levels and almost completely abolished PARP activation [64].

Previous studies implicated that inosine also exerts anti-inflammatory effects in many human cell types [65], inhibits pro-inflammatory cytokine and chemokine production *in vitro* and *in vivo*, while it can enhance the production of the protective interleukin-10. Purin nucleotides in general and inosine in particular may reduce neutrophil accumulation and neutrophil-mediated injury. In an experimental model of endotoxin shock, inosine treatment improved vascular reactivity, endothelial function and reduced the inflammatory response in the gastrointestinal tract [66, 67].

In vivo, inosine is metabolized to hypoxanthine and to urate. Urate is a potent scavenger of peroxynitrite or peroxynitrite-derived reactive nitrogen species, therefore these urate-mediated antioxidant effects may also contribute to the protection seen with adenosine and inosine [68, 69].

2.3 ENDOTHELINS

The vascular endothelium plays a key role in the regulation of vascular tone. Several important vasodilator and vasoconstrictor substances are produced by endothelial cells, such as nitric oxide, prostacyclin, bradykinin, endothelium-derived hyperpolarizing factor (EDHF) and endothelins acting primarily on vascular smooth muscle cells in a paracrine manner.

Endothelin (ET) was initially isolated and sequenced from porcine aortic endothelial cell cultures in 1988 by Yanagisawa *et al* [70]. Endothelin represents the most potent and long-lasting vasoconstrictor in humans, being 100-fold more potent than norepinephrine. This peptide, endothelin, does not belong to any previously known peptide family. Three distinct genes of ET were demonstrated during the analysis of the human genom encoding the later termed endothelin-1 (ET-1) endothelin-2 (ET-2) and endothelin-3 (ET-3), as the three isoforms of the endothelin family [71]. All these isoforms of endothelin show a high degree of primary amino acid sequence identity; all consist of 21 amino acid polypeptide residues including two intramolecular disulfide bonds [72]. However, ET-1 represents the predominant and the biologically most relevant isoform. Although the vascular endothelium is the main physiological source of ET-1 in humans, but vascular smooth muscle cells, cardiomyocytes, macrophages, leukocytes, fibroblasts, tubular epithelial and mesangial cells and podocytes in the kidney also produce and release ET-1 [73]. In contrast, ET-2 and ET-3 seem to be implicated in renovascular function, lung alveolarization, ovulation and neurotransmission based on their respective localizations in the renal medulla, bronchial and intestinal epithelial cells, and nerve endings [74]. The genes that encode the three endothelin isopeptides are expressed in a wide variety of cell types, which suggests that these peptides may participate in complex regulatory mechanisms across many organs. Endothelins exert a number of physiological functions, including both beneficial and detrimental effects [75]. ET-1 is a potent vasoconstrictor, has inotropic and mitogenic properties, influences homeostasis of salt and water, and stimulates the renin-angiotensin-aldosterone and the sympathetic nervous system [73, 74, 76].

Synthesis, release and regulation of endothelin-1 production

Endothelin-1 is generated from precursor peptides via a two-step proteolytic pathway (Figure 3.). Transcription of the ET-1 gene on the chromosome 6 generates mRNA encoding the 212-amino-acid peptide, preproET-1. PreproET-1 is secreted into the cytoplasm and further cleaved by a furin-like endopeptidase to the 38-amino-acid precursor peptide proendothelin-1 (big-ET-1) [77]. Big ET-1 can be found in the systemic circulation but however, does not possess significant biological activity [78]. ET-converting enzyme (ECE) catalyses the second and rate-limiting step in ET-1 synthesis [79]. By removing the further 17 COOH-terminal residues from big ET-1 ECE forms the mature 21-amino-acid ET-1. In humans there are two functionally distinct isoenzymes of ECE, ECE-1 and ECE-2. ECE-1 is expressed in the Golgi apparatus of the "producer" cells, such as endothelial cells and appears to be responsible for intracellular processing of big ET-1 to ET-1. ECE-2 is expressed in the "responder" cells, such as vascular smooth muscle cells, and located in the plasma membrane. It cleaves extracellular big ET-1. The generation of ET-1 is regulated principally at the level of ET-1 mRNA transcription and influenced by many stimuli, including hypoxia, vasoactive hormones, growth factors, shear stress, free radicals and endotoxins [80]. Low shear stress, turbulent blood flow, ischemia, hypoxia, angiotensin II, norepinephrine, bradykinin, vasopressin, insulin, cortisol, TNF-α, TGF-β, IL-1 thrombin and low density lipoprotein have been shown to enhance the transcription of ET-1 gene. In contrast, production of ET-1 is inhibited by stimuli which can increase intracellular level of cGMP including NO, vasodilator prostaglandins, ANP, BNP and heparin. Interestingly, ET-1 can increase its own synthesis in endothelial cells in an autocrine manner by activating ET_B receptors [81]. ET-1 is released from endothelial cells through both constitutive and regulated (rapid release) pathways. In the endothelial cells vesicles containing big ET-1, ECE and ET-1 have been recently identified and beside their transport and storage function, they also serve as site of ECE-regulated ET-1 synthesis [82].

The circulating ET-1 is stable in bloodstream but is eliminated rapidly from circulation, with a half-life of 7 minutes [83]. ET-1 clearance occurs through both receptor-mediated and enzymatic processes. The lungs, kidney and the liver have significant role in the process of eliminating ET-1 from the systemic organism [84, 85]. Clearance of

ET-1 from plasma occurs primarily in the lungs by endocytosis and degradation of the ET_B receptor-ligand complex [86]. Enzymatic degradation also contributes to the clearance of ET-1 by neutral endopeptidase enzyme (NEP) [87, 88]. NEP is an endothelial, membrane-bound metallopeptidase with zinc at its active site that functions to cleave endogenous peptides at the amino side of hydrophilic residues. The membrane-bound metalloproteinase has a similar catalytic unit to angiotensin convertase enzyme (ACE). NEP is widely distributed in endothelial cells, vascular smooth muscle cells, cardiac myocytes, renal epithelial cells, and fibroblasts. It catalyses the degradation of a number of endogenous vasodilator peptides, including ANP, BNP, CNP, substance P, and bradykinin as well as vasoconstrictor peptides, including endothelin-1 (ET-1) and angiotensin II .

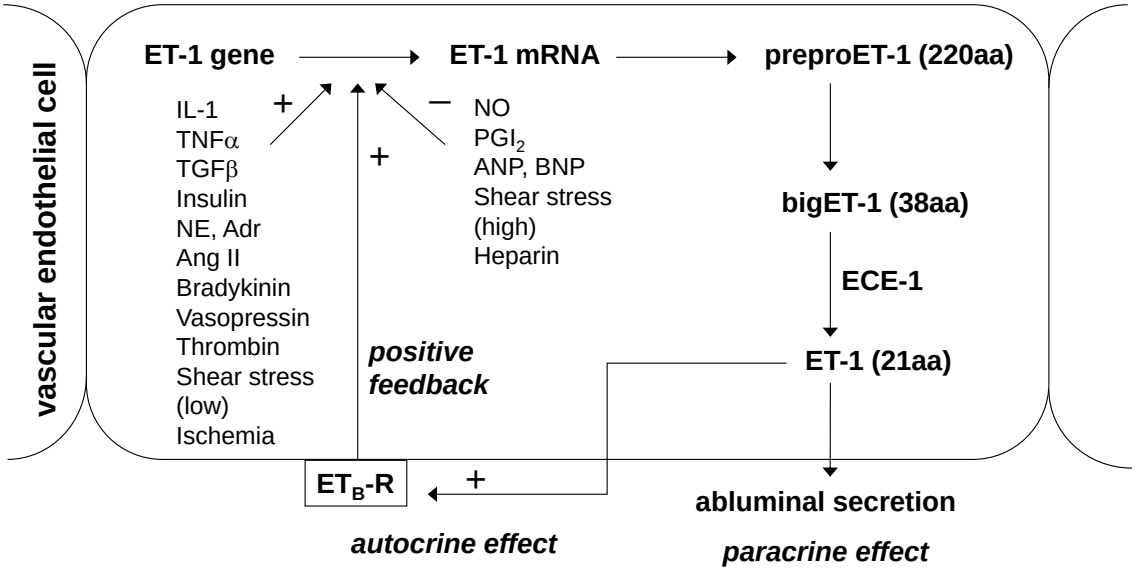

Figure 3. The synthesis of ET-1 in vascular endothelial cells and the summary of factors regulating the transcription of ET-1 mRNA.

(ET-1: endothelin-1, mRNA: messenger ribonucleic acid, aa: amino acid, ECE: endothelin converting enzyme, ET_B-R: B type endothelin receptor, IL-1: interleukine-1, TNFα: tumor necrosis factor-α, TGFβ: transforming growth factor-β, ANP: atrial natriuretic peptide, BNP: brain natriuretic peptide, Ang II: angiotensin II, NE: norepinephrine, Adr: adrenaline)

Receptors and signal transduction of endothelin-1

Endothelins act on two distinct G-protein coupled receptor subtypes (ET_A and ET_B receptors) characterized according to the relative binding affinities of the three endothelin isopeptides [71, 89]. The ET_A receptor shows subnanomolar affinities for ET-1 and ET-2, while 100-fold lower affinity for ET-3 [75]. The ET_A receptor expressed mostly in vascular smooth muscle cells and mediates contraction by increasing intracellular calcium [90]. Depending on tissue distribution, ET_A receptor mediates vasoconstriction, bronchoconstriction and uterine smooth muscle contraction. The ET_B receptor is equally activated by ET-1, ET-2 and ET-3, primarily located on endothelial cells, mediates vasodilatation through the generation of nitric oxide and prostaglandin I_2 (PGI_2) [91]. This endothelium dependent vasodilator effect can manifest only before the development of vasoconstrictor effect of ET-1 after administration of ET-1 in low doses and can be attenuated by NOS and COX inhibitors. However, ET_B receptors also appear on vascular smooth muscle cells where they mediate vasoconstriction [92]. It might have clinical significance during pathological conditions, such as ischemic heart disease, atherosclerosis and congestive heart failure, when ET_B receptors have been observed to be up-regulated on vascular smooth muscle cells [93-97].

ET_A receptor on vascular smooth muscle cells also can be considered as growth-promoting, mitogenic receptor, whereas the ET_B receptor inhibits cell growth [98-101].

Both ET_A and ET_B receptors produce G-protein dependent activation of phospholipase C (PLC) [102, 103]. This leads to generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes rapid increase in intracellular concentration of calcium ($[Ca^{2+}]_i$), through its release from intracellular stores, including the endoplasmic reticulum. A more sustained rise in $[Ca^{2+}]_i$ occurs through opening of membrane Ca^{2+} channels. In case of smooth muscle cell the prompt increase of intracellular free Ca^{2+} levels result in contraction of myofilaments via the formation of Ca^{2+}-calmodulin complex and activation of myosin light chain kinase. DAG activates PKC, which leads to increasing Ca^{2+} sensitivity of the contractile apparatus and change in intracellular pH (alkalinization) via stimulation of Na^+-H^+ exchange [104, 105]. There is also evidence that ET-1 closes membrane K^+ channels and prevents efflux of K^+ from the cell, which enhances depolarization of membrane and contraction of

smooth muscle cell. Furthermore, ET-1 induced prostacyclin and thromboxane A_2 production via phospholipase A_2 activation also contributes to the vasoconstrictor effect.

Actions of endothelin-1

ET-1 is an extremely potent vasoconstrictor peptide derived from vascular endothelial cells (Figure 4.). When administered exogenously *in vivo*, ET-1 evokes potent, long-lasting coronary constriction and marked flow reduction, which may result in ischemia. In concord with previous investigations [106], Juhász-Nagy and his co-workers have had confirmed earlier the tremendous sensitivity of the coronary vascular bed to ET-1 [107]; upon intracoronary administration, ET-1 yields a full spastic coronary dose response curve in a comparatively narrow concentration range. Interestingly, the receptor distribution and thus the pharmacological effects of ET-1 in the coronary vascular bed seem to vary between different species. In humans, after intracoronary administration of ET-1, only the coronary flow reduction can be observed due to the coronary constrictor effect of ET-1. However in rat both vasoconstrictor ET_A and ET_B receptors as well as vasodilator ET_B receptors appear to be present. In the pathophysiology of vasospasm and spastic ischemia the role of ET-1 was suggested [108]. The continuous production of endothelin-1 in the endothelium may have an important role in the control of basal vascular tone, even in the coronary circulation [109]. However, the overall cardiovascular action of endogenous ET-1 depends on the balance between ET_A and ET_B receptor mediated effects. Activation of vascular smooth muscle cell ET_A receptors causes vasoconstriction and tends to elevate blood pressure. Activation of endothelial and renal ET_B receptors promotes vasodilatation and natriuresis and tends to decrease blood pressure. Obviously, the cardiovascular effects of endogenous ET-1 may change in cardiovascular diseases. ET-1 has also been demonstrated to exert positive inotropic effects in various isolated heart preparations and in vivo accompanied by ET_A receptor activation [110-112]. Apart from myocardial ischemia induced arrhythmogenic effect, ET-1 has direct arrhythmogenic effect as a result of the prolongation of the action potential in the ventricular myocardium [26, 113].

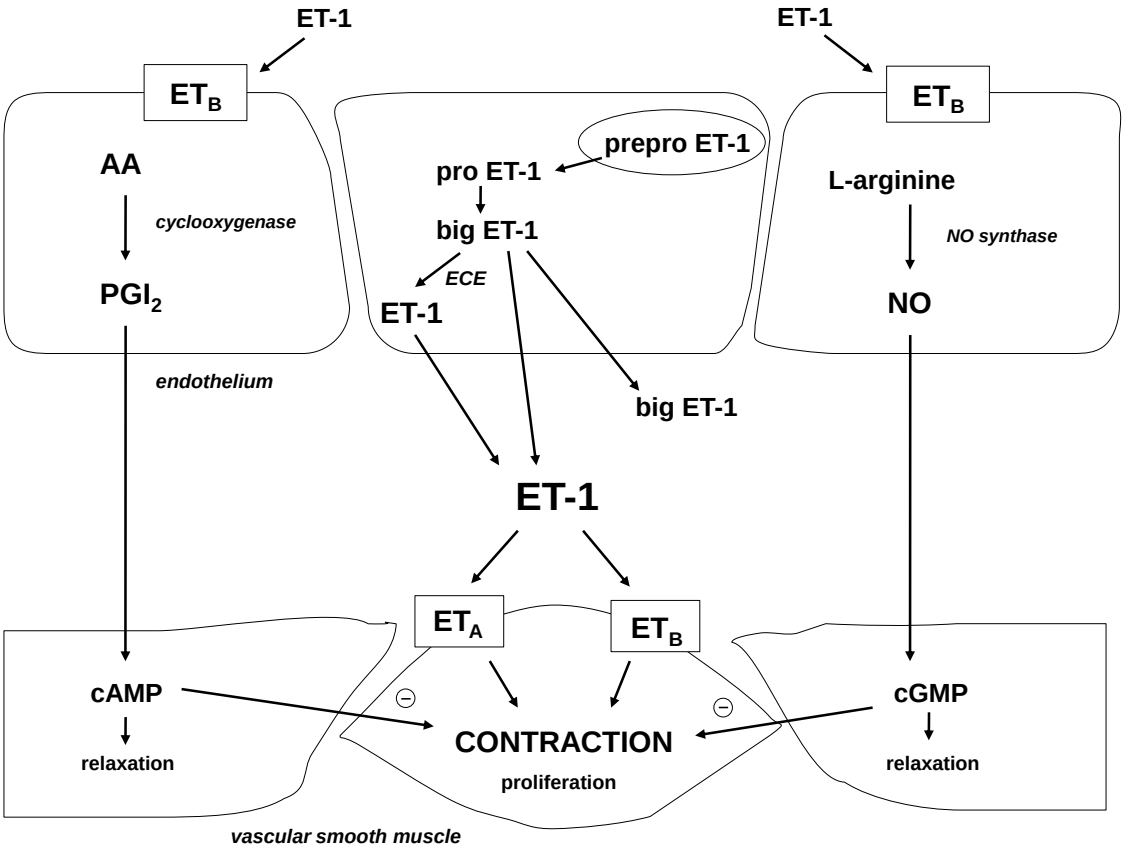

Figure 4. Endothelium derived factors, including endothelin-1, that regulate vascular smooth muscle tone [80].

(ET-1: endothelin-1, ECE: endothelin converting enzyme, ET_A: A type endothelin receptor, ET_B: B type endothelin receptor, AA: arachidonic acid, NO: nitrogen monoxide, PGI_2: prostacyclin, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate)

ET-1 stimulates regional changes in renal blood flow, typically inducing vasoconstriction in the renal cortex and vasodilatation in the medulla [114]. ET-1 contracts both afferent and efferent arterioles and thus reduces renal plasma flow and glomerular filtration rate [115, 116]. Medullary vasodilatation appears to be solely mediated by ET_B receptor activation, while effects on the larger renal vessels and glomerular afferent and efferent arterioles are both ET_A and ET_B receptor dependent [117]. ET-1 also controls water and sodium excretion and acid-base balance in the kidney under physiologic conditions [118]. It was demonstrated that ET-1 blocks reabsorption of Na^+ by inhibiting tubular Na^+/K^+ ATPase activity in the proximal

tubule, blocks reabsorption of water in the collecting duct and inhibits the effects of antidiuretic hormone (ADH) on tubular osmotic permeability [119-121].

Furthermore, ET-1 has contrasting effects on the renin-angiotensin-aldosterone system, inhibiting the release of renin from isolated rat glomeruli, but stimulating endothelial ACE activity [122, 123] and the tissue renin-angiotensin system (RAS) in the rat isolated mesenteric bed [124]. In addition, angiotensin II increases ET-1 tissue levels and ECE activity *in vivo*, but ET-1 stimulates release of aldosterone from isolated cortical zona glomerulosa cells and adrenalin from medullar chromaffin cells [125, 126]. ET-1 has been proposed to be an important activator of natriuretic peptide release from the heart. Furthermore, ET-1 stimulates the production and release of ANP and BNP *in vitro* and *in vivo* [127, 128].

The discovery of endothelin-1 was rapidly followed by the realization that its release, perhaps from the dysfunctional endothelium could have a major role in the pathogenesis of a variety of cardiovascular disorders. The actions of ET-1 including vasoconstriction, increasing sympathetic nervous system, renin-angiotensin-aldosterone system activity and mitogenesis, make ET-1 a plausible mediator in the pathogenesis of hypertension, arteriosclerosis and its complications. Several studies have demonstrated that increased production of endothelin, not solely but is responsible for the maladaptive cardiac and subcellular remodeling depending upon the type and stage of congestive heart failure [129]. Furthermore, significantly increased plasma concentrations of endothelin-1 have been observed in pathophysiological states associated with increased vascular resistance, vasospasm, and vascular injury (including myocardial injury or infarction, cardiogenic shock, hypertension, subarachnoid hemorrhage, diabetes)[122, 130-134]. Plasma endothelin-1 levels have been shown to be increased in liver cirrhosis, septic shock, renal failure, pulmonary diseases and hepato-renal syndrome [135].

In the lung, the endothelin system regulates bronchial tone and the proliferation of pulmonary airway blood vessels and thus promotes the development of pulmonary hypertension [136, 137].

ET-1 is not merely one of the most potent and characteristically sustained vasoconstrictors described but a multifunctional peptide with cytokine-like activity that affects almost all aspects of cell function.

In vascular smooth muscle and cardiac muscle, ET-1 is a potent mitogen in cultured vascular smooth muscle cells, cardiac myocytes, glomerular mesangium and other cells [98, 138]. It also induces the expression of several proto-oncogenes. A variety of mitogenic stimuli activate the intracellular kinase cascade, including mitogen-activated protein kinase (MAPK). It is generally accepted that the ET_A receptor mediates cell growth regulation. The ET_B receptor also mediates some of the growth responses as well as the growth inhibitory effects associated with apoptosis [139].

3 AIMS

The present study was proposed to investigate the cardiac interaction of adenine nucleosides and endothelin-1. Regarding the growing body of evidence about the potential regulatory role of pericardial fluid, we aimed to utilize the intrapericardial space for pharmacological interventions examining the aforementioned important regulatory couple.

Considering the fact that the intracoronary administration of the potent vasoconstrictor peptide, endothelin-1 evokes coronary vasoconstriction and consequent regional myocardial ischemia, we hypothesized that the ischemic effect of ET-1 may be also provoked from the pericardial space. The myocardial production and release of the main vasodilator mediators, adenine and inosine during the activation of coronary metabolic autoregulation may be mirror reflected in the pericardial space.

Regarding the basic principle in cardiovascular physiology, that the excitatory and inhibitory agents always enhance the production of each other, we hypothesized that adenine nucleosides applied intrapericardially may also influence the production of endothelin-1 in the heart.

Moreover, we have no information about the pericardial turnover and pericardial effects of adenine nucleosides compared to the intravascular action of these agents.

Therefore, we aimed

- to investigate whether characteristic cardiovascular effects of ET-1 can be achieved from the pericardial space by its intrapericardial administration to the *in situ* dog heart;
- to confirm myocardial ischemia caused by ip. ET-1 by evaluating left ventricular (endo- and epicardial) monophasic action potential tracings;
- to prove the activation of coronary metabolic adaptive mechanisms evoked from the pericardial space after ip. administration of ET-1;
- to measure the pericardial concentrations of adenine nucleosides before and after administration of ET-1 into the pericardial space using the “closed pericardium model”

- to investigate the alteration of pericardial ET-1 concentrations in response to pericardial administration of adenine nucleosides on the *in situ* dog hearts;
- to estimate the turnover rate of adenine nucleosides in the pericardial fluid compared to the plasma;
- to observe the effects of adenine nucleosides on the physiological cardiovascular function in the *in situ* dog heart using both intrapericardial and intravenous administration of the agents.

4 MATERIALS AND METHODS

4.1 GENERAL PREPARATION

Fifty four experiments were performed on anesthetized dogs of either sex. Animal procedures were carried out in accordance with the relevant *Hungarian National Legislation* and with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (National Research Council, Washington, DC, U.S.A., 1996). Each animal was anaesthetized with intravenous pentobarbital sodium (Nembutal; CEVA; 30 $mg \cdot kg^{-1}$, i.v.) intubated and ventilated with room air (Cape; CV24224 ventilator, Cape Engineering Co. Ltd. Warwick, UK). Body temperature was kept at 37°C using a heating pad. The right femoral artery was cannulated with a polyethylene catheter to monitor arterial blood pressure with pressure transducer (Statham P23Db, Experimetria HG01 modem and Electromedics XD003, Electromedics Inc., Englewood, CO, USA). A 4F pigtail catheter (Cordis, Miami, FL) was inserted into the left ventricle via the right carotid artery to measure left ventricular pressure. Needle limb electrodes served for standard ECG recordings (CU12, Madaus Schwarzer; München, Germany). Hemodynamic data were registered with a computerized data acquisition system (SPEL Adv Haemosys 2.46, Experimetria Ltd., Budapest, Hungary). The chest was opened transsternally at the fifth intercostal space.

Each pericardial experiment was carried out using a "closed pericardium model" developed in our research laboratory. Firstly, the pericardial sac was cannulated in a watertight way via a small, approximately 3 mm diameter incision made by a microcauter. A soft, 2.5 mm diameter wide, flexible rubber catheter was inserted through the incision and its distal end was positioned in the oblique sinus, which is the most inferior region of the pericardial sac in supine position. The pericardium was tightened around the catheter entry site with a purse-string suture to prevent fluid escape. This pericardial catheter was used to obtain pericardial fluid samples and to apply endothelin-1 and adenine nucleosides into the pericardial space.

In a series of experiments (n=5) investigating the actions of intrapericardial administration of ET-1, the general animal preparation was followed by electrophysiological analysis. For monophasic action potential (MAP) recording

fractally coated screw-in MAP catheter (V177; Biotronik G.m.b.H., Germany) was fixed on the anterolateral epicardial surface of the left ventricle. In these experiments, one more incision was prepared on the pericardial sac for the left ventricular epicardial MAP catheter. The pericardium was tightened around the MAP catheter in the same way detailed above. For recording the left ventricular endocardial MAP signals, fractally coated electrophysiology catheter (V177, Biotronik G.m.b.H., Germany) was inserted via the left femoral artery into the left ventricular apex.

In another series of experiments (n=10) when ADO and INO were administered intravenously, after the sham thoracotomy, the right femoral vein was cannulated for the intravenous application of the agents. In these experiments, the arterial blood samples were collected from the left brachial artery.

At the end of each experiment the weight of the heart was measured.

The closed pericardium model and the pericardial sampling technique

Each step of the pericardial sampling process is illustrated in Figure 5. After a twenty-minute stabilization period, the native pericardial fluid was removed and substituted with standard quantities of pericardial infusate fluid (0.75ml/kg body mass). We have previously investigated the hemodynamic effects of the different volumes of the pericardial infusates used in this closed pericardium model. However we have demonstrated that 0.75ml/kg of pericardial infusate fluid did not depress myocardial contractility and right ventricular filling, while the lubrication of the whole cardiac surface was warranted. This infusate fluid consisted of phosphate buffered saline containing 0.5% bovine serum albumin (PBSA, pH 7.4) in order to be more similar to the native pericardial fluid. Each infusate fluid was incubated for 15 minutes in the pericardial space and removed completely allowing a reliable and repetitive sampling method. The 15-minute long incubation period was chosen based on previous studies from our laboratory, which indicated that 15 min is sufficiently long to achieve a near equilibrium. Between each incubation step the pericardial sac was rinsed repeatedly with 5x5ml PBSA in order to decrease the possibility of any kind of contamination.

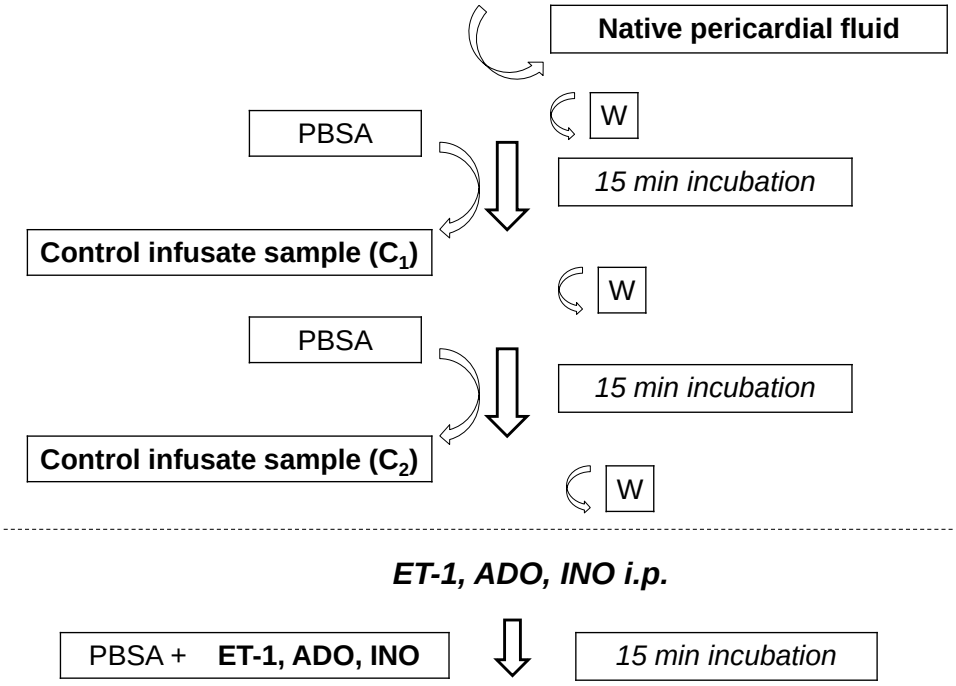

Figure 5.
Repetitive pericardial sampling process using in each pericardial experiment.
(PBSA= phosphate buffered saline containing 0.5% bovine serum albumin, W=the pericardial sac was rinsed repeatedly with 5x5ml PBSA, ET-1=endothelin-1, ADO=adenosine, INO=inosine, i.p.=intrapericardial)

4.2 DETAILED EXPERIMENTAL PROTOCOL

Protocol 1: Intrapericardial administration of endothelin-1

The effects of intrapericardial administration of ET-1 were studied in nine experiments (body weight: 24±1 kg). In the first phase of the experiments after removal of native pericardial fluid (N), two control samples (C_1, C_2) were taken. Afterwards, standard volume of PBSA containing 150pmol/kg ET-1 (Sigma-Aldrich) was incubated for 15 minutes in the pericardial space and withdrawn (sample ET-1_I). Then the incubation was repeated with ET-1-free PBSA two more times (samples ET-1_{II} and ET-1_{III}). Thus, three consecutive pericardial samples were obtained after intrapericardial ET-1 administration. (Figure 6.). Arterial (femoral artery) blood samples were taken in

parallel with pericardial sampling. The concentrations of adenosine, inosine and hypoxanthine were determined in the consecutive pericardial and arterial blood samples.

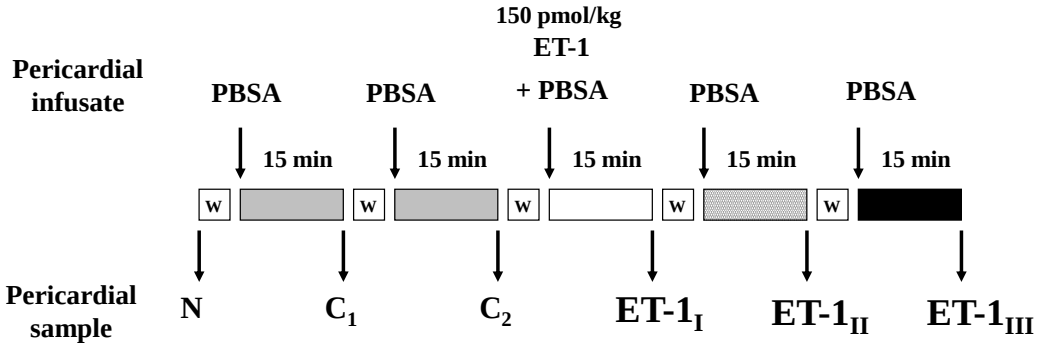

Figure 6.
Time table of ET-1 administration and pericardial sampling procedure in the experiments.
(PBSA= phosphate buffered saline containing 0.5% bovine serum albumin, W=the pericardial sac was rinsed repeatedly with 5x5ml PBSA, ET-1=endothelin-1,N=native pericardial fluid, C_1,C_2=control pericardial infusate sample, ET-$1_{I\text{-}II\text{-}III}$=pericardial samples after administration of 150 pmol/kg ET-1 intrapericardially)

Protocol 2: Intrapericardial administration of adenine nucleosides

The effects of increasing doses of ADO and INO on pericardial concentrations of ET-1 were investigated in sixteen experiments (body weight: 17±1 kg). Following control sampling (sample C_1, C_2), standard volumes of PBSA solutions containing increasing doses of ADO (10-100-1000μM, n=8) or INO (1-10-100mM, n=8) were given intrapericardially. (These doses represented 0.27-2.7-27μg adenosine per 1g myocardial tissue mass and 0.027-0.27-2.7 mg inosine per 1g myocardial tissue mass.) After the incubation periods - each 15 minutes long – three pericardial infusate samples ($ADO_{10\text{-}100\text{-}1000}$ and $INO_{1\text{-}10\text{-}100}$) were obtained (Figure 7.). Arterial blood samples were collected parallel with each pericardial sample. ET-1 levels of pericardial infusate and arterial plasma samples were measured.

Almost the same sampling procedure was used in those pericardial experiments (n=14, body weight: 15±1 kg) when the elimination and cardiovascular effects of intrapericardially administered adenine nucleosides were compared to their intravenous application. After 15 minute long pericardial incubation, the remaining fractions of

adenosine and inosine were measured in the three consecutive pericardial infusate samples ($ADO_{10\text{-}100\text{-}1000}$ and $INO_{1\text{-}10\text{-}100}$).

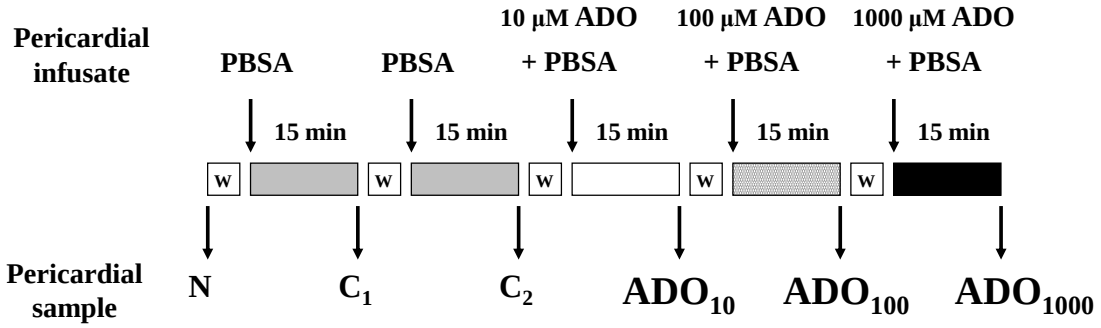

Figure 7.

Time table of ADO administration and pericardial sampling procedure in the experiments.

(PBSA= phosphate buffered saline containing 0.5% bovine serum albumin, W=the pericardial sac was rinsed repeatedly with 5x5ml PBSA, ADO=adenosine, N=native pericardial fluid, C_1,C_2=control pericardial infusate sample, $ADO_{10\text{-}100\text{-}1000}$=pericardial samples after administration of 10-100-1000µM ADO intrapericardially)

Protocol 3: Intravenous administration of adenine nucleosides

The elimination and hemodynamic effects of intravenous administration of adenine nucleosides were studied in experiments (n=10, body weight:15±1 kg) using a different sampling procedure. Before intravenous administration of adenosine and inosine, control arterial blood sample (C) was taken from the left brachial artery. Afterwards, physiological saline (1.5ml/kg body mass) containing ADO (0.27-2.7-27mg adenosine/kg body mass, n=5) or INO (0.027-0.27g inosine/kg body mass, n=5) was applied intravenously into the right femoral vein. These doses could provide the initial plasma concentrations of ADO (10-100-1000µM) or INO (1-10mM) respectively, which made the results of the pericardial and intravenous experiments comparable to each other. Supposing the quick elimination of nucleosides from the plasma, arterial blood samples were collected from the brachial artery at 1, 2, 5 and 15 minutes after the iv. boluses of ADO and INO (Figure 8.).

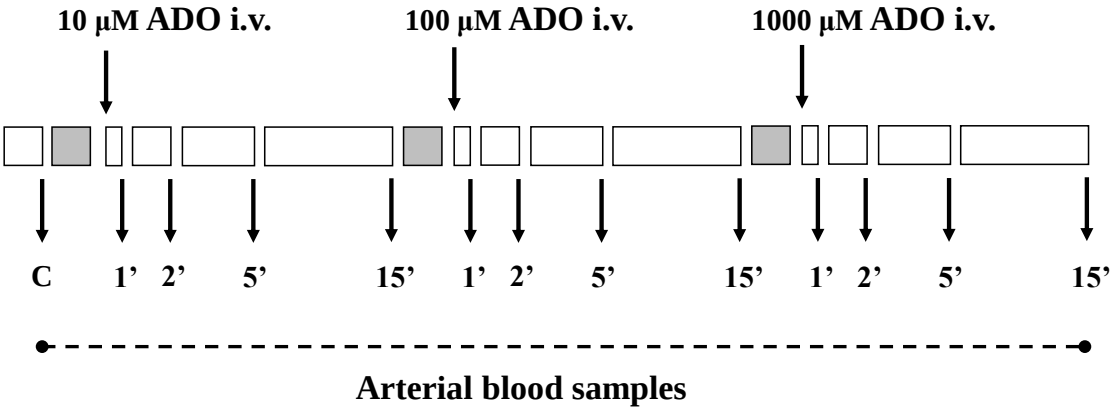

Figure 8.

Time table of intravenous ADO administration and arterial blood sampling procedure in the experiments. (ADO=adenosine, i.v.=intravenously, C=control sample, 1',2',5',15'=1-,2-,5-,15-minute plasma samples after intravenous administration of 10-100-1000μM ADO respectively)

4.3 MEASUREMENTS OF PERICARDIAL AND PLASMA ADENOSINE, INOSINE AND HYPOXANTHINE LEVELS

Production and degradation of the adenine nucleosides in the pericardial and plasma samples were blocked with dipiridamole, the inhibitor of 5'-nucleotidase enzyme (inhibitor of AMP to ADO conversion, 400μmol/l), and also with EHNA [eritro-4-(2-hidroxy-3-nonil) adenine-hydrochloride, 10 or 100μmol/l], the inhibitor of adenosine deaminase enzyme. Since pericardial concentrations of adenine nucleosides were expected to be higher than plasma concentrations, 10 times higher concentrations of EHNA (100μmol/l) were given to the withdrawn pericardial samples. All samples were centrifuged immediately (4˚C with 3000U/min for 15 min) and stored at -20˚C until biochemical analysis was performed. Concentrations of ADO, INO and hypoxanthine (HXA), the metabolite of INO, were measured by a high-performance-liquid-chromatography-ultraviolet (HPLC-UV) technique. When the production and intrapericardial concentrations of ADO, INO and HXA were investigated after intrapericardial administration of ET-1, we had to make some corrections to the sample volumes and heart mass. The pericardial adenosine, inosine and hipoxanthine

concentrations were corrected by the volume of the native pericardial fluid of each animal and to the total volume of pericardial infusates without enzyme blockers and then standardized for 100 g myocardial tissue.

High-performance-liquid-chromatography

Immediately after sampling, pericardial and plasma samples were mixed with stop solutions containing enzyme blocker dipiridamole and EHNA (see above) and centrifuged. Plasma proteins above 25.000 molecular mass were removed by ultrafiltration. Chromatographic analysis was carried out with an LKB chromatograph with a dual-wavelength UV detector. Separation was performed with a 3µm Microspher C18 column with a 5µm Chromsep C18 guard column. Isocratic elution was at a flow-rate 1.0 ml/min. The mobile phase was a mixture of 0.05M Na_2HPO_4 buffer (pH 3.7) and acetonnitrile (97.5:2.5, v/v), filtered through a 0.45µm filter and degassed by helium before the analysis. Detection was at 260nm. Standards were used to determine the retention time and the required run-time. Plasma and pericardial ADO, INO and HXA identity were verified by retention time and by co-elution with the standards.

4.4 MEASUREMENTS OF PERICARDIAL AND PLASMA ET-1 LEVELS

EDTA treated pericardial and plasma samples were centrifuged (4˚C with 3000U/min for 15 min) immediately after sampling procedures and stored at -20˚C until biochemical analysis was performed. Pericardial and plasma ET-1 levels were measured by enzyme-linked immunosorbent assay (Biomedica GmbH, Wien, Austria). ET-1 levels were corrected to the native pericardial fluid volumes and to the heart mass.

Enzyme immunoassay for the quantitative determination of endothelin-1

A polyclonal antibody specific for ET-1 has been pre-coated onto a microplate. Standards, samples, control and conjugate are pipetted into the wells and any ET-1 present is sandwiched by the immobilized antibody and the enzyme-linked antibody specific for ET-1. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, substrate is added to the wells and color develops in proportion to the amount of ET-1 bound. The color development is stopped and the

intensity of the color is measured. ELISA reader capable of measuring absorbance at 450 nm was used.

4.5 HEMODYNAMIC AND ELECTROPHYSIOLOGICAL PARAMETERS

Hemodynamic parameters, such as arterial blood pressure and left ventricular pressure were measured continuously during all experiments. Statham Db 23 pressure transducers were used for recording peripheral arterial blood pressure via the femoral artery and for recording left ventricular pressure by a pig-tail catheter via the carotid artery. Experimetria HG 01 modem and SPEL Adv Haemosys 2.46 software were used for the analysis of hemodynamic data, such as mean arterial blood pressure, dP/dt_{max} and heart rate. The dP/dt_{max} was calculated from left ventricular systolic pressure during the heart circle and used as a parameter of left ventricular contractile force.

In case of intrapericardial ET-1 administration elecrophysiological analysis was also performed. During ECG recordings the alterations of ST-segments were observed. PQ segment was considered as the isoelectric line. During electrophysiological analysis endocardial and epicardial left ventricular monophasic action potential (MAP) were recorded. The duration of MAP was evaluated at 90% of repolarization ($MAPD_{90}$) and the upstroke velocity (UV) was defined as the quotient of the repolarization amplitude and the repolarization duration of the MAP curve (Figure 9.). MAP parameters were digitalized by a 12-bit analogue-to-digital converter after being amplified and filtered. The analogue signals were recorded on a 12-channel direct chart recorder (Madaus Schwarzer CU12).

4.6 STATISTICAL ANALYSIS

Adenine nucleoside and hypoxanthine levels were corrected for the volume of the enzyme blocker solutions. The statistical analysis was conducted using Wilcoxon signed-rank test, Mann-Whitney-U test for biochemical and Student’s t-test for hemodynamic variables. All data are expressed as mean ± S.E.M. P value <0.05 was considered statistically significant.

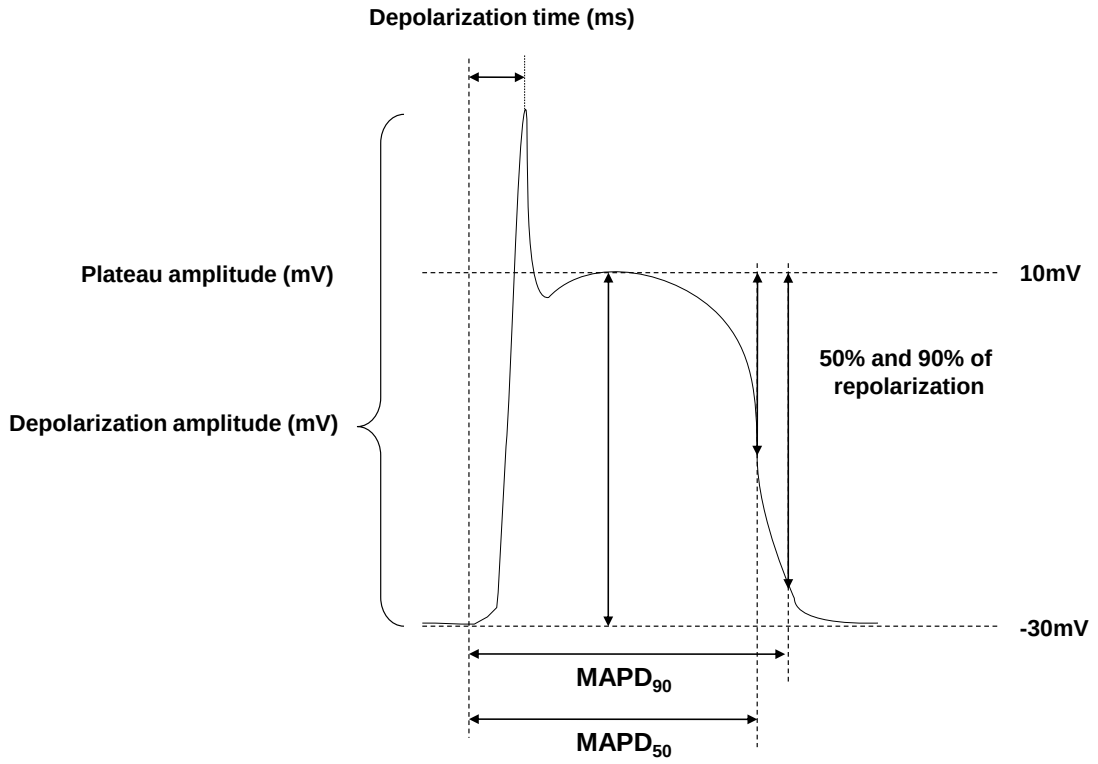

Figure 9. Monophasic action potential of the cardiomyocytes

(MAP: monophasic action potential, $MAPD_{90}$: duration of MAP at 90% of repolarization, $MAPD_{50}$: duration of MAP at 50% of repolarization)

5 RESULTS

5.1 INTRAPERICARDIAL ADMINISTRATION OF ENDOTHELIN-1

The effects of intrapericardial administration of ET-1 were studied in nine experiments using the "closed pericardium model". In additional five experiments we analyzed the electrophysiological actions of ET-1 applied intrapericardially.

Regarding the baseline pericardial levels of adenine nucleosides, we found that the native pericardial fluid contained a higher concentration of adenosine compared to the peripheral arterial blood. The concentration of ADO accumulated in the control sample (C_1) was found lower than in the native pericardial fluid. However, no significant difference was found between pericardial infusate samples C1 and C2 taken consecutively thus confirming the reproducibility of the incubation method (Table 5.).

Intrapericardial administration of ET–1 induced myocardial ischemia with significant ST segment elevations recorded in the surface ECG (ST_{max}: 0.68±0.01 mV, $p<0.001$ at the time point of obtaining sample I), while the overall hemodynamic parameters and the systemic plasma concentrations of the purine metabolites have not changed significantly (Table 6.). The positive inotropic effect of ET–1 was not observed.

The plasma levels of the purine metabolites remained unchanged during the myocardial ET-1 effect however, the concentrations of all the three purine metabolites increased in the pericardial infusate samples. The adenosine concentration showed a 9-fold, the inosine showed a 20-fold elevation following the intrapericardial administration ET–1. The most marked change was measured in the pericardial infusate sample (PI sample II) 30 minutes after ET–1 administration (Figure 10.).

In the electrophysiologically analyzed 5 dogs the left ventricular epicardial $MAPD_{90}$ and the upstroke velocity of MAP decreased significantly at the time point II. vs. control (C2). Endocardial $MAPD_{90}$ and UV did not exhibit significant changes. Considering that ischemia and ET-1 have opposite effects on $MAPD_{90}$ and UV, i. e. the ischemia decreases, while ET-1 increases the values of these variables, an unequivocal ischemic change was not proven in the endomyocardial layer of the heart (Table 7.)

Table 5.: Reproducibility of basal values of the purine metabolites in the pericardial and plasma samples.

	Pericardial samples (μmol/l)		
	Native PF	**C1**	**C2**
Adenosine	3,19±0,53	0,51±0,1*****	0,54±0,1*****
Inosine	1,73±0,70	1,26±0,20	0,87±0,15
Hypoxanthine	7,26±1,81	14,10±3,16	13,43±2,64
	Arterial plasma samples (μmol/l)		
Adenosine	0,04±0,01	0,04±0,01	0,04± 0,02
Inosine	0,19±0,04	0,19±0,06	0,25±0,06
Hypoxanthine	3,55±0,62	4,13±0,94	4,51±0,66

(C1 and C2: control 1 and control 2 pericardial infusate samples; PF: pericardial fluid; C1 and C2 were compared to native PF; Values are means ± S.E.M. * $p<0,05$, n=9)

Table 6: Changes in hemodynamic variables following pericardial administration of endothelin-1

Parameter	Control	Sample I	Sample II	Sample III
MABP (mmHg)	116±1	112±1	114±1	110±1
HR (beats/min)	157±1	155±1	161±1	161±2
dP/dt_{max} (mmHg/s)	2827±65	2680±36	2812±79	2837±69
ST segment (mV)	-0.01±0.00	0.68±0.01*	0.44±0.01*	0.26±0.01*

(MABP: mean arterial blood pressure, HR: heart rate, dP/dt_{max}: left ventricular contractility. Control data are from the C2 sample time point. Sample I, II, III: time points after intrapericardial administration of ET-1 parallel with pericardial and plasma sampling respectively. Values are means ± S.E.M., n=9, *$p<0.05$ vs. control)

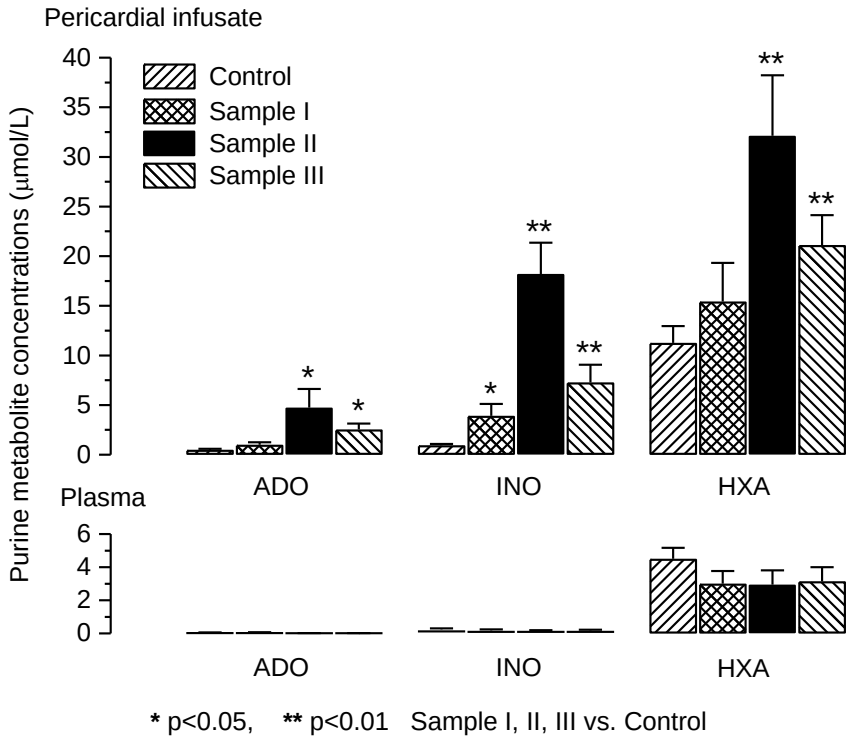

Figure 10. Concentrations of purine metabolites in pericardial infusate and plasma samples following intrapericardial administration of endothelin-1

(Control : purine metabolite concentrations in control 2 (C2). Sample I, II, III: time points after ip. administration of ET-1 parallel with hemodynamic measurements respectively. Values are means ± S.E.M., n=9, *p<0.05, **p<0.01 compared to control. ADO: adenosine, INO: inosine, HXA: hypoxanthine)

Table 7.: Changes in electrophysiological data following pericardial administration of endothelin-1

	MAPD90 (ms)		**Upstroke velocity (V/s)**	
Time point	**Endocardial**	**Epicardial**	**Endocardial**	**Epicardial**
Control	215.7±2.4	214.6±2.9	1.43±0.25	1.76±0.1
Sample I	217.8±3.0	196.5±2.7*	1.44±0.22	1.22±0.08*
Sample II	214.1±3.1	193.0±3.2*	1.66±0.29	0.83±0.06**
Sample III	218.6±1.7	186.0±5.6*	1.45±0.26	1.13±0.08

(Control data are from the C2 sample time point. Sample I, II, III: time points after intrapericardial administration of ET-1 parallel with pericardial and plasma sampling respectively. Values are means ± S.E.M., n=5, *p<0.05, **p<0.01 vs. control)

5.2 INTRAPERICARDIAL ADMINISTRATION OF ADENINE NUCLEOSIDES

In the second series of experiments we aimed to determine the alteration of pericardial concentrations of endothelin-1 after intrapericardial administration of increasing doses of adenosine and inosine. The endothelin-1 concentrations measured in the pericardial infusate fluid and in the plasma under control conditions are shown in Table 8. Very similar basal endothelin-1 concentrations were found in the consecutive pericardial infusate samples (C_1, C_2) in both experimental groups (Group I., II.). Intrapericardial administration of increasing doses of ADO resulted in dose-dependent and significant elevations in pericardial ET-1 concentrations (Figure 11.) and a more than twofold increase of the pericardial ET-1 level was detected following the highest dose of the agent. In the second experimental group when i.p. INO was applied the pericardial ET-1 concentrations continuously increased and these elevations were significant except the lowest dose of INO. During the administration of adenine nucleosides the arterial plasma levels of ET-1 remained unchanged. The i.p. nucleosides elicited only slight but significant alterations in the heart rate and left ventricular contractility (dP/dt_{max}). A weak negative chronotropic effect of ADO was observed at the highest dose of the agent (HR: –4±1 %; $p<0.05$) while the mean arterial pressure and dP/dt_{max} did not change. After i.p. INO administration (100 mM) a moderate positive inotropic effect (dP/dt_{max}: +7±3 %; $p<0.05$) with small elevation of blood pressure was detected without any alteration of the heart rate.

Table 8.: Basal ET-1 concentrations in pericardial infusate and plasma samples

	ET-1 concentrations			
	Group I. ADO i.p. (n=8)		**Group II. INO i.p. (n=8)**	
	Control 1	**Control 2**	**Control 1**	**Control 2**
Pericardial infusate (pg/ml)	23.2 ± 7.3	20.2 ± 7.3	20.4 ± 9.2	20.6 ± 8.7
Arterial plasma (pg/ml)	19.7 ± 3.9	18.6 ± 3.8	16.6 ± 4.5	16.4 ± 4.5

(Data are standardised for 100 g of heart muscle. Values are presented as means ± S.E.M., ET-1 – endothelin-1, ADO – adenosine, INO – inosine, i.p. – intrapericardial)

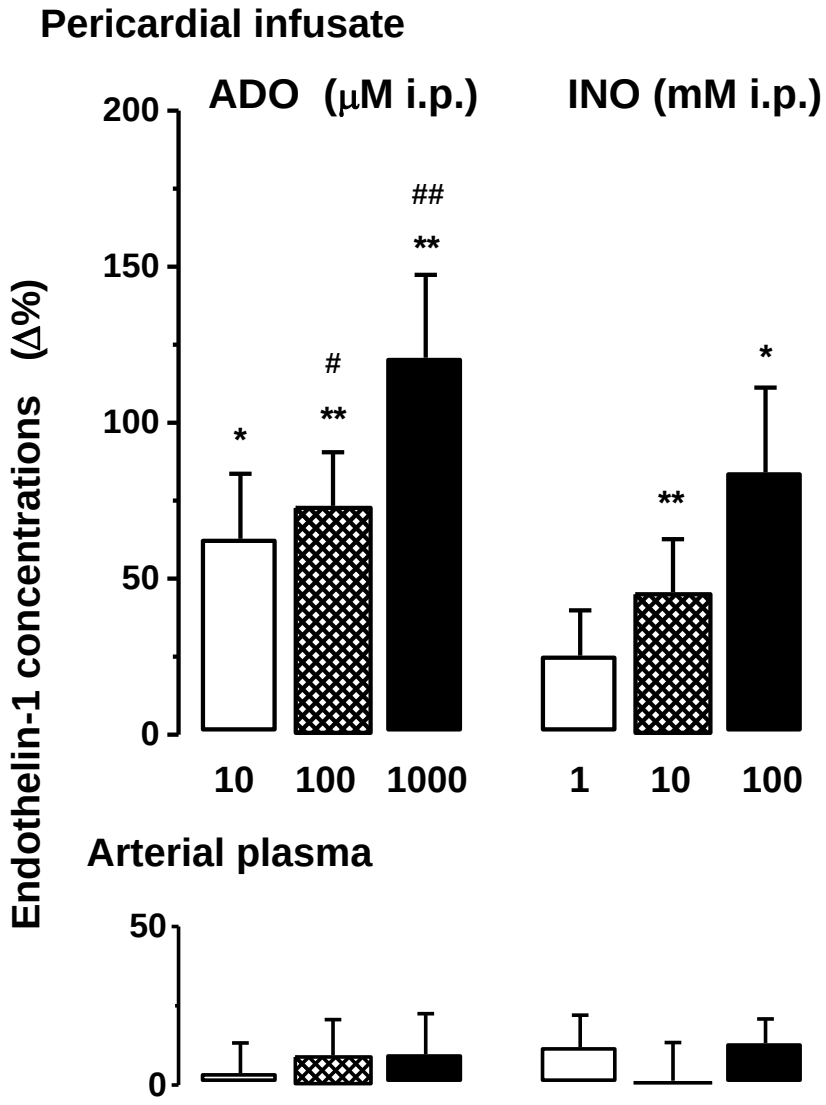

Figure 11.: Endothelin-1 concentrations in pericardial fluid and plasma samples following intrapericardial administration of increasing doses of adenosine and inosine.

(Values are means ± S.E.M., n=8, *$p<0.05$, **$p<0.02$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs plasma. ADO: adenosine, INO: inosine, i.p.: intrapericardially. Control: ET-1 concentrations in control 2 (C2).

5.3 PERICARDIAL ELIMINATION AND CARDIOVASCULAR EFFECTS OF ADENOSINE AND INOSINE

Table 9 summarizes the adenosine, inosine and hypoxanthine concentrations found under physiological conditions in the native pericardial fluid (N) and in the control (C_1 and C_2) pericardial infusate samples obtained by using a repetitive pericardial sampling technique. Concentrations of adenosine and inosine were extremely similar in both control samples with no significant differences, which validated our sampling method. Control adenosine levels were found to be slightly lower, while hypoxanthine concentrations were higher than their levels in the native pericardial fluid indicating a slightly increased breakdown of adenosine in the pericardial infusates. However, concentrations of all the purine derivates proved to be significantly higher in the pericardial than in the respective plasma samples.

Table 9.: Basal concentrations of adenosine, inosine and hypoxanthine in native pericardial fluid, pericardial infusate and arterial plasma samples.

	Concentrations (μM)					
	Baseline		**Control 1**		**Control 2**	
	Native PF	**Plasma**	**Peric. infusate**	**Plasma**	**Peric. infusate**	**Plasma**
ADO	**2.71±0.32****	**0.12±0.03**	**1.38±0.24** #**	**0.09±0.02**	**1.45±0.47****	**0.09±0.02**
INO	**3.34±1.48****	**0.28±0.07**	**3.26±1.12****	**0.18±0.05**	**3.26±1.39****	**0.21±0.07**
HXA	**4.21±0.53****	**1.90±0.20**	**12.43±2.62** ##**	**2.07±0.24**	**10.28±1.38** ##**	**1.92±0.20**

(Values are presented as mean values ± SEM; n=14, ** $p<0.01$ pericardial vs. plasma; # $p<0.05$, ## $p<0.01$ control vs. baseline, , ADO: adenosine, INO: inosine, HXA: hypoxanthine, PF: pericardial fluid, peric.: pericardial)

In order to study the pericardial elimination of adenine nucleosides, increasing doses of adenosine and inosine were applied into the pericardial space. Following intrapericardial administration of ADO pericardial concentrations decreased markedly during the 15-minute incubation (Figure 12/A.). However, at the end of the incubation periods significant fractions (10-19 %) of the i.p. applied ADO remained in the pericardial fluid samples. Thus, the residual ADO levels were much higher than the basal ADO levels measured in the native pericardial fluid. In contrast, in the course of intravenous administration, adenosine was rapidly metabolized within the systemic circulation; no more than 0.1-2 % of the intravenously applied ADO was measured in the 15^{th}-minute blood samples (Figure 12/B.). The time course of ADO elimination from the arterial blood (Figure 13.) shows, that after a few minutes plasma ADO concentration declined exceedingly and returned to the basal level. Similar results were found when increasing doses of INO were administered intrapericardially and intravenously (Figure 14.). In the case of inosine, the degree of elimination was less than that of adenosine and was also significantly slower from the pericardium than from the plasma; 36-45% of the i.p. INO was present in the pericardial space at the end of the incubation periods (Figure 14/A.). However, 2 minutes after intravenous application of INO, plasma concentrations decreased by 95-82% (INO $_{1mM}$ i.v. - 0.05±0.01 mM, INO $_{10mM}$ i.v. 1.8±0.7 mM, $p<0.05$). Only 0.03-1 % of INO was detected in the plasma samples 15 minutes after its intravenous bolus injections (Figure 14/B.).

Intrapericardial application of adenosine resulted in moderate, but characteristic hemodynamic effect, e.g. the negative chronotropic action of the agent. The diagrams on Figure 15/A. show significant bradycardia, a slight decrease of myocardial contractility as secondary anti-adrenergic response and a concomitant small decrease of mean arterial blood pressure occurring within a few minutes after intrapericardial administration of adenosine. These effects, except for the decrease of blood pressure, sustained throughout the incubation period; the negative chronotropic and negative inotropic effects were even significant at the 15th minute. The systemic (i.v.) application of adenosine caused dose-dependent and significant changes in all hemodynamic variables (Figure 15/B.). Within the first minutes, significant bradycardia occurred and the mean arterial pressure decreased remarkably, by 35-85% as a consequence of the peripheral vasodilatory effect of adenosine. Left ventricular

contractility was also significantly reduced (by 55%, at the highest dose of ADO). By the end of the 15-minute examination periods these peak effects gradually faded away. After intrapericardial application of inosine we could only observe a weak, non-significant positive inotropic effect as a response to the highest dose of the agent (Figure 16/A.). However, intravenous inosine evoked marked and significant increase of left ventricular contractility which was then transitionally diminished for a few minutes by the remarkable fall of blood pressure as a consequence of peripheral vasodilatation immediately after the intravenous administration of the highest INO dose (Figure 16/B.).

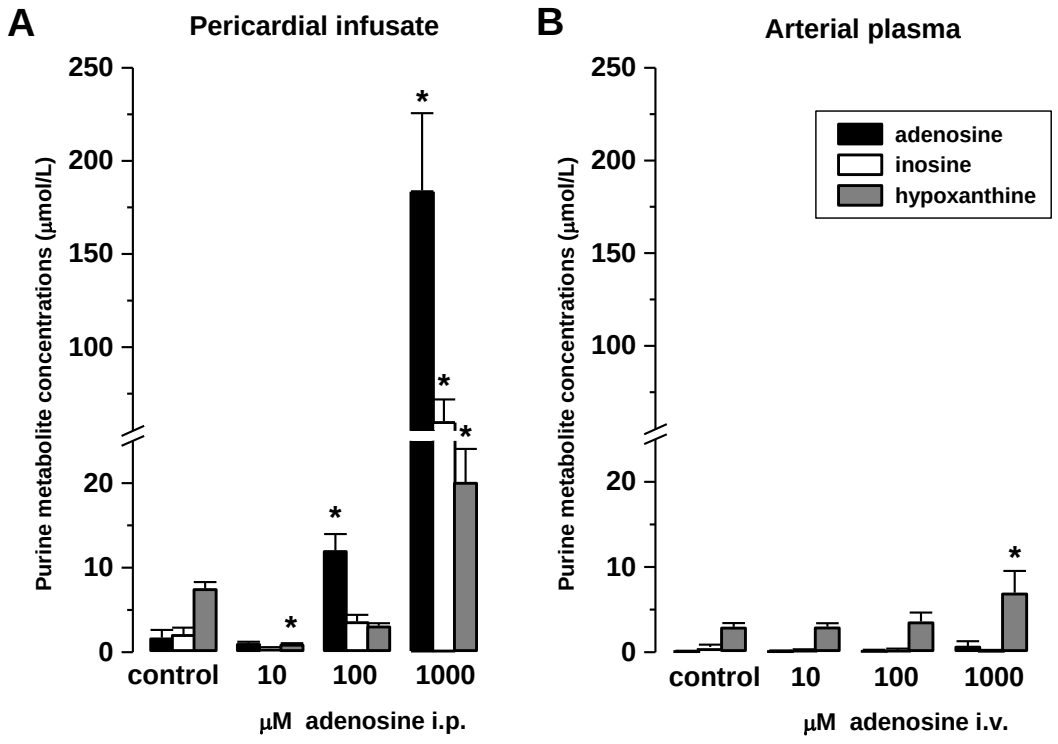

Figure 12.: Purine metabolite concentrations in the pericardial infusate and plasma samples 15 minutes after ip. (A) and iv. (B) administration of increasing doses of adenosine.

(Control: purine metabolite concentrations in control 2 (C2). Values are presented as means±S.E.M., n=7 (A), n=5 (B), *p<0.05 vs. control.)

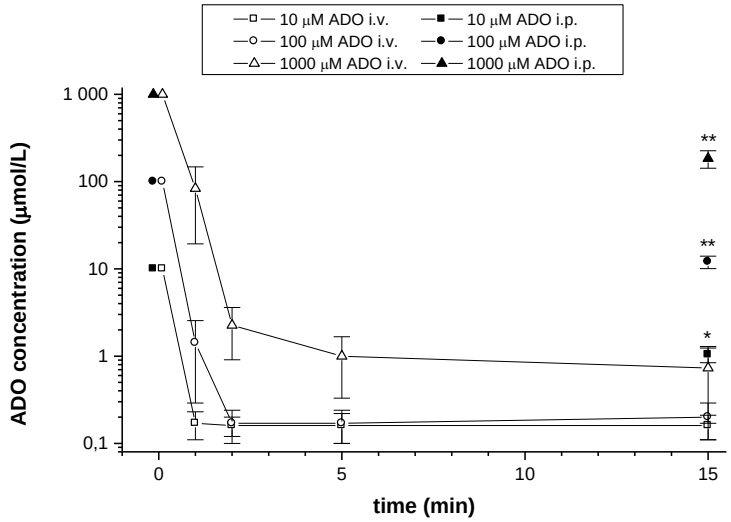

Figure 13.: Time course of plasma adenosine levels after intravenous administration of increasing doses of adenosine (open symbols).

(The pericardial concentrations 15 minutes after intrapericardial application of the three adenosine doses are also presented for reference (solid symbols). ADO: adenosine; i.p.: intrapericardial; i.v.: intravenous. Values are presented as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ pericardial vs. plasma at 15 minutes.)

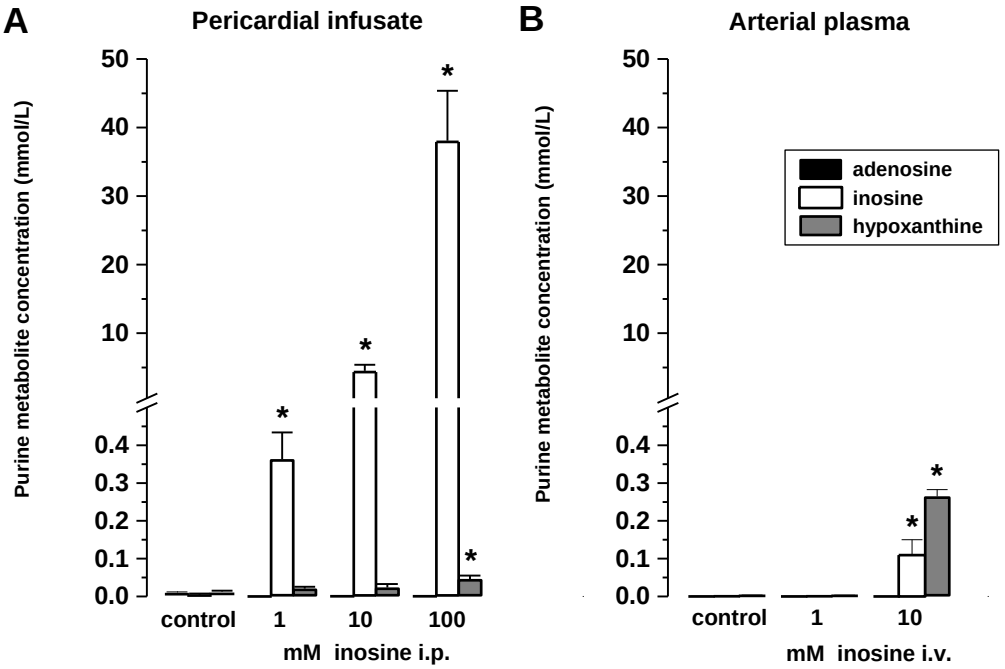

Figure 14.: Purine metabolite concentrations in the pericardial infusate and plasma samples 15 minutes after intrapericardial (A) and intravenous (B) administration of increasing doses of inosine.

(Control: purine metabolite concentrations in control 2 (C2). Values are presented as mean ± SEM, n=7 (i.p.), n=5 (i.v.); * $p<0.05$ vs. control.)

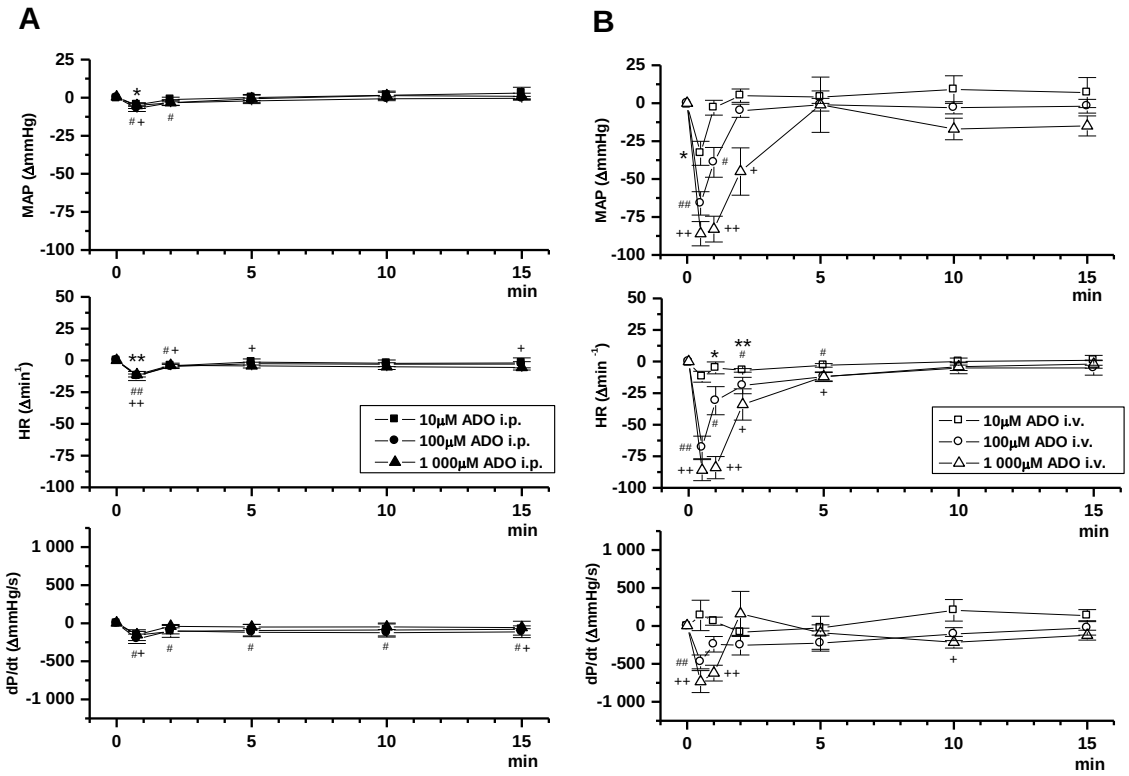

Figure 15.: Time course changes of hemodynamic parameters after intrapericardial and intravenous administration of adenosine.

(A: ip ADO, B: iv.ADO, ADO: adenosine, i.p.: intrapericardial; i.v.: intravenous; MAP, mean arterial blood pressure; HR, heart rate; dP/dt_{max}, left ventricular contractile force. Values are presented as mean ± SEM; n=7 (i.p.); n=5 (i.v.)., 10μM ADO i.p.(A) or i.v.(B): * p<0,05, ** p<0,01 vs. control; 100μM ADO i.p.(A) or i.v.(B): # p<0,05, ## p<0,01 vs. control; 1000 μM ADO i.p.(A) or i.v.(B): + p<0,05, ++ p<0,01 vs. control. Control data are from the C2 sample time point in pericardial experiments.)

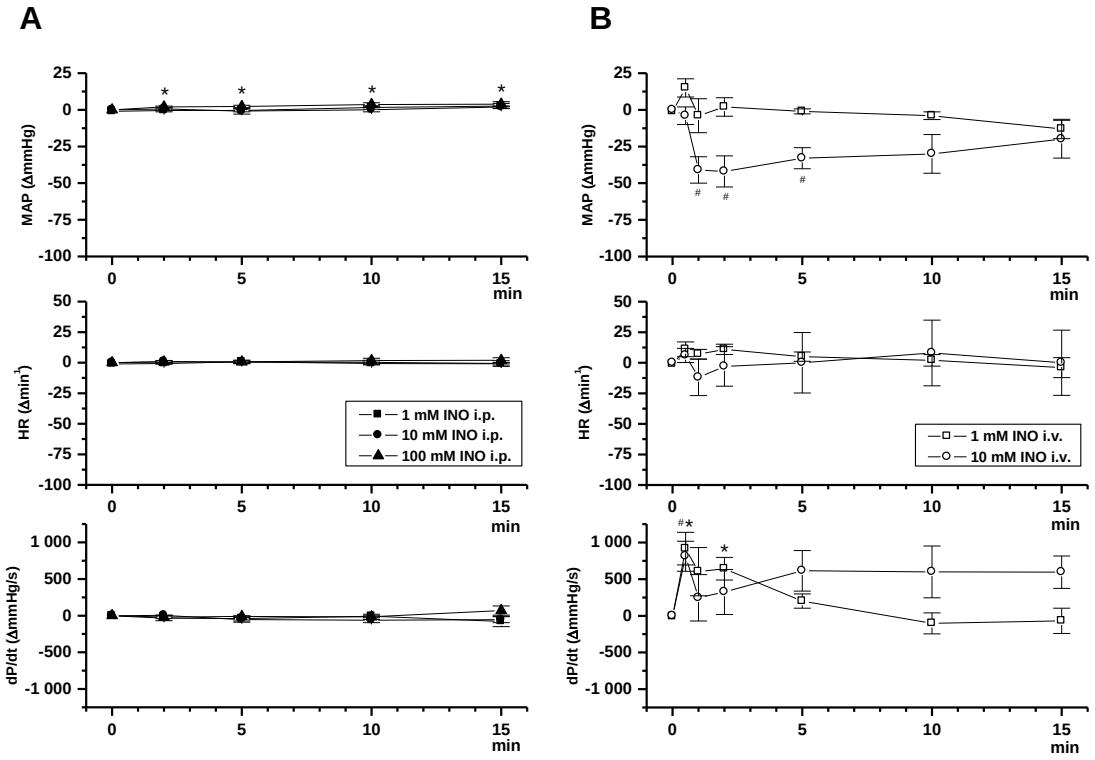

Figure 16.: Time course changes of hemodynamic parameters after intrapericardial and intravenous administration of inosine.

(A: ip.INO, B: iv.INO, INO: inosine, i.p.: intrapericardial; i.v.: intravenous; MAP, mean arterial blood pressure; HR, heart rate; dP/dt_{max}, left ventricular contractile force. Values are presented as mean ± SEM; n=7 (i.p.); n=5 (i.v.)., 100mM INO i.p.(A): * p<0,05, vs. control; 1mM INO i.v.(B): * p<0,05 vs. control; 10mM INO i.v.(B): # p<0,05 vs. control. Control data are from the C2 sample time point in pericardial experiments.)

6 DISCUSSION

Most of the cardio- and vasoactive agents function locally in either an autocrine or paracrine manner in the heart. Their levels in the circulating blood can be technically extremely difficult to detect due to the relatively small range of concentrations presented in the systemic circulation. Moreover, the plasma levels of these paracrine/autocrine agents lack to provide relevant information about the possible ongoing regulatory mechanisms in the myocardium, where their actions have been developed. However, the pericardial fluid, which is a myocardial transudate, contains numerous important cardioactive regulatory agents in such concentrations, which are several magnitudes higher, than their plasma levels. Therefore, our group has turned its attention in the past decades to investigate this exceedingly promising and challenging compartment because of two critical principles: 1. the pericardial fluid has the potential to mirror reflect the levels and alterations of the compounds of myocardial interstitium; 2. cardioactive regulatory agents may act backward towards the myocardium, by which the pericardial space could behave as a potentially active fluid compartment of the heart. Hence, the pericardial space and fluid became a novel inspiring field for experimental and clinical cardiovascular research.

The present studies were performed on *in situ* dog heart after developing a successful technique of cannulation of the pericardial sac *in vivo* and working out a reliable pericardial sampling method in order to investigate the compounds of the pericardial fluid.

From the 1980s a large number of animal models were developed to estimate the local changes of interstitial concentrations of cardiac regulatory agents, in vivo. Many early studies relied on measurements of coronary sinus blood or ventricular biopsy could represent at best a rough qualitative estimation, for instance, of the left ventricular interstitial adenosine levels.

In case of adenosine, the vast majority of total myocardial adenosine is contained within the cells and its extremely short plasma half-time also render more difficult to evaluate its interstitial concentrations. Arterio-venous differences of adenosine are only a rough index of capillary adenosine concentrations. Adenosine release from isolated perfused hearts into venous effluent cannot be an accurate index of interstitial adenosine, as the endothelium is recognized as a highly active metabolic barrier [140, 141].

Cardiac microdialysis [142], epicardial disk [143], epicardial well [144] and pericardial superfusate [145] techniques all aimed to give a better estimation of the interstitial concentrations of local regulatory agents, such as adenine nucleosides (Table 10.). Even one magnitude higher adenosine concentrations were evaluated by all of these aforementioned techniques, than it could be measured in coronary sinus venous blood.

Each of these methods has advantages, disadvantages and limitations. Pericardial superfusion might underestimate interstitial adenosine levels because of the great dilution, while cardiac microdialysis probe could only be inserted in one certain region of the whole heart. Previous studies have confirmed that investigation of pericardial infusate fluid samples acquired by complete removal show the steady-state pericardial concentrations of the investigated agents more accurately than fractional sampling. The reliability of pericardial infusate technique is supported by the evidence that ADO accumulates in the pericardial fluid in an exponential fashion which may approach equilibrium with interstitial fluid ADO concentration [146]. Adenosine can penetrate by simple diffusion through the visceral pericardium into the pericardial fluid, so the interstitial adenosine concentration is the main factor that determines the amount of ADO accumulating over a fixed period of time. In different experimental conditions, among different animal models the steady state concentration of ADO varied within 4.5 to 30 minutes [37, 145]. It is likely that between the interstitial and pericardial fluid compartments the time which is needed for an agent to reach the total equilibrium may be too long for experimental investigations. However, the “time to near-equilibrium” could be constant under standardized experimental protocol.

The closed pericardium model using pericardial infusate technique with standard incubation time may be a reliable method for repetitive sampling. The “closed pericardium model” has been successfully established in our research laboratory in order to investigate the interstitial concentrations of endogenous regulators, such as adenine nucleosides, endothelin-1, natriuretic peptides, angiotensin II and catecholamines [27, 147-150].

In the “closed pericardium model” the pericardium is cannulated in a water-tight way via a small incision and the original pericardial fluid is replaced by standard volume of pericardial infusate. This fluid serves two purposes: can freely contact the entire surface

of the heart without hampering the cardiac contractility and filling, and provides vehicle for the released agents from the myocardium and the intrapericardially administered agents can be taken up by the heart.

Table 10.

Summary of different methods evaluating the concentration of adenine nucleosides

Adenosine concentrations	Inosine concentrations	Methods	References
0.57 –1.07 μM	0,65 -0,8 μM	Cardiac microdialysis	[142, 151, 152]
0.4 – 2.0 μM	0.80 μM	Pericardial infusate	[153-155]
0.25 - 0.55μM	-	Epicardial transudate	[156-158]
0.03 – 0.08 μM		Intravascular (sinus coronarius blood)	[159-161]

In the present study, in the beginning of every experimental setup, we tested the reliability of the repetitive pericardial sampling technique of the "closed pericardium model".

Regarding the baseline pericardial levels of adenine nucleosides, we found that the concentrations of ADO accumulated in the control infusate samples were lower than ADO levels of the native pericardial fluid. The duration of incubation period is well-proportioned with the amount of ADO accumulated in the pericardial samples and in case of the native pericardial fluid the equilibration period lasted for years. The slightly lower ADO levels of the pericardial infusate samples may be the consequence of the limited time of incubation.

In an experimental model of regional myocardial ischemia the time course of changes of dialysate ADO concentration – attained using cardiac microdialysis technique – was increased by 15 minutes of ischemia and decreased towards pre-ischemic levels by 45 minutes of ischemia [151]. Inosine and hypoxanthine accumulated in the dialysate samples after 20 minutes of ischemia.

In our experiments, no significant difference was found between the control pericardial infusate samples taken consecutively, which validated the reproducibility of our sampling method.

Considering the difference between plasma and pericardial levels of adenine nucleosides our data also confirm that under physiological conditions, the concentrations of these endogenous regulators are several magnitudes higher in the pericardial fluid than in the peripheral venous blood.

Previous studies elegantly demonstrated that certain regulatory agents applied into the pericardial space may exert characteristic cardiovascular effects [23, 29, 162]. Formerly we discovered that intrapericardially administered low doses of dopamine induced immediate increase of heart rate, while inotropic response could not develop during the effect of dopamine, indicating definite penetration of the atrial wall by the agent [27]. Other investigators found that beta-blocking agents, sotalol and atenolol infused intrapericardially exerted more pronounced antitachycardiac, negative chrono-, and inotropic effects compared to intravenous delivery in rats [18]. Another experimental study demonstrated that esmolol applied into the pericardial space caused prolonged antitachycardiac effect without affecting left ventricular contractility and blood pressure compared to intravenous application [19].

In the present study we focused on one of the most potent vasoconstrictors, ET-1. We hypothesized that ET-1 applied into the pericardial space can penetrate through the epicardium towards the myocardial interstitium and can cause myocardial ischemia by its coronary vasoconstrictor effect. While a detectable ischemia, significant ST-segment elevation was recorded in the surface ECG, hemodynamic parameters were preserved after intrapericardial administration of ET-1. During electrophysiological analysis, significant shortening of the epicardial but not the endocardial MAP duration was observed. It means that ET-1 did not cause transmural ischemia, which was demonstrated by the different alterations of the endocardial and epicardial MAP tracings. Considering the transmural penetration of these agents, in case of the intrapericardially administered dopamine, characteristic tachycardiac effect indicated its definite penetration of the atrial wall [27].

Our laboratory has previously found that intrapericardial infusion of ET-1 induced ventricular arrhythmias associated with prolongation of QT time and MAP duration. This finding revealed the direct arrhythmogenic effect of ET-1 that could be inhibited by ET-A receptor blockade [26, 113]. On the other hand, acute myocardial ischemia itself can shorten MAP duration and may be dependent mainly on the activation of

K(ATP) channels [163]. In this study the ip. ET-1 showed no arrhythmogenic effect in the dosage of 150 pmol/kg and in the development of the shortening of MAP duration the ischemic effect of the vasoconstrictor ET-1 may be mainly involved. Since ET-1 was discovered and its numerous cardiovascular effects have been demonstrated one after the other, several *in vitro* studies have been performed in various species with conflicting results regarding the inotropic effect of ET-1. A few years ago a possible *in vivo* positive inotropic and negative lusitropic actions of ET-1 were published [112]. In anesthetized pigs, intracoronary infusion of ET-1 increased myocardial contractility through a possible ET-A receptor dependent mechanism, while intravenous administration of ET-1 failed to increase the cardiac contractility. In our study, positive inotropic effect of intrapericardial ET-1 could not have been demonstrated. During intrapericardial administration of adenine nucleosides, we have found moderate, but characteristic cardiac effects. I.p. ADO elicited only slight but significant negative chorontropic effect and i.p. INO caused moderate increase of dP/dt_{max}.

Apart from the cardiovascular effects evoked from the pericardial space, multiple interactions among ET-1, adenine nucleosides and other endogenous regulatory agents may be more important in the modulation of cardiac function. We have previously demonstrated that ET-1 applied intracoronarially caused significant elevations in the concentrations of pericardial but not plasma purine metabolites, suggesting a local effect of ET-1 on myocardial production and release of adenine nucleosides [14, 19]. Furthermore, the pericardial concentration of the vasoconstrictor ET-1 and the vasodilator ADO increased in response to intrapericardial administration of catecholamines (dopamine and norepinephrine) [27].

In the present study we investigated the interaction of ADO and ET-1 and aimed to validate the concept that the changes of their interstitial concentrations would be reflected by the composition of the pericardial infusate samples. First, we hypothesized that ET-1 applied into the pericardial space can cause myocardial ischemia and can activate the coronary metabolic adaptation of the heart. In the *in situ* dog heart, using the ‘closed pericardium model’ we have demonstrated that increased intrapericardial concentrations of ET-1 elicited significant elevations of the pericardial purine metabolite levels. The ET-1 induced myocardial release of adenine nucleosides was revealed by the significant and continuous increase of purine metabolite levels in

pericardial infusate sample I and II. At the time point of sample III also significantly elevated, but lower pericardial purine metabolite levels were observed, than in Sample II, suggesting slow elimination of the ET-1 action. However, during intrapericardial administration of ET-1 the plasma concentrations of purine metabolites were not changed, which also confirm the local effect of ET-1 on myocardial production and release of adenine nucleosides. The rapid local appearance of the cardioprotective endogenous regulatory products in the pericardial fluid indicates that the entire pericardial space may serve as the milieu of the cardiac self-defence mechanisms against myocardial ischemia.

In the second series of experiments we investigated further the adenosine-endothelin-1 interaction from the opposite way. We aimed to determine the alteration of pericardial concentrations of endothelin-1 after intrapericardial administration of increasing doses of adenosine and inosine. We demonstrated for the first time that i.p. adenosine and inosine stimulated the myocardial release of endothelin-1, which resulted in significant elevations of pericardial concentrations of ET-1. During the intrapericardial administration of adenine nucleosides the arterial plasma levels of ET-1 remained unchanged. The only data elucidating such an interaction between adenosine and endothelin-1 have been obtained on rat thyroid cells [164]. Adenosine-induced activation of endothelin-1 secretion on the FRTL-5 cells was mediated by A_1 purinergic receptors. The marked elevations of pericardial endothelin-1 concentrations without alterations of the plasma endothelin-1 levels and without any considerable changes in the systemic hemodynamic variables indicate the local action of the pericardial adenine nucleosides on the endothelin-1 release from the *in situ* dog heart.

Since ET-1 also provokes adenine nucleoside release from the heart muscle, the present results indicate that this interaction is bi-directional, which may reveal a novel type of endothelin-1 function in adenosine - endothelin-1 interaction. All these interactions demonstrated in the present study could be provoked by and detected in the pericardial space, reflecting probably parallel changes in the interstitial fluid compartment of the heart.

Adenosine and its metabolite inosine play a crucial role in the regulation of vascular reactivity, metabolic adaptation, inflammation, monocyte/macrophage/lymphocyte function, ischemic preconditioning and angiogenesis (for references see [165]).

Furthermore, numerous investigators have provided compelling evidence demonstrating protective actions of adenine nucleosides on the ischemic heart [34, 166]. Myocardial protection of ADO and INO is based on multiple underlying protective mechanisms including nucleotide repletion and the decrease of oxidative stress, Ca^{++} overload, platelet and neutrophil activation, glycolytic flux and apoptosis mediated by a broad range of intracellular signaling pathways (for references see [36, 45, 167]). However, the use of adenine nucleosides is sturdily restricted by their extremely rapid metabolism and elimination by cardiomyocytes, endothelial cells, red blood cells and monocytes upon intravenous application. Adenosine has a plasma half-life of a few seconds and inosine has only 1-2 minutes [168]. During the intravenous administration of adenosine, systemic circulation can be adversely affected and disadvantageous hemodynamic effects can arise. Thus far, the pericardial elimination rate of ADO and INO remained elusive. Regarding the significantly higher pericardial adenine nucleoside concentrations compared to the plasma, a substantially lower pericardial elimination rate of adenosine and inosine could be hypothesized.

The third series of experiments aimed to investigate the pericardial elimination of intrapericardially applied adenosine and inosine and to observe their effects on the physiological cardiovascular function in the *in situ* dog heart compared with their intravenous administration. The current data verified a significantly slower elimination rate of ADO and INO from the pericardial fluid, supporting the possibility of their potential therapeutic usage for cardioprotection. Since significant amounts of ADO and INO were measured in the pericardium 15 minutes after their intrapericardial application, a new dynamic metabolic characteristic of the adenine nucleosides within the pericardial environment could be suggested; reuptake and/or metabolic processes may be reduced in the pericardial fluid. These findings are in concert with the results of recent experimental study indicating low turnover rate of atrial natriuretic peptide in the pericardial space [8].

In the current study, only weak cardiovascular effects were observed during intrapericardial administration of adenine nucleosides. One possible explanation to this finding is that no transmural penetration through the left ventricular wall occurred by the agents' intrapericardial administration. From a different perspective, balanced cardiac actions and the lack of drastic systemic hemodynamic effects measured after

intrapericardial application of ADO and INO may be beneficial and suggest a promising therapeutic use of intrapericardial adenine nucleosides.

The cardioprotective feature of adenosine and inosine affecting predominantly the ischemically damaged myocardium, are widely accepted and well demonstrated. The question arises, whether locally elevated levels of adenine nucleosides can influence myocardial function and whether cardioprotective effects of nucleosides can be provoked from the pericardial space? Our most current data suggest that the residual pericardial concentrations of ADO and INO measured at the end of the incubation periods are at the same order of magnitude or interestingly, even higher than their concentrations under severe myocardial ischemia [147]. The low pericardial turnover rate offers the opportunity of pericardial administration of adenine nucleosides for local pharmaco-therapeutic interventions.

Several investigations also suggest that pericardial delivery of different endogenous or exogenous agents can influence myocardial function in certain cardiovascular pathologies. It has been demonstrated that intrapericardial administration of the NO donor nitroglycerin suppressed ischemia-induced cardiac electrical instability and exerted a potent antifibrillatory effect during acute myocardial ischemia in the porcine heart [20]. In a recent study, pericardial infusion of docosahexaenoic acid (DHA), one of the main omega 3 (n-3) polyunsaturated fatty acids (PUFA), significantly reduced infarct size and associated malignant arrhythmias (including ventricular fibrillation) in an ischemia-reperfusion porcine model [21].

The therapeutic myocardial angiogenesis – a novel approach to the treatment of myocardial ischemia – has been studied, using a single intrapericardial injection of basic fibroblast growth factor (FGF-2) in a model of chronic myocardial ischemia. Intrapericardial FGF-2 resulted in a significant increase in angiographic collaterals and myocardial perfusion in the ischemic region supplied by the experimentally occluded coronary artery [28]. It has also been demonstrated that intrapericardial administration of different gene carriers could be a promising approach to target the myocardium for gene delivery as an alternative therapy for the failing heart [30]. The aforementioned experimental pericardial approaches all aim to develop a beneficial, regional (free of systemic effect) and effective therapeutic option to manage and ultimately cure cardiovascular disorders.

Intrapericardial delivery of pharmacologic agents represents a promising novel route of administration in a variety of heart diseases. The possible benefits include reduced systemic absorption and prolonged exposure of the myocardium to the therapeutic material.

Until very recently, the delivery of pharmacologic agents into the pericardial space has been limited by the inability to access this distinct compartment without invasive thoracotomy. Surgical pericardial window and needle pericardiocentesis are usually performed only in the settings of significant pericardial effusion. However, there are some new, percutaneous approaches using a catheter system for transatrial [169, 170] or subxyphoid access [171, 172] to the pericardial space, which advocate a new possibility for both diagnostic purpose and pharmaco-therapeutic usage. Transatrial delivery of nitroglycerine into the pericardial space in five Yorkshire pigs has been recently reported and sustained coronary vasodilatation could be achieved [22]. In another animal study digoxin and procainamide was administered intrapericardially using a pericardial access device and electrophysiological properties, like reduction of QTc interval were observed [24]. The percutaneous approach via the right atrial appendage provided a rapid, safe route to access the normal pericardial space for diagnostic sampling in large animal model [170]. In 53 porcine experiments the efficacy and safety of the percutaneous, subxyphoid technique has also been demonstrated even in a 1-month follow-up [171]. The possible adverse effects and safety of these different access routes need to be explored further to provide the best possible technique and approach for both diagnostic and therapeutic procedures.

7 CONCLUSION

In conclusion,

- Intrapericardially applied ET-1 induced subepicardial myocardial ischemia and markedly enhanced myocardial release of adenine nucleosides due to the activation of coronary metabolic adaptive mechanisms in the *in situ* dog heart.
- We demonstrated for the first time that intrapericardial administration of adenine nucleosides stimulated the myocardial release of ET-1 under physiological condition of the *in situ* dog heart.
- The closed pericardium model and our pericardial sampling method were suitable for characterizing the alteration of pericardial concentrations of adenine nucleosides and endothelin-1.
- These findings support the hypothesis that the pericardial fluid may have the potential to mirror reflect the compounds of myocardial interstitium and allow a new possibility to investigate the local interaction of ADO and ET-1.
- Our results confirm significantly slower elimination of ADO and INO in the pericardial fluid than in the systemic circulation.
- Considering the balanced cardiac actions and the lack of adverse systemic hemodynamic effects, the intrapericardial administration of adenine nucleosides may suggest a promising approach in the local treatment of the diseased heart.

8 ÖSSZEFOGLALÓ

A jelen tanulmány a perikardiális folyadéktér felől kiváltható farmakológiai hatások vizsgálatával foglalkozik, az adenozin és az endotelin-1 regulátor-pár perikardiális hatásainak jellemzése révén. Számos, szív eredetű, többségében parakrin/autokrin úton érvényesülő szabályozó ágens van jelen a perikardiális térben, olykor a plazmaszinteket többszörösen meghaladó töménységben, sőt különböző kardiális kórállapotokban szintjeik tovább emelkednek. Mindez arra engedett következtetni, hogy a szív interstícium koncentrációs viszonyai hűen tükröződnek a perikardiális folyadéktérben. A kardioaktív regulátor ágensek magas perikardiális koncentrációja felvetette továbbá azok szívműködésre való visszahatásának lehetőségét, vagyis hogy a perikardiális tér nem csupán „követő kompartmentje" a szív interstíciumának, hanem valóságos hatótérként is működhet. Vizsgálatainkban arra kerestük a választ, hogy az endotelin-1 (ET-1) és az adenin nukleozidok az intraperikardiális térbe juttatva hogyan hatnak a szívműködésre, és befolyásolják-e egymás felszabadulását. Mindezekkel összefüggésben tanulmányoztuk az adenin nukleozidok perikardiális térben, valamint a szisztémás keringésben végbemenő eliminációját és kardiovaszkuláris hatásaik karakterisztikáját. *In situ* kutyaszíven, az általunk korábban kidolgozott zárt perikardiális inkubációs módszert alkalmaztuk és egyértelműen igazoltuk a perikardiális tér felől az adenin nukleozidok miokardiális koncentrációjának gyors és jelentős mértékű növekedését ET-1 hatására. A perikardiális adenin nukleozid – ET-1 kölcsönhatásban elsőként sikerült bizonyítani az intraperikardiális tér felől az adenin nukleozidok ET-1 felszabadulást elősegítő hatását. Kimutattuk, hogy az adenin nukleozidok eliminációja szignifikánsan lassabban megy végbe az intraperikardiális térben, mint a szisztémás keringésben. Ezáltal a patológiás szintekkel összevethető, vagy azokat akár meg is haladó adenin nukleozid koncentrációk hozhatók létre a perikardiális térben anélkül, hogy az intravénás alkalmazáskor tapasztalt mellékhatásokkal számolni kellene. Összegzésként megállapíthatjuk, hogy a jelen dolgozatban ismertetett kísérleti adatok egyértelműen bizonyítják a perikardiális tér felől kiváltható és ugyanott detektálható hatásokat: az ET-1 és adenin nukleozidok kölcsönös pozitív egymásra hatását a szív interstíciális terével folyamatos kapcsolatban álló intraperikardiális folyadéktéren keresztül. Az adenin nukleozidok lassabb perikardiális eliminációja megteremti az elvi lehetőséget ezen ágensek sokrétű jótékony hatásainak kiaknázására és egyúttal felveti a perikardium felőli potenciális farmakoterápiás beavatkozások lehetőségét.

9 SUMMARY

The present study aimed to investigate the adenosine - endothelin-1 regulatory couple and their cardiovascular and pharmacological effects evoked from the pericardial space. The pericardial fluid contains numerous important cardioactive regulatory agents that function in an autocrine or paracrine manner in the heart and their levels were found to be several magnitude higher in the pericardial fluid than in the venous plasma. Moreover, in certain cardiovascular pathologies their pericardial levels could increase further. Therefore the pericardial fluid may reflect the levels and alterations of the compounds of myocardial interstitium. On the basis of the high pericardial concentrations of these cardioactive regulators the question arose, whether these agents may act backward towards the myocardium, by which the pericardial space could behave as a potentially active fluid compartment of the heart. The aim of the present study was to investigate the interaction and cardiovascular effects of adenine nucleosides and endothelin-1 (ET-1) evoked from the pericardial space, determine the pericardial elimination of intrapericardially applied adenosine and inosine and observe their effects on the cardiovascular function compared with their intravenous administration. The present studies were performed on *in situ* dog heart using the "closed pericardium model" worked out previously in our research laboratory. Our present data have demonstrated the significant increase of the concentrations of adenine nucleosides after intrapericardial administration of ET-1. Regarding the adenine nucleosides - ET-1 interaction, we have demonstrated ET-1 liberation provoked from the pericardial space after intrapericardial administration of adenosine and inosine. The present study confirmed the significantly slower elimination of adenine nucleosides in the pericardial space than in the systemic circulation. Thus, the same order of magnitude or even higher adenine nucleoside levels could be achieved in the pericardial space with local administration than the endogenous concentrations measured under severe myocardial ischemia without adverse hemodynamic effects. In conclusion, the present study confirmed that characteristic cardiovascular effect and bi-directional interaction of adenine nucleosides and ET-1 could be provoked by and detected in the pericardial space, reflecting probably parallel changes in the interstitial fluid compartment of the heart. The low pericardial turnover rate of adenine nucleosides offers the opportunity of utilizing their multiple beneficial effects for local pharmaco-therapeutic interventions.

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