

Natriuretic Peptides as Therapy in Cardiac Ischaemia/Reperfusion

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THE TWO ORIGINAL RESEARCH PAPERS ARE

1. Natriuretic Peptide Infusion Reduces Myocardial Injury During Acute Ischemia/Reperfusion. Manuscript submitted.
2. B-type Natriuretic Peptide Increases Cardiac Triglyceride Accumulation after Myocardial Ischemia in Pigs. Manuscript submitted.

INTRODUCTION

Despite extraordinary advances in both preventive and clinical medicine acute myocardial infarction (AMI) is a leading cause of mortality in modern societies (1-3). On top of this, a vast majority of the surviving patients are still at risk of developing chronic heart failure which apart from personal consequences for the patient also poses an additional burden to society. Interestingly, from being an illness often seen in developed countries, the disease is now common in developing countries as well (4).

The most common cause of AMI, accounting for 70 % of fatal events, is coronary artery occlusion from atherosclerotic ruptured or eroded plaques (5). The underlying pathology is characterized

by a chronic inflammatory process of the arterial wall. Atherosclerotic plaques result from a complex cellular interaction in the lamina intima of the arteries where smooth muscle cells and endothelial cells in the vessel wall in concert with cells of the immune system, local flow disturbances, and lipids form atheromas. Apart from occlusive atherosclerosis other causes of AMI include coronary spasm, emboli or dissection of the coronary artery. Several risk factors are associated with AMI such as hyperlipidemia, hypertension, diabetes, insulin resistance, atherosclerotic disease, and also age (6). Importantly, once AMI develops, the infarct size per se is the primary determinant of post-infarct remodelling, and thereby morbidity and mortality (7). The application of coronary thrombolysis or immediate percutaneous coronary intervention (PCI) to reperfuse the ischaemic myocardium has dramatically improved the outcomes of patients presenting with AMI. Nevertheless, reperfusing the myocardium can lead to irreversible tissue damage and thus increase mortality and morbidity. This type of myocardial injury may partly explain why mortality after AMI approaches 10 % and morbidity (development of heart failure) is close to 25 % (8). Hence, focus remains on improving the beneficial effects of reperfusion and limiting the irreversible myocardial injury (9). In the light of this, natriuretic peptides are pursued as adjunctive therapy in AMI (10).

THE NATRIURETIC PEPTIDE FAMILY

The discovery of natriuretic peptides as a hormonal entity was first reported in 1981. The Canadian physiologist Adolfo de Bold and his colleagues injected atrial tissue extracts into rats and registered a rapid and potent natriuretic response (11). Later that year, the same authors identified atrial natriuretic peptide (ANP) (known then as atrial natriuretic factor) as the causal peptide hormone (12). Two related natriuretic peptides, B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), were later purified and sequenced from porcine brain tissue by Sudoh and

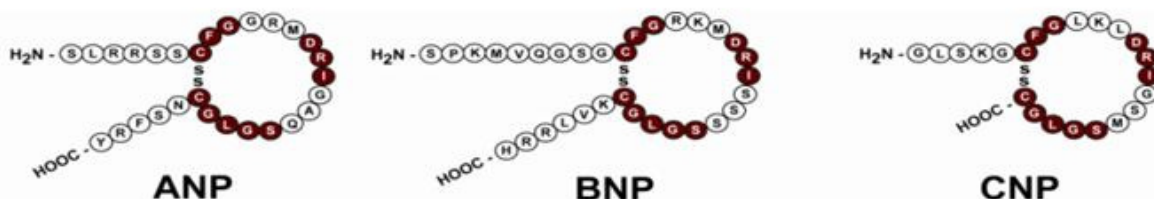


Figure 1

The natriuretic peptide family ANP, BNP, and CNP. Note that the three members contain the conserved ring-sequence CFGXXXDRIXXSGLGC where X represents a non-conserved amino acid residue. (Modified with permission from J.P. Goetze).

colleagues in 1988 and 1990 (13-16). Although first identified in brain tissue, BNP was soon shown to be expressed mainly in the myocardium (16,17).

Other related peptides include human urodilatin which is a kidney-specific form of ANP and DNP from the venom of the green mamba (18,19). Furthermore, uroguanylin and guanylin expressed in the intestinal epithelium are structurally related (20). Here, however, the term natriuretic peptide family will only refer to ANP, BNP and CNP.

Primary structure

Each peptide comprises a ring structure of 17 aminoacids (aa) linked by a cysteine disulphide bridge. The ring structure is essential for receptor binding and bioactivity (figure 1). In contrast to the homology between the bioactive peptide regions, the prohormonal structures differ greatly, both within and across species. The impact of this is not resolved, but major differences in peptide maturation and bioactivation must be expected, which in turn will have an impact on peptide physiology.

Localisation, secretion and synthesis

The genes encoding human BNP and ANP are closely localised on chromosome 1 whereas the gene encoding CNP is located on chromosome 2 (21,22). All three peptides are synthesised as prepropeptides and biochemically modified during translation and post-translational processing. In humans and pigs, the biologically active BNP in the circulation comprises a 32 amino acid residue hormone, whereas BNP represents a 45-residue hormone in mice and rats (23-25).

BNP expression is most abundant in the atrial chambers of the normal porcine heart (26). However, in cardiovascular disease such as acute myocardial ischemia, the gene expression is up-regulated in the cardiac ventricles, where peptide products are released constitutively in response to cardiac stretch and hypoxia (27-29). In contrast, ANP is normally stored as proANP in cellular granules in atrial cardiomyocytes and rapidly released to the circulation in response to myocardial stress.

Unlike the gene expression of cardiac ANP and BNP, CNP is primarily expressed in the vascular endothelium. Moreover, CNP is diffusely expressed in other tissues such as nervous tissue, bone cells, and the male reproductive glands (30). CNP is involved in the local regulation of vascular tone, acting as a potent vasodilator and does not possess significant natriuretic and diuretic effects. CNP is released by stimulation of growth factors and endothelial sheer stress (31,32). In healthy humans the CNP plasma concentration is very low compared with ANP and BNP levels. However, it is released in minor concentrations in patients with cardiovascular disease, which suggests that CNP acts in a paracrine manner as an important modulator of extracellular matrix, antiproliferation, antifibrosis, and collagen suppression (22).

In the circulation, BNP has a half-life of ~20 minutes in contrast to ANP and CNP which have even shorter half-lives (2 minutes). In heart failure patients, BNP concentrations increase 200- to 300-fold whereas ANP concentrations may be elevated 10- to 100-fold (33-35). For both ANP and BNP, normal plasma concentrations lie between 0-10 pmol/L, depending on physical exercise, salt intake, gender, body mass index, and age. Hence, several biological factors influence natriuretic peptide expression and secretion apart from cardiovascular disease.

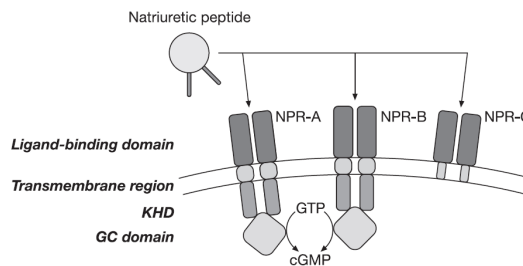


Figure 2

Structure of natriuretic peptide receptors. Ligand-binding catalyses conversion of GTP to cGMP which leads to activation through protein kinases, ion channels, and phosphodiesterases. NPR-A: natriuretic peptide receptor type A, NPR-B: natriuretic peptide receptor type B, NPR-C: natriuretic peptide receptor type C, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate.

NATRIURETIC PEPTIDE RECEPTORS

Natriuretic peptides act via the particulate guanylyl cyclase receptors NPR-A, NPR-B, and NPR-C (figure 2). All three receptors consist of an extracellular binding domain and a single membrane spanning region whereas only NPR-A and NPR-B contain an intracellular domain consisting of a kinase homology domain, a dimerization domain, and a carboxy-terminal guanylyl cyclase domain. Homodimerization is required for activation of NPR-A and NPR-B (36,37). The ligand-binding catalyse conversion of intracellular guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The increase in cGMP mediates intracellular and physiological responses through interaction with cGMP-dependent PKG (38), cGMP-gated ion channels (39), and cGMP-regulated cyclic nucleotide phosphodiesterases (40). NPR-C lacks guanylyl cyclase activity and contains a smaller intracellular domain of only 37 aa. It acts mainly as clearance peptide via receptor-mediated internalisation and degradation, although a few reports have also described signalling functions (41,42). The receptors bind the ligands with different affinities. NPR-A has greater affinity for ANP \geq BNP \gg CNP, whereas the affinity for NPR-B is CNP $>$ ANP \geq BNP (43). NPR-C binds all 3 natriuretic peptides with almost similar affinity (44). The receptors are expressed in diverse tissues such as heart, kidney, lung, adrenal, bone, and, adipose tissue (45,46). This highlights the diversity of natriuretic peptide physiology. Interestingly, the cardiac natriuretic peptide receptor expression even seems to be chamber-dependent (26,47).

So far, a specific blocker of NPR-A, -B or C, has not been reported. HS-142-1, a polysaccharide derivative from the *Aureobasidium* sp. Culture, blocks both ANP and CNP, which indicates a blockage of both NPR-A and -B (48). Assessment of the precise receptor-ligand relation is therefore complicated. Furthermore, blockage of natriuretic peptide receptors in one species could differ compared with the situation in another species.

SYNTHETIC NATRIURETIC PEPTIDES

Synthetic peptides represent a new generation of natriuretic peptide therapeutics. In this respect, there are two types of peptides: alternatively spliced peptides and designer or chimeric peptides. Alternatively spliced peptides are multiple protein

isoforms, whereas chimeric natriuretic peptides are hybrid structures engineered from native natriuretic peptides through addition, deletion, or substitution of particular amino acid residues (49-52). A number of chimeric natriuretic peptides are currently being investigated and in the next section the chimer CD-NP will be described.

CD-NP

CD-NP is a CNP analogue consisting of human CNP-22 extended by the 15-residue COOH-terminus of dendroaspis natriuretic peptide (DNP) (figure 3) (53). DNP itself was isolated from green mamba snake venom (19). Interestingly, immunoreactivity to DNP in human plasma has been reported, although no further peptide identification has been performed in man (54). DNP consists of 38 residues comprising a 17-amino-acid ring structure, a 6-aa N-terminal, and a 15-aa C-terminal region. The C-terminal extended CNP form gives CD-NP a different receptor activity, binding the NPR-A with 200-fold greater affinity and the NPR-B receptor with 5-fold lesser affinity than CNP (55). Theoretically, this should allow CD-NP to retain the antifibrotic and anti-proliferative effects of CNP, whereas the natriuretic and diuretic properties of DNP are supposedly maintained with less hypotensive effect (53,56).

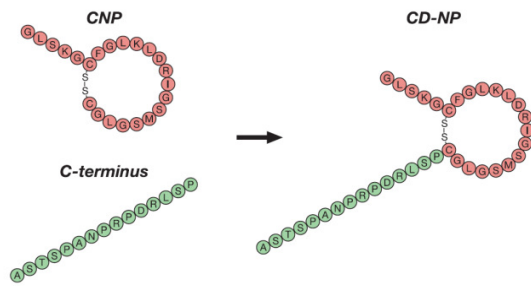


Figure 3

Structure of CD-NP: CD-NP is produced by fusion of CNP (red shade) and the C-terminus (green shade) of DNP. Moreover, the C-terminal extension seems to introduce resistance to degradation by neutral endopeptidase (57). Conclusively, these properties render CD-NP an interesting candidate as adjunctive therapy in myocardial ischemia.

THE PHYSIOLOGICAL RATIONALE FOR NATRIURETIC PEPTIDES AS THERAPY

Natriuretic peptides possess several physiological effects within the body. They affect cardiovascular homeostasis, myocardial contractility (58) and bone growth, induce peripheral lipid metabolism (59), decrease gastric emptying (60), influence the immune response (61), and renal function (62). The main hormonal effects are, nevertheless, cardiovascular, with dramatic changes in cardiac pre- and afterload (figure 4). The focus in this section will thus be physiological effects in the cardiovascular system together with the effects on lipid metabolism in adipose and muscle tissue.

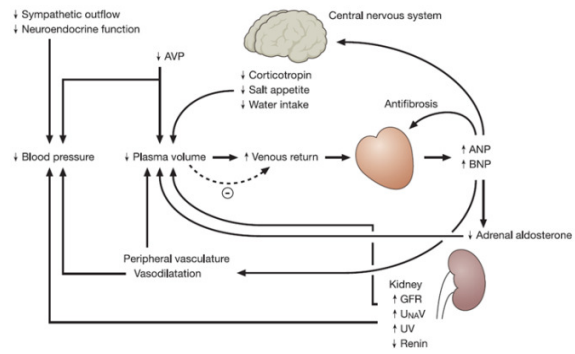


Figure 4

Physiological effects of natriuretic peptides. Increased secretion of natriuretic peptides reduces blood pressure and plasma volume through a coordinated interplay between heart, brain, kidney, adrenal, and vasculature. The minus sign indicates that a decrease in plasma volume leads to a decrease in venous return, which in turn decreases the secretion of the natriuretic peptide. GFR: glomerular filtration rate, UNaV: urinary sodium excretion, UV: urinary volume, AVP: arginine vasopressin (Modified from Levin et al, NEJM 1998).

Roles of ANP and BNP in blood pressure and cardiac remodelling

Natriuretic peptides (ANP and BNP) in the circulation cause vasodilation, increase diuresis and natriuresis, and act counter-regulatory to the renin-angiotensin-aldosterone system (RAAS) (62-64). All these mechanisms aim to reduce body fluid and blood pressure, which leads to a reduction in both pre- and afterload. Genetic disruptions of the natriuretic peptides and their receptor genes in mice have disclosed several interesting aspects of natriuretic peptide physiology. For instance, BNP knockout mice develop pressure-dependent cardiac fibrosis (65), and ANP knockout mice develop hypertension and ventricular hypertrophy (66,67). Overexpression of ANP or BNP genes leads to marked hypotension (25,68). However, BNP gene overexpression also leads to, e.g., skeletal overgrowth, which highlights a dual activation potential that both peptides possess. Overexpression of the common ANP and BNP receptor (NPR-A) leads to hypotension and protection against salt-sensitive hypertension (69), whereas lack of NPR-A is associated with cardiac hypertrophy, hypertension and cardiac interstitial fibrosis (70,71). NPR-A targeted cardiomyocyte knockout mice develop cardiac hypertrophy, hypotension, and an increase in hypertrophy marker (72). In NPR-A targeted smooth muscle cell knockout mice there is a loss of ANP response and volume-dependent hypertension (73), and in NPR-A targeted vascular endothelium knockout mice arterial hypertension, cardiac hypertrophy, and increased plasma volume are seen (74). In conclusion, this corroborates the importance of BNP and ANP in blood pressure regulation and cardiac ventricular remodelling.

Roles of ANP and BNP in lipid metabolism

Natriuretic peptides are involved in lipid metabolism where they promote peripheral mobilisation and oxidation, and affect adipose tissue derived hormones such as adiponectin (75). A recent report suggests that obesity per se down-regulates cardiac natriuretic peptide gene expression in mice (76). These findings imply the existence of a negative feedback mechanism and are sup-

ported by the observation that B-type natriuretic peptide concentrations in plasma are inversely correlated to body mass index (77). NPR-A activation in adipose tissue increases intracellular cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G (PKG), which phosphorylates hormone-sensitive lipase (HSL) and perilipin. This induces the breakdown of triglycerides to free fatty acids and glycerol (figure 5). This lipolytic pathway represents an adrenergic-independent lipolytic pathway (78,79). In healthy humans, ANP infusion corresponding to ANP plasma concentrations seen in heart failure patients increases both lipolysis in adipose tissue and fat oxidation in skeletal muscle (59).

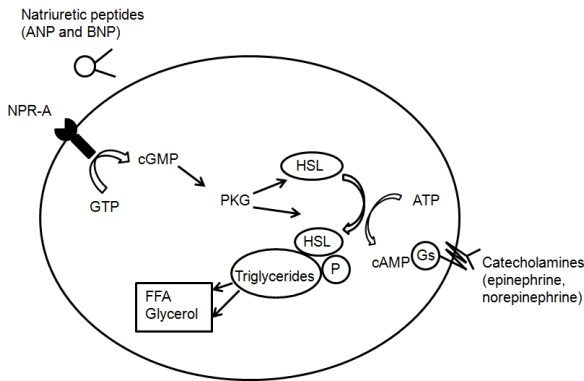


Figure 5
Lipolytic pathway of natriuretic peptides in the adipocyte. NPR-A activation in adipose tissue increases cyclic guanosine monophosphate (cGMP) leading to activation of protein kinase G (PKG). PKG in turn phosphorylates hormone-sensitive lipase (HSL) and perilipin (P), and induces the breakdown of triglycerides to free fatty acids (FFA) and glycerol in a cyclic AMP-independent manner

Furthermore, lipid mobilisation during repeated physical exercise and during head-down bed rest may, at least to some extent, be caused by increased endogenous ANP concentration (80,81). Since natriuretic peptides act lipolytic in adipose tissue and increase β -oxidation in skeletal muscle, they may also affect cardiac lipolysis during myocardial ischaemia. In acute myocardial infarction the decrease in myocardial oxygen delivery induces prompt activation of several intracellular transduction systems including the hypoxia inducible factor (HIF) system. HIF is a transcription factor that regulates several genes involved in cellular metabolism where glucose becomes the major energy substrate, whereas fatty acid oxidation is suppressed (82-84). Concomitantly the gene expression of ANP and BNP is up-regulated. Interestingly, a recent study has shown that hyper-lipidaemia per se leads to the deterioration of cGMP/PKG-dependent cardioprotection in rats (85). However, the metabolic role of natriuretic peptides in normal and hypoxic myocardium is still unresolved.

MYOCARDIAL ISCHAEMIA/REPERFUSION AND PEPTIDE THERAPY

The pathophysiology of ischaemia/reperfusion injury is highly complex and is currently still extensively studied. However, ischaemia/reperfusion injury can be alleviated by "conditioning" the myocardium (86). The recognition that additional myocardial tissue can be salvaged by use of either mechanical or adjunctive therapeutic means either before ischaemia (preconditioning), during ischaemia (perconditioning) or at the onset of reperfusion (postconditioning) have highlighted several molecular mecha-

nisms and signalling pathways to target (87). Reperfusion is a scheduled event and for that reason far easier modifiable than ischaemia which onset is unpredictable. With regard to adjunctive therapy administered just prior to and in the early reperfusion phase the supposed effect on infarct size (IS) is illustrated in figure 6.

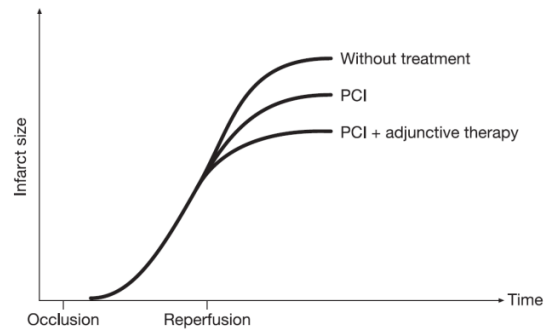


Figure 6
A simplified description of infarct size as function of time with and without percutaneous coronary intervention (PCI) and postconditional adjunctive treatment. Additional cardiac tissue can be salvaged when PCI is complemented with adjunctive therapy, such as natriuretic peptide infusion.

Necrosis and apoptosis

Ischaemia/ reperfusion injury involves two forms of cell death: necrosis and apoptosis (88). Necrosis is characterised by massive cell swelling (oncosis), cell membrane disruption, cell lysis and fragmentation which is associated with an acute inflammatory response. In contrast, apoptosis is a regulated energy dependent process that results in chromatin condensation DNA fragmentation and apoptotic body formation with preserved cell membrane integrity and hence without an associated inflammatory response. The requirement of energy in apoptosis has led to the presumption that apoptosis require a period of reperfusion in order to proceed (89). However, ischaemia has been reported to increase apoptotic cell numbers although only in a very minor proportion (90). On this basis, it seems reasonable to suggest that the apoptotic signalling pathway is triggered by ischaemia but manifests itself during reperfusion (91). The relationship between apoptosis and necrosis in ischemia/reperfusion injury is still unresolved (92). Most likely, there is an overlap between the early signalling pathways of necrosis and apoptosis since they occur simultaneously during reperfusion. Zhao et al showed, however, that necrosis peaks after 24 hours of reperfusion while apoptosis increases up to 72 hours after onset of reperfusion (93).

Reperfusion injury

Although ischaemia by itself causes myocardial injury, focus has turned to the pathophysiology taking place during reperfusion. In 1960, Jennings and colleagues described several histological features of the reperfused ischaemic canine myocardium such as cell swelling (oncosis), contracture of myofibrils, disruption of the sarcolemma, and the appearance of intra-mitochondrial calcium phosphate particles. They proposed that reperfusion can hasten the progression of necrosis (94). During the next decades, it was debated whether cardiomyocytes suffer irreversible injury during ischemia or if additional injury occurs during reperfusion (95,96), where current studies points toward the latter (88). The reperfu-

sion injury phenomenon has been eagerly studied and according to Piper et al “refers to a causal event associated with reperfusion that had not occurred during the preceding ischaemic period...” (97). Reperfusion injuries lead to four types of cardiac dysfunction:

1. *Myocardial stunning* which is defined as the mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near to normal coronary blood flow (98).
2. *The no-reflow phenomenon* which is the inability to reperfuse a previously ischaemic region and is defined by the impedance of microvascular blood flow encountered during opening of the occluded coronary artery (99).
3. *Reperfusion arrhythmias* defined as disturbances of rhythm that arise as consequence of total or partial restoration of flow in tissue which has been previously ischaemic (100).
4. *Lethal reperfusion injury* which is defined as the death of cardiomyocytes that were viable and only reversibly injured during ischemia and just prior to reperfusion (101).

“Paradoxes” of reperfusion injury and the mitochondrial permeability transition pore

The overall contributors to reperfusion injury are the oxygen paradox, calcium paradox, and pH paradox (101). The oxygen paradox denotes when re-oxygenation generates myocardial injury that exceeds the injury induced by the ischaemic event. Oxidative stress is part of the oxygen paradox and reduces the bioavailability of the intracellular signalling molecule nitric oxide (NO) which in the experimental setting acts cardioprotective. NO inhibits neutrophil accumulation, inactivate superoxide radicals and improve coronary blood flow (102). Oxygen radicals mediate Ca²⁺ loading through the inhibition of the sarcoplasmic reticulum calcium ATP-ase and the Na⁺ + K⁺ ATP ase leading to Na⁺ mediated Ca²⁺ gain.

The calcium paradox denotes the abrupt increase in intracellular Ca²⁺ which is due to sarcolemmal membrane damage and oxidative stress-induced dysfunction of the sarcoplasmic reticulum. These actions overwhelm the normal mechanisms that regulate Ca²⁺ in the cardiomyocytes. This leads to intracellular and mitochondrial calcium overload and induces cardiomyocyte death by causing hypercontracture of the cardiomyocytes and mitochondrial permeability transition pore opening (97).

The pH paradox is associated with the rapid restoration of physiologic pH during reperfusion. The pH restoration follows the wash-out of lactic acid and the activation of the sodium-hydrogen exchanger and the sodium- bicarbonate symporter (103). The Na⁺ influx may induce a Na⁺/Ca²⁺ exchange mechanism transporting sodium outwards and calcium inwards and this may enhance Ca²⁺ overload of the cells (97).

The consequence of all three paradoxes centres around the mitochondrial permeability transition pore (mPTP) in the cardiomyocytes. MPTP is a non-selective channel of the inner mitochondrial membrane. The contribution of mPTP to ischaemia/reperfusion injury was first proposed in 1987 (104). The opening of mPTP has been shown to result in both necrotic and apoptotic cell death. During ischaemia, the mPTP remains closed due to lactate acid induced pH < 7.0. Concurrently, there is a rise in tissue Ca²⁺ and P concentration as well as ATP depletion due to lack of oxygen supply. Within the first few minutes of reperfusion oxidative stress, mitochondrial calcium overload due to Ca²⁺ influx, and restoration of pH leads to opening of the mPTP (105). This causes an efflux of solutes and H₂O which leads to swelling

of the mitochondrial matrix. The inner mitochondrial membrane can accommodate this due to its folding, in contrast to the outer membrane which ruptures and releases the contents of the intermembranous space into the cytosol and apoptosis is initiated.

Targeting reperfusion injury

To attenuate ischaemia/reperfusion several intracellular molecular pathways can be targeted during reperfusion (87). A few targets will be briefly mentioned here. The reperfusion injury salvage kinase (RISK) pathway plays a pivotal role in cardioprotection and has been shown to reduce infarct size by up to 50% (106). This group of protein kinases mediates a form of programmed cell survival and prevents lethal reperfusion injury when activated during reperfusion. Key components of the RISK pathway include the PI3K/Akt and MEK/ERK cascades with several potential downstream substrates to PI3/Akt and MEK/ERK. The RISK pathway centres on inhibiting mPTP.

The cGMP-PKG signal transduction pathway also appears interesting in survival signalling (9). The cardioprotective effect of cGMP and PKG activation depends upon regulation of Ca²⁺ homeostasis and thereby attenuating hypercontracture as well as an indirect and perhaps direct interaction with the mitochondria to attenuate mPTP opening. The control of calcium homeostasis is mediated by several targetpoints such as sarcolemmal L-type Ca²⁺ channel, the Ca²⁺ activated potassium channel, and the Ryanodine receptors.

Natriuretic peptides as adjunctive therapy in ischaemia/reperfusion injury

It is worth noting that peptide therapy in myocardial infarction is not a novel intervention strategy. In 1962, Sodi-Pallares introduced insulin (glucose-insulin-potassium infusion) as a cardioprotective agent in acute myocardial ischaemia (107). Further studies suggest that insulin possess both an indirect metabolic, as well as a direct, cardioprotective effect within the myocardium (108). Today, several peptide hormones are investigated as adjunctive treatment in the early reperfusion phase of myocardial ischaemia. These include the kinins, urocortins, adrenomedullin, incretins and the natriuretic peptides (109,110). This section will however focus on the natriuretic peptides as adjunctive therapy in acute myocardial ischemia.

Speculation has been whether the cardioprotective actions of natriuretic peptides are due to direct actions on the heart or indirect actions through peripheral vasodilation, and thereby a theoretically desirable decrease in pre- and afterload – or both. However, clear evidence exists on the beneficial infarct-limiting effects of natriuretic peptides directly mediated in the cardiomyocytes. Here focus has been the very central cGMP – PKG signalling pathway. Nevertheless, when studying the literature it becomes clear that the cardioprotective actions of natriuretic peptides must be due to several other intracellular actions as well (9)

Natriuretic peptide infusion is well-described in the Langendorff heart preparation. In the isolated rat heart, BNP infusion during early reperfusion limits infarct size (111). D’Souza et al reported this cardioprotective action to be mediated through mitochondrial but not sarcolemmal K-ATP-channel opening (111,112). Furthermore, Insete et al showed that urodilatin limited acute reperfusion injury in the isolated rat heart by attenuating cGMP depletion (113). Beneficial effects, i.e. a reduced release of myocardial creatinine kinase isoform MB by ~ 15%, an increase in left ventricular ejection fraction by ~5 % and a de-

crease in reperfusion injuries (defined as malignant arrhythmias, re-elevation of ST-segment or worsening of chest pain) have also been reported in a clinical placebo-controlled trial on ANP infusion during myocardial infarction. (114). Furthermore, infusion of urodilatin in a porcine open chest model of acute myocardial ischaemia normalized cGMP and decreased infarct size. Notably, the infarct-limiting effect was dose-dependent with no limiting effect when cGMP concentration was very high (335% times higher than the cGMP concentration in the control myocardium) (115). In rodents, CD-NP infusion via osmotic pumps for 2 weeks post myocardial infarction lowered plasma aldosterone, increased renal blood flow, decreased proteinuria, and decreased left ventricular fibrosis formation (116). Yang et al. reported that ANP infusion in the isolated rabbit heart given just prior to and during early reperfusion limits infarct size possibly through the RISK pathway involving activation of PI3/Akt and Erk1/2 kinases (117). It is believed that this action could be mediated by NPR-C which is believed to be G-protein coupled (9). NPR-C as potential mediator of cardioprotection is further corroborated by Hobb et al. who showed that CNP interacts with NPR-C and hence protects against ischaemia/reperfusion injury maybe by mediating opening of a G protein coupled inwardly rectifying K⁺ channel (GIRK) (42)

In vitro, Gorbe et al reported a cytoprotective response via a downstream signalling pathway involving an increase in cGMP activating a PKG-dependent response (118). This study was further corroborated by Giricz et al who showed that PKG acted as a critical mediator of natriuretic peptide-/cGMP-induced cytoprotection in rats (85). As early as 1997, Hempel et al showed that ANP and urodilatin protected cardiomyocytes subjected to anoxia/reoxygenation against reoxygenation hypercontracture (119). In addition, Abdallah et al reported that the cardioprotective actions of urodilatin was due to inhibition of Ca²⁺-induced hypercontracture caused by an increase in sarcoplasmic reticulum ATPase (SERCA) activity (120). It is worth noting that, ANP has been reported to act through a cGMP-independent signalling pathway in chronic cardiac hypertrophy, which probably counteracts the outcome of the cGMP-dependent PKG mechanism (121). This curious as well as the mentioned study by Giricz et al (85) highlights the challenge of co-morbidities in cardioprotective adjunctive therapy.

HYPOTHESIS AND AIM

HYPOTHESIS

Natriuretic peptides are up-regulated in ischaemia where they act through NPR's to protect cardiac tissue. Furthermore natriuretic peptides induce lipolysis in adipose tissue and this could influence cardiac lipid accumulation and the cardioprotective action of natriuretic peptides (78,85). This forms the rationale for the hypotheses:

B-type natriuretic peptide protects reversible injured myocardium both in the re-modelling phase after myocardial infarction and in the initial phase of cardiomyocyte damage and injury in a large animal model of regional cardiac ischaemia (111,112,114,116,122). This effect may be mediated directly in the heart tissue (65).

B-type natriuretic peptide infusion depresses the cardiac expression, production, and release of endogenous natriuretic peptides (122).

B-type natriuretic peptide infusion increases lipid metabolism during ischemia/ reperfusion (76).

AIM

To study whether natriuretic peptides (BNP and CD-NP) possess myocardium- protective effects when administered in the acute reperfusion phase in an in vivo porcine model of regional cardiac ischaemia, supplemented by a BNP stimulation study in an in vitro murine cellular model of hypoxia and reperfusion.

To study whether exogenous natriuretic peptide infusion (BNP and CD-NP) affects the endogenous production and secretion of natriuretic peptides in a porcine model of regional cardiac ischaemia and reperfusion.

To study whether exogenous natriuretic peptide infusion (BNP) affects cardiac lipid accumulation and lipid mobilisation to plasma in a porcine model of regional cardiac ischaemia and reperfusion.

METHODOLOGICAL CONSIDERATIONS

Whether a drug alleviates ischaemia/reperfusion injury can be tested in different settings such as intact animal models (mouse, rat, rabbit, dog, and pig), isolated hearts (the Langendorff model), in vitro (in cells and organelles), and in clinical studies. Infusions of natriuretic peptides in the Langendorff preparation of ischaemia/reperfusion have been thoroughly performed. In principle, it would be most interesting to study BNP infusion in human patients presenting with myocardial infarction. One such trial of ANP infusion in myocardial infarction (114) has indeed pursued this strategy but left several questions unanswered. Urodilatin has been investigated in an open chest porcine model of ischaemia and reperfusion (115). However, a study dealing with BNP infusion in a larger animal model of ischaemia/reperfusion was not found in the current literature. This formed the rationale for using a porcine model of myocardial ischaemia and reperfusion.

THE PORCINE MODEL

The pig is a suitable model for cardiovascular physiology and pathophysiology studies since it shares most anatomical characteristics with human heart anatomy and physiology. Apart from size and morphology, the coronary anatomy and blood supply shares similarities (123). Studies of ischaemia/reperfusion in the pig can be investigated in a closed chest model or an open chest model. In a closed chest model, occlusion of the coronary artery is induced e.g. by placing and inflating a balloon dilatation catheter in the coronary artery e.g. the left anterior descending artery (LAD). In the open chest model a sternotomy is first performed and a snare or clips is placed around the coronary artery and tightened to induce and maintain ischaemia. The advantages and disadvantages of the two models are listed table 1.

Briefly, the choice of a closed chest model minimises the interference of inflammatory effects, since sternotomy is not performed until the end of the experiment. However, it is impossible to visually observe the occlusion site and monitor both the development of tissue oedema during occlusion as well as the hyperaemia that develops during reperfusion. Consequently, the persistence of occlusion must be validated by use of contrast fluoroscopy and confirmation of ischaemia can be verified by detection of ST-segment elevation/depression > 1.0 mm. Additionally, myocardial temperature influences infarct size in pigs and dogs during ischaemia (124-126). A stable myocardial temperature is easier to maintain in a closed chest model compared with an open chest model. However, in the open chest model, the myocardium is easily accessible to instrumentation e.g. an oxygen tension probe or micro dialysis probe can easily be placed within the myocardial tissue.

Table 1: Advantages and disadvantages in a closed- versus an open chest porcine model of regional cardiac ischemia and reperfusion.

MODEL	Advantages	Disadvantages
Closed-chest	<p>Minimally invasive</p> <p>Less stressful with less activation of the inflammatory system</p> <p>Myocardial temperature parallels body core temperature</p>	<p>Occlusion must be confirmed by contrast injection</p> <p>Occlusion site must be rediscovered before delineating the area at risk</p> <p>Endothelial trauma can cause re-occlusion</p>
Open-chest	<p>Occlusion is visually easily confirmed</p> <p>Occlusion site is unquestionable</p> <p>Instrumentation of vessels and myocardium is easily obtained</p>	<p>Difficult to maintain epicardial temperature</p> <p>Altered ambient pressure in the thorax</p> <p>Major activation of the inflammatory system due to major surgery</p>

Infarct development

Due to the lack of collaterals in coronary blood supply in pigs, transmural ischaemia develops quickly. The ischaemic lesion proceeds in a wave-front pattern from the subendocardium towards the epicardium. In addition, the development of the infarcted area as a function of time is fairly predictable with the majority of the myocytes dying after 30 to 60 minutes of ischaemia followed by reperfusion (127).

Gender, age and natriuretic peptides

Ischaemic tolerance changes with age, where piglets have an enhanced capacity for aerobic glycolysis which makes them less susceptible to ischaemic damage from low-flow ischaemia but more susceptible to no-flow ischaemia (128). However, it is not feasible to use adult pigs due to the fast growth rate in production swine (approximately 350 grammes daily in pigs 0-30 kg and approximately 950 grammes daily in pigs > 30 kg). Moreover, natriuretic peptides are known to interact with sex hormones and the outcome of the experiments could be influenced by gender (129).

The in vitro model

On the basis of the results in the porcine model, an attempt to answer the major question on cardioprotection regarding indirect versus direct mechanisms was attempted in an in vitro study. Paradoxically, the advantage of a cell culture, "simplicity", is also the largest drawback. The interplay with other cell types and systems is lost. Therefore, a cell culture study does not per se allow for deduction of in vivo physiology. However, it introduces an opportunity to change one parameter at a time without interaction from unknown factors. The used cell model was chosen to

investigate whether there was a direct cardioprotective effect of BNP stimulation on isolated cardiomyocytes.

HL-1 Cell line versus primary cardiomyocytes

The murine atrial tumour cell line HL-1 was chosen based on earlier experiments performed on HL-1 and concerning ANP and BNP (76). Notably, this cell line has previously been used as a surrogate model for short-term ischaemia (130). The advantages are e.g. the homogeneity, stability, and the unlimited availability of cells. Using immortalised cells, however, leaves some difficulties in interpretation; as the cells are immortalised and thus do not readily represent true cardiomyocytes. Ideally, primary cultured cells would have been the best choice, as they will express the same receptors and intracellular pathways as native cells in vivo. Nevertheless, primary cells are not as stable and homogeneous as immortalised cells.

STUDY DESIGN

This thesis is divided into three substudies based on natriuretic peptides and their physiological effects during ischaemia and reperfusion (figure 7).

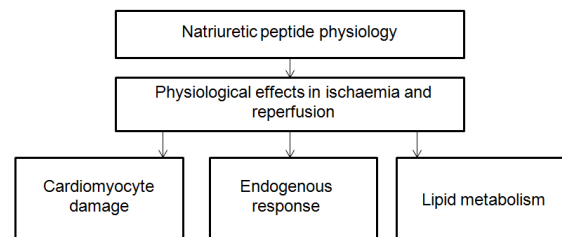


Figure 7

The thesis structure is based on natriuretic peptides and their physiological effects in ischaemia and reperfusion.

For this purpose three different types of models were designed, comprising 1) an in vivo acute model of regional cardiac ischaemia and reperfusion, 2) an in vivo 48-hour model of regional cardiac ischaemia and reperfusion and 3) an in vitro model of total "ischaemia and reperfusion". In sections 4.1 and 4.2 the three different model types will be described.

IN VIVO MODEL

The in vivo model was a porcine closed chest model with induction of regional cardiac ischaemia by use of a balloon catheter guided through the right carotid artery. Two different model types were studied:

1. An acute model of regional cardiac ischaemia and reperfusion. The LAD was occluded for 1 hour just distal of the second diagonal branch followed by 3 hours of reperfusion (figure 8).
2. A 48-hour model of regional cardiac ischaemia and reperfusion. The LAD was occluded as mentioned above followed by 47 hours of reperfusion (figure 9).

Conventional pigs were used and the inclusion criterias were as follows: the pigs had to be in good condition, aged 3-4 months (which is prior to sexual maturity), and weigh 25 ± 2 kg. Furthermore, contrast fluoroscopy had to verify zero-flow occlusion and confirm reperfusion. Also ST-segment elevation/depression had to be $> \pm 1.0$ mm. During the experiment myocardial temperature had to be $38 - 39$ °C. Due to urinary catheter placement via ure-

thra all pigs were female (this method is not feasible in male pigs). All pigs were fasted from the day before the experiment but had access to water.

Acute model

Danish Landrace/Yorkshire pigs were allocated to one of three groups: A BNP-32 infusion, a CD-NP infusion, and a control group. The BNP-32 and CD-NP groups received synthetic porcine BNP-32 (Phoenix Peptides, Germany) or CD-NP (Nile Therapeutics, USA). Both peptides were infused in a 0.05 µg per kg per min dose. The control group received an equal volume of isotonic saline infusion. The outline of the study is presented in figure 8.

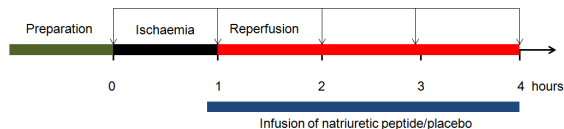


Figure 8

Outline of the in vivo study of acute regional cardiac ischaemia and reperfusion. The arrows indicate blood sampling every hour. Five minutes before reperfusion an infusion of natriuretic peptide (BNP or CD-NP) or placebo (isotonic saline) was started.

48-hour model

To validate troponin T (TnT) release to plasma, four Danish Landrace/Yorkshire pigs were induced with ischaemia for 1 hour and surveyed for a total of 48 hours. The outline is presented in figure 9.

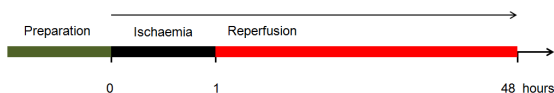


Figure 9

Outline of the 48-hour in vivo study of regional cardiac ischaemia and reperfusion. The arrow indicates the period where blood samples for TnT measurements were obtained. During the first 4 hours, blood samples were collected each hour and then every 4th hour for a total of 48 hours.

In vitro model

The in vitro model study was performed in a HL-1 cell culture where anoxia was induced by use of a specially developed hypoxia box containing an anaerobic sachet and a hypoxic medium deprived of substrates (130). The outline of the study is presented in figure 10.

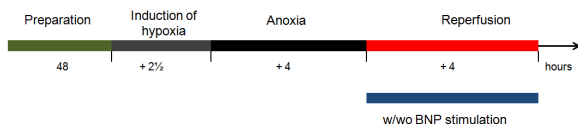


Figure 10

Outline of the in vitro study of ischaemia/reperfusion. The timeframe is preparation 48 hours, induction of hypoxia 2½ hours, anoxia 4 hours, reperfusion 4 hours with (w) or without (wo) BNP stimulation (10 -6 or 10 -7 M) (blue line) during "reperfusion".

MATERIALS AND METHODS

OUTLINE OF THE STUDY PROTOCOLS

In vivo - acute model (substudy I + II + III)

A total of 28 pigs were intramuscularly premedicated with midazolam and S-ketamine. Anaesthesia and analgesia were achieved with pentobarbital and fentanyl, respectively. The pigs were orally intubated and ventilated. A potassium and isotonic saline infusion was started. Intravascular sheaths were placed in the right and left external jugular vein and carotid artery. Heparin and amiodarone were administered. A Swan-Ganz catheter was positioned with the tip placed in the pulmonary artery. Regional cardiac ischaemia distal to the second diagonal branch of the LAD was induced by placement (via the right carotid artery) and inflation of a coronary balloon dilation catheter (131). Ischaemia was maintained for 1 hour. Contrast fluoroscopy verified a zero-flow occlusion and subsequent reperfusion (figure 11).

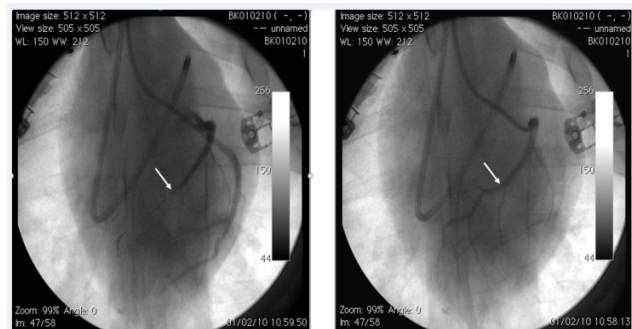


Figure 11

Occlusion and reperfusion angiograms. Occlusion and reperfusion of the LAD was verified by contrast fluoroscopy. The white arrow indicates occlusion (left panel) and reperfusion (right panel).

Five minutes before reperfusion, an infusion of BNP-32, CD-NP or placebo was started. Reperfusion lasted 3 hours (figure 8). A sternotomy was performed and the LAD re-occluded at the previous occlusion site. Evans blue dye was injected directly into the left auricle. The heart was excised and transmural tissue biopsies collected from the appendage of the left and right atria, from the right ventricle, and from the ischaemic and non-ischaemic areas of the left ventricle. The biopsies were snap frozen in liquid nitrogen and stored in a -80 °C freezer until later analysis. The heart was cut into slices perpendicular to the long-axis and pictures of the section planes scanned. Slices were then incubated for 10 minutes in 37 °C phosphate-buffered triphenyl-tetrazolium chloride (TTC) and rescanned. Blood was collected before induction of ischaemia and every hour and stored at -80 °C until later analysis. The animals were kept at a steady body temperature throughout the procedure. Cardiac output, SvO₂, mean arterial pressure and mean pulmonary arterial pressure were recorded throughout the experiment.

In vivo - 48 hour model (substudy I)

Four Danish Landrace/Yorkshire pigs were used. The pigs were premedicated as described for the acute model. Anaesthesia was initiated with propofol and maintained with sevoflurane after the pigs were orally intubated and ventilated. Analgesia, fluids, anti-arrhythmic and anti-thrombotic treatment was initiated as described in the acute model. A transcutaneous central venous catheter

was placed into the right external jugular vein for blood sampling. Intravascular access to the left carotid artery was achieved by sheath insertion and regional cardiac ischaemia lasted 1 hour and was induced as previously described in the acute model. The balloon catheter was removed, the carotid artery ligated and the wound closed lege artis. After 1½ hours of reperfusion, anaesthesia was stopped. Before waking and 24 hours later, flunixin was administered to sustain analgesia. The pigs were moved to regular stable facilities upon waking. Blood samples were collected every hour during the first 4 hours and every 4th hour during the remaining 44 hours (figure 9).

After a total of 48 hours, the animals were sedated using midazolam. Anaesthesia and analgesia were induced and maintained as just described. Sternotomy, LAD ligation and Evans Blue dye tissue staining were performed as described in the acute model. The heart was excised and sliced into 5 mm slices and scanned and stained as described above.

Verification of myocardial ischaemia in the porcine model

Patent myocardial ischaemia was verified by ST-segment depression/elevation > 1.0 mm and contrast-fluoroscopy. In addition, vascular endothelial growth factor (VEGF) mRNA analysis in a defined area of the ischaemic area was measured at the end of the experiment to confirm the ischaemic event as previously described by Goetze et al (28).

Temperature

To minimize the impact of temperature variation, pigs were kept at a steady body core temperature (38-39 °C) using a warming unit and blanket covered by tissue drapes.

Anti-thrombotic and anti-arrhythmic drugs

Heparin was administered as a bolus after instrumentation and every hour. The model is, however, prone to arrhythmia including ventricular fibrillation. To avoid such complications, amiodarone was given as a bolus prior to ischaemia (1mg per kg). The dose was based on previous own unpublished experimental data on amiodarone titration (1-4 mg per kg iv). Furthermore, a potassium infusion was given throughout the experiment to keep K⁺ concentrations between 4-5 mmol/L.

In vitro model – (substudy I)

Murine HL-1 cells were cultured in Claycombs medium supplemented with foetal calf serum, penicillin/streptomycin, L-glutamine, and mM noradrenalin. Flasks and plates were all pre-coated with fibronectin in 0.02 % gelatine (132). The cell concentrations and viability were measured using a cell counter. An experimental set-up simulating ischaemia/reperfusion was established (figure 10). HL-1 cells were grown with a density of 3 x 10⁵ cells per well (six-well plates). After 48 hours, cell medium was substituted with a hypoxia medium as previously described (130). The plates were immediately transferred to a hypoxia box containing an AnaeroGen sachet and incubated at 37 °C. Anoxia was determined by an Oxoid Anaerobic Indicator and achieved after approximately 2½ hours. After 4 hours of anoxia, the cells were removed from the hypoxia box, and the medium was changed to supplemented Claycombs media without NA. Cells were then stimulated with murine BNP-45 (10⁻⁶ M or 10⁻⁷ M) and incubated at normal O₂ conditions for 4 hours. Control cells were incubated with supplemented Claycombs medium without NA from the beginning of the experiment. For further details please consult manuscript I materials and methods (132).

MEASUREMENTS

Baseline measurements in vivo (substudy I+II+III)

Systemic (mean arterial pressure; MAP) and pulmonary (mean pulmonary artery pressure; MPAP) pressures were measured in the carotid and pulmonary artery respectively. Furthermore, heart rate (HR), cardiac output (CO), mixed venous saturation (SvO₂), core temperature (Swan Ganz), lactate, bicarbonate, glucose, and pH as well as plasma lipids were recorded as baseline values to ensure the comparability of the animals prior to natriuretic peptide/placebo intervention.

Peptide infusion – in vivo (substudy I+II+III)

Plasma concentrations of the infused peptides were quantified by radioimmunoassay for porcine BNP-32 and CNP-22, (Phoenix Peptides, Germany) in order to validate the concentration of infused peptide.

Diuresis – in vivo (substudy I+II+III)

A catheter was placed through the urethra into the bladder to measure accumulated diuresis during the natriuretic peptide infusion experiment.

mRNA analysis – in vivo and in vitro

For confirmation of ischaemia in cardiac tissue/HL-1 cells, VEGF mRNA was measured in the acute model and the in vitro model using real time PCR.

Intracellular transduction activity mediated by BNP stimulation – in vivo (substudy I)

Intracellular cGMP was measured using a commercial ELISA kit (Sigma-Aldrich). Briefly, HL-1 cells were stimulated with murine BNP-45 for 15 min and subsequently lysed in 0.1 mM HCl at 37°C for 20 min. The supernatant was then centrifuged 20 min at 600 G at room temperature, and the cGMP content was quantified according to the kit insert.

Infarct size over area at risk – in vivo (substudy I)

The measurement infarct size (IS) over area at risk (AAR) was measured by use of planimetry, where quantitative information about three-dimensional materials is extracted from two-dimensional planar sections such as myocardial tissue cut into slices of known thickness (48-hour model) or weight (4-hour model). The area at risk was delineated by Evans Blue Dye (figure 12A). The delineation of IS was made by use of TTC (figure 12B). The latter staining method is dependent on the leakage of active dehydrogenases and co-factors such as NADH from the non-viable myocytes and is dependent on sufficient reperfusion time (133).

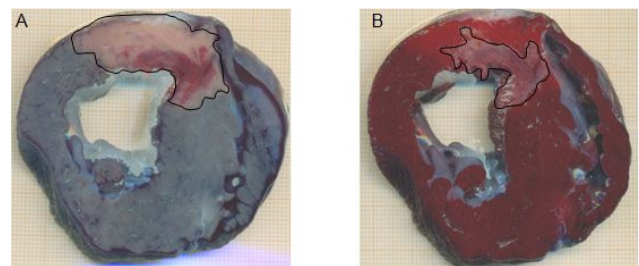


Figure 12 Myocardial tissue staining of a similar tissue slide. Panel A: Evans Blue delineates the area at risk (marked by the black line). Panel B: TTC delineates the infarct size (marked by the black line).

ates the area at risk (marked by the black line). In the pig, first the slice (A) is scanned and the area at risk is measured. Then the slice is immersed in TTC and rescanned and the infarct size is measured.

TnT – in vivo and in vitro (substudy I)

Plasma Troponin is used as a diagnostic marker of myocardial damage in humans. In this study, troponin T was measured in plasma and in cell media from HL-1 cells using an automated assay (Modular P, Roche Diagnostics, Germany). This assay utilizes monoclonal antibodies directed against conserved epitopes in pig, mouse, and human TnT. The interassay coefficient of variation is <3 %.

Apoptosis – in vitro (substudy I)

Apoptosis was evaluated by caspase activity, assessed using a Caspase-Glo 3/7 Assay (Promega, Sweden)(134). This luminescent assay measures caspase-3 and -7 activities in cultures of adherent or suspension cells. HL-1 cells were harvested from the plates and the cell concentrations were determined. The caspase activity in 1000 cells was then determined according to the manufacturer's protocol.

mRNA analyses in vivo and in vitro (substudy I + II)

To detect changes in gene expression associated with the induced stimuli ("ischaemia, reperfusion" and natriuretic peptide stimulation), real-time PCR (RT-PCR) was used as a transcriptional measure. Total RNA was isolated with TRIzol (Invitrogen, Taastrup, Denmark). The RNA integrity was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Germany). The integrity was expressed as RNA Integrity Number (RIN) values based on the ratio of 28S to 18S of ribosomal RNA. The mRNA was thereafter evaluated by RT-PCR. The contribution of contaminating DNA was earlier found to be negligible. cDNA and subsequent RNA amplification was performed as earlier described for BNP, VEGF, beta actin (β -actin), and glyceraldehyde 3-phosphate-dehydrogenase (GADPH) (28). For ANP mRNA amplification, we used the following primers: 5'-ACGACGCCAGCATGAGCTCCTTC-3' and 5'-GCTGTTATCTTCAGTACCGGAA-3'. All data were normalised to β -actin and GADPH prior to data analyses.

RNA extraction and analysis of mRNA from HL-1 cells was performed as described above. The primers were mouse-specific and selected based on previous experience (76). The primers used for PCR were as follows: VEGF, 5'- AGTCCCATGAAGTGATCAAGTTCA-3' and 5'-ATCCGCAT GATCTGCATGG-3'; 18S, 5'-CGCGTTCTATTTTGTGGT-3' and 5'- AGTCGGCATCGTTTATGGTC-3'; ANP, 5'-CCTGTGTAC AGTCGGGTGC-3' and 5'-AGATCTATCGGAGGGTCCCA-3'; and BNP, 5'-CTGAAGGTGCTGTCCAGAT-3' and 5'-GTTCTTTT GTGAGGCCTTGG-3'. All data were normalised to 18S before data analyses.

ProANP in plasma – in vivo (substudy II)

ProANP was measured using the new processing independent proANP assay (PIA) which allows measurement of proANP-derived peptides irrespective of changes in post-translational maturation (135). Specifically, this assay is calibrated against porcine proANP and measures all forms of N-terminal proANP fragments; the analytical principle has been reviewed by Rehfeld and Goetze (136). The inter-assay CV was always <15 %.

Analysis of free fatty acids, cholesterol and triglycerides – in vivo (substudy III)

Plasma lipid concentrations were determined with enzymatic kits: free fatty acids (Wako® NEFA C kit, TriChem Aps, Frederikssund, Denmark), triglycerides (GPO-TRINDER, Sigma; this kit also measures free glycerol), and total cholesterol (CHOD-PAP, Roche Molecular Biochemicals). Plasma analysis were performed in all three groups; BNP-group (n=7), CD-NP-group (n=5), and untreated group (n=10, except for triglyceride analysis here n = 7).

Cardiac tissue lipid analysis – in vivo (substudy III)

Myocardial tissue biopsies from the non-ischaemic part of the ventricle (right ventricle) of BNP-infused and untreated animals were analysed. Lipids were extracted from the cardiac biopsies (60 - 112 mg) with chloroform/methanol. For enzymatic quantification of triglycerides, cholesterol esters, and cholesterol the same kits were used as those for plasma analyses as previously described (137). Cardiac tissue lipid analyses were performed in the BNP-group (n=6) and the untreated group (n=5). This analysis was not performed in the CD-NP infused animals due to lack of sufficient cardiac tissue.

STATISTICS

Substudies I + II: Data are expressed as mean \pm standard error of the mean (SE) unless otherwise stated. For comparison between groups, we used one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. For comparison between two groups, we used student's t-test with Welch correction or the Mann-Whitney test when appropriate. P-values < 0.05 were considered statistically significant.

Due to lack of a few data points, hemodynamics were pooled in groups: time = 0-55 minutes and time = 180 – 240 minutes. These data were analysed using one-way ANOVA with Tukey's multiple comparison test.

Substudy III: Data are expressed as mean \pm standard error of the mean (SEM) unless otherwise stated. For comparison between groups, we used one-way ANOVA with Tukey's multiple comparison test and the Mann-Whitney test when appropriate. Correlation was tested using Spearman's rank correlation coefficient. P-values < 0.05 were considered significant. GraphPad Prism 5.0 software was used for all calculations (GraphPad Software Inc, CA, USA).

MAIN RESULTS

Haemodynamics and standard blood parameters - acute model

Baseline haemodynamic measures and body core temperature are shown in table 2. There was no difference between the groups at baseline. Cardiac output, arterial pH, temperature, bicarbonate, lactate, and glucose were unchanged between the groups over time.

Natriuretic peptide infusion - acute model

Infusion regimens were well-tolerated by animals during the BNP/CD-NP peptide infusion period. Peak plasma concentrations were comparable in the BNP-32 and CD-NP groups ([1003] plasma pmol/L versus [981] plasma pmol/L, CI 95%, P = 0.93) 2 hours after reperfusion start (figure 13).

Table 1

Haemodynamics and standard blood parameters (venous blood) for the pigs included in the 4-hour acute infusion experiment. HR heart rate; MAP mean arterial pressure; MPAP mean pulmonary pressure; SvO₂ mixed venous oxygen saturation; CO cardiac output; HCO₃⁻ bicarbonate.

Time	0-55 min	0-55	0-55	180-240	180-240	180-240
Group	BNP	CD-NP	Control	BNP	CD-NP	Control
HR (beats/min)	96.8 ± 2.1 ns	91.6 ± 2.0 ns	100.6 ± 8.6	104.5 ± 2.2 ns	120 ± 5.2 ns	109.8 ± 5.0
MAP (mmHg)	83.7 ± 1.0 ns	87.8 ± 4.8 ns	87.0 ± 5.8	47.0 ± 0.7 ***	49.8 ± 2.8 *	61.7 ± 2.8
MPAP (mmHg)	15.9 ± 0.4 **	17.6 ± 0.9 ns	18.6 ± 1.2	15.3 ± 0.2 ***	14.6 ± 0.3	18.6 ± 0.3
SvO ₂ (%)	74.3 ± 0.9 ns	73.6 ± 2.1 ns	70.8 ± 3.1	60.2 ± 1.0 *	63.2 ± 3.9 ns	68.5 ± 2.5
CO (L/min)	2.7 ± 0.1 ns	2.3 ± 0.1 ns	2.4 ± 0.1	1.8 ± 0.1 ns	1.8 ± 0.1 ns	1.8 ± 0.1
Temperature (C°)	38.4 ± 0 ns	38.5 ± 0.1 ns	38.2 ± 0.1	38.3 ± 0 ns	38.5 ± 0.1 ns	38.3 ± 0.1
Lactate (mmol/L)	2.2 ± 0.2 ns	2.3 ± 0.2 ns	2.0 ± 0.2	2.9 ± 0.3 ns	3.6 ± 0.2 ns	2.5 ± 0.7
HCO ₃ ⁻ (mmol/L)	28.7 ± 0.7 ns	29.3 ± 0.9 ns	30.1 ± 1.2	24.1 ± 0.4 ns	23.7 ± 0.4 ns	25.8 ± 1.5
Glucose (mmol/L)	4.0 ± 0.1 ns	4.5 ± 0.4 ns	3.6 ± 0.2	4.3 ± 0.2 ns	4.0 ± 0.2 ns	4.1 ± 0.5
pH	7.5 ± 0 ns	7.5 ± 0 ns	7.4 ± 0	7.4 ± 0 ns	7.4 ± 0 ns	7.4 ± 0

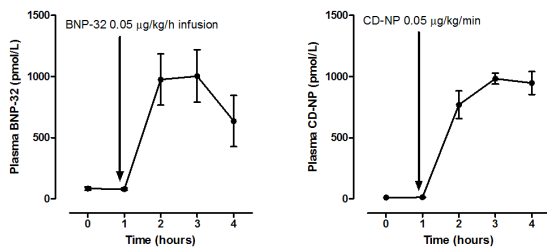


Figure 13
Peptide concentrations in plasma. The left and the right panels show the BNP-32 and CD-NP concentrations in plasma, respectively. The black arrows indicate the infusion start, i.e. 5 minutes before reperfusion.

Blood pressure and diuresis - acute model

The mean arterial pressure (MAP) in the BNP-32 group decreased during the final hour of reperfusion compared with controls (~15 mmHg, P < 0.0001, figure 14 A). A similar decrease was found in the CD-NP group (~12 mmHg, P = 0.0052, figure 14 B).

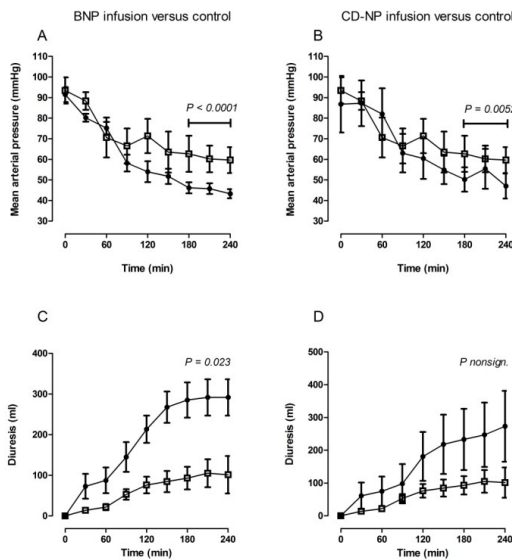


Figure 14
Mean arterial pressure and diuresis. Panels A and C depict the BNP infusion animals versus control animals whereas panels B and D depict CD-NP infused animals versus control animals. -●-: the natriuretic peptide group and -□-: the control group.

A reduction in mean pulmonary artery pressure (MPAP) was also found in both groups compared with controls (table 2). As expected, BNP- and CD-NP-treated animals displayed increased diuresis throughout the experiment (figures 14 C and D, respectively).

Intracellular cGMP and VEGF – In vitro model

To ascertain an intracellular effect of BNP-45 in HL-1 cells, we assessed the cGMP response in a dose-dependent manner. BNP stimulation at 100 nM increased cGMP by ~3-fold (P = 0.0037), which corroborates the functional presence of natriuretic peptide receptors on HL-1 cells.

Furthermore, VEGF mRNA contents in HL-1 cells increased 2.3-fold in the hypoxia/reperfusion set-up compared with control cells (P < 0.0001). Moreover, we observed a 2.5-fold increase (P < 0.0001) in BNP mRNA content in the hypoxia/reperfusion set-up cells. Taken together, the experimental setup confirms hypoxia-induced transcriptional activation in HL-1 cells, as previously shown for human cardiomyocytes in culture (138). Furthermore, the data parallel the in vivo findings in the porcine model with regard to hypoxia-induced VEGF and BNP mRNA stimulation.

Substudy I: Cardiomyocyte damage in vivo and in vitro.

25 pigs were included in this study. Three animals developed cardiac arrest upon induction of ischaemia, i.e. before initiation of peptide infusion, and were excluded. Furthermore, 3 animals (one in the BNP-32 group and two in the CD-NP group) were excluded after the experiments due to unsuccessful peptide infusion. The study thus comprised 19 animals. In the 48-hour model, one pig was excluded due to failure to induce sufficient ischaemia. The remaining three animals tolerated the setup fairly well.

IS/AAR

Estimation of infarct size determined as IS/AAR in the acute model evaluated by planimetry did not reveal significant differences between the peptide infused animals and the control group (range 35-44 %, n=14). However, Evans Blue staining quality varied for the area at risk and could only be clearly defined in 14 animals (figure 15). In addition, calculation of coefficient of variation (CV) for this method (IS/AAR) in the acute model was > 20 %. Estimation of IS/AAR in the 48-hour model was 30.3 ± 2 %. The latter result was based on slice thickness in contrast to measurements in the acute model which were based on the weight of the slices.

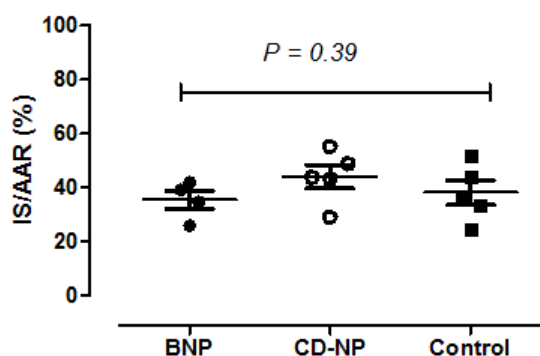


Figure 15
Infarct size over area at risk (IS/AAR) in percent in the acute model.

TnT acute model

The total TnT release was assessed as area under the curve (AUC) during the peptide infusion period. A 46% decrease ($P = 0.0015$, figure 16A) was noted in the BNP-32 infusion group compared with controls. In parallel, a 40% decrease was found in the CD-NP group ($P = 0.0194$, figure 16B), with no significant difference between the BNP-32 and CD-NP groups.

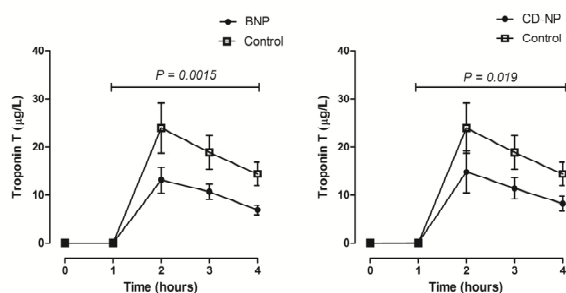


Figure 16
Cardiac troponin T in plasma in the acute model of cardiac regional ischaemia. Panel A shows the cardiac troponin T ($\mu\text{g/L}$) response in the BNP-infused group (-●-) compared with the control group (-□-). Panel B shows the cardiac troponin T ($\mu\text{g/L}$) in the CD-NP-infused group (-●-) compared with the control group (-□-).

TnT 48 hour model

The TnT release (figure 17) after ischaemia was confirmed to be single phased with no subsequent washout or release. The infarct size, defined as IS/AAR, was $30.3 \pm 2\%$ after 48 h, which parallels earlier experiments using porcine models.

TnT HL-1Cells

In cell media, TnT increased after hypoxia/reperfusion ($100 \pm 12\text{ng/L}$) compared with control cells ($25 \pm 2\text{ng/L}$). No TnT changes were observed for the intervention groups (BNP $10\text{-}6\text{ M} = 91 \pm 7\text{ng/L}$, $P = 0.29$; BNP $10\text{-}7 = 83 \pm 10\text{ng/L}$, $P = 0.50$).

Caspase

Apoptosis was evaluated after hypoxia/reperfusion with ($10\text{-}6\text{ M}$ or $10\text{-}7\text{ M}$) and without post-conditional BNP stimulation in HL-1 cells. Caspase activity increased 2.9-fold (12807 ± 2703 relative light units, RLU) compared with control cells (4457 ± 612 RLU) ($P = 0.03$). However, no effect on caspase activity was observed after BNP stimulation (BNP $10\text{-}6\text{ M} = 9178 \pm 973$ RLU, $P = 0.25$; BNP $10\text{-}7$

$7\text{ M} = 10688 \pm 1736$ RLU, $P = 0.53$) compared to controls (12807 ± 2703).

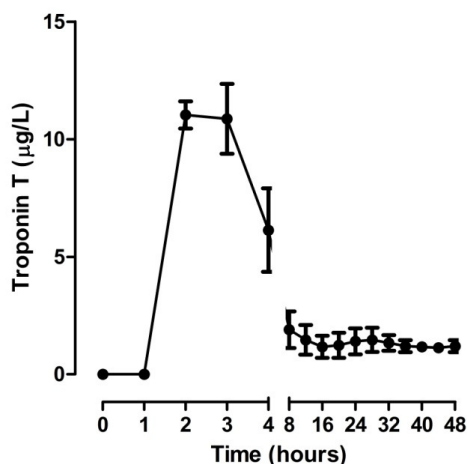


Figure 17
TnT $\mu\text{g/L}$ in plasma as marker of cardiac necrosis in the 48-hour model of cardiac regional ischaemia and reperfusion

RIN integrity

RNA integrity was better preserved in the infusion groups; RIN values were on average 1.2 to 1.4 higher than in the control animals (figure 18). The RNA integrity number in all samples generally suggested ongoing RNA degradation reflected as relatively reduced RIN values.

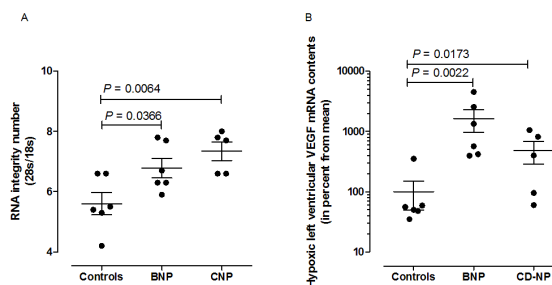


Figure 18
Effects of natriuretic peptide infusion on cardiac RNA integrity and VEGF mRNA expression. Panel A shows the RNA integrity number in the three groups. Panel B shows the VEGF mRNA expression in the ischaemic/hypoxic lesion.

Substudy II: Endogenous response

Natriuretic peptide infusion was validated as previously described in section 6.1. The study group comprised the animals previously described in substudy I.

ProANP in plasma

An expected increase in plasma proANP concentrations after induction of myocardial ischaemia was observed in the porcine model. In contrast to both the control animals and CD-NP-infused animals, BNP infusion resulted in an inverse plasma profile with decreased proANP concentrations after ischaemia and throughout the reperfusion period (figure 19).

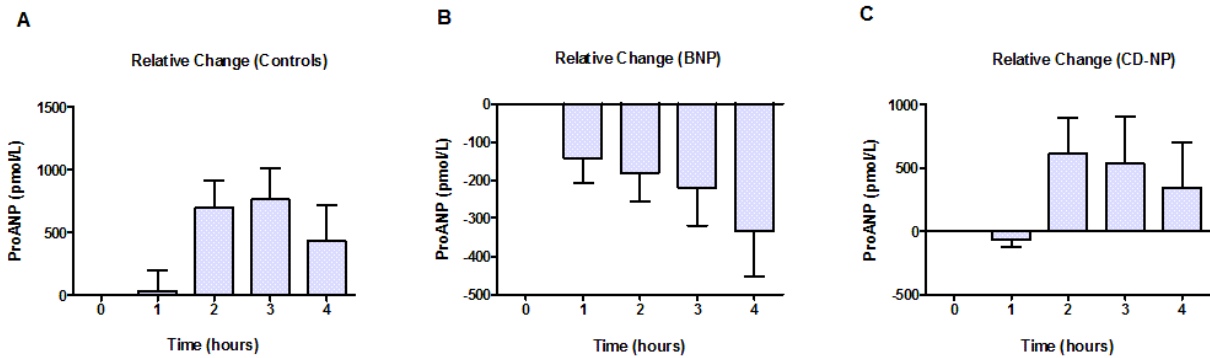


Figure 19 Endogenous ProANP concentration in plasma. Panel A shows the proANP response in plasma to acute ischaemia and reperfusion; panel B shows the same setup with BNP-32 infusion, and panel C shows the profile in CD-NP-infused animals.

BNP and ANP mRNA

The mRNA values were evaluated by RT-PCR (figure 20). The ANP mRNA content in the normoxic region was marginally reduced in the BNP-infused animals compared with controls. In contrast, there was no effect on BNP mRNA contents in the normoxic myocardium. Specific mRNA analyses of ANP and BNP transcripts in the hypoxic myocardium disclosed no difference in ANP mRNA content, whereas BNP mRNA content was higher in the BNP-infusion group compared with controls.

Substudy III: Lipid metabolism

The study included 28 pigs. Six pigs were excluded as mentioned in substudy I-II. No difference in plasma lipid baseline values was noted, except for cholesterol (table 3).

Table 2 Plasma lipid profile at baseline (T = 0). There was no difference in baseline values except for cholesterol in the BNP-infused animals.

Groups	Control (n=10)	CD-NP (n=5)	BNP (n=7)
Free fatty acids (nmol/L)	0.19±0.02	0.23±0.03 ns	0.16±0.02 ns
True triglyceride (nmol/L)	0.10±0.01	0.11±0.03 ns	0.10±0.01 ns
Glycerol (nmol/L)	0.09±0.01	0.10±0.01 ns	0.09±0.01 ns
Cholesterol (nmol/L)	2.21±0.11	1.99±0.11 ns	3.05±0.13***

Plasma lipids

Lipids in plasma were increased in both of the intervention groups compared with the untreated group during reperfusion. A 2.8-fold ($P < 0.0001$, figure 21A) free fatty acid increase was found in the BNP-infusion group (0.59 ± 0.03 mmol/L) compared with the control group (0.19 ± 0.02 mmol/L) at the end of the experiment. In the CD-NP group, a 2.1-fold ($P < 0.0001$) increase was found in the CD-NP group (0.45 ± 0.07 mmol/L) compared with the control group, with no differences between the BNP-32 and CD-NP groups. In extension, we observed a 2.1-fold ($P < 0.0001$, figure 21B) increase in plasma glycerol concentrations in the BNP-infused group (0.22 ± 01 mmol/L) and a 1.9-fold ($P < 0.01$) increase in the CD-NP infused animals (0.20 ± 0.02 mmol/L)

compared with the control animals (0.10 ± 0.02 mmol/L), with no significant differences between the BNP and CD-NP groups. There were no differences ($P < 0.31$) in triglyceride concentrations between the three groups at the end of the experiment [BNP group: 0.17 ± 0.02 mmol/L; CD-NP group 0.15 ± 0.02 mmol/L; and the untreated group: 0.13 ± 0.01 mmol/L]. Moreover, there was no difference in plasma cholesterol between the CD-NP group and the control group (data not shown). Finally, a difference in the BNP- versus the CD-NP-infused animals and the control group was noted also but with differences in baseline values (table 3).

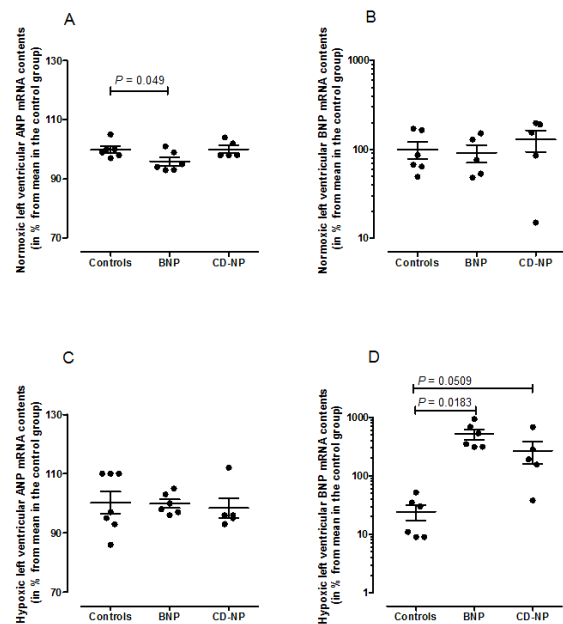


Figure 20 Effects of natriuretic peptide infusion on cardiac mRNA expression in percent from mean in the control group. Panels A and B show the ANP and BNP mRNA contents in the non-ischaemic/normoxic left ventricle. Panel C and D show the content of ANP and BNP mRNA in the ischaemic/hypoxic lesion. The horizontal bars mark the mean values in each group \pm SEM. Please note that some tissue samples were inadequate for RNA measurements.

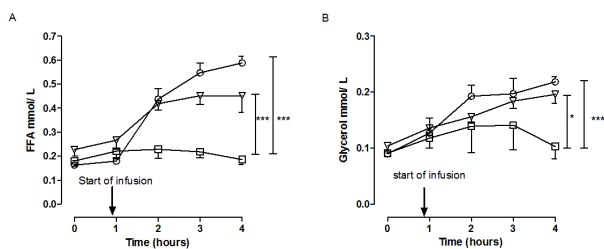


Figure 21
Plasma lipid concentrations in the BNP (-○-), the CD-NP (-△-) and the control group (- -). A: free fatty acids (FFA) in mmol/L and B: glycerol in mmol/L. * P < 0.05; *** P < 0.0005.

Cardiac lipid accumulation (triglycerides)

Thin layer chromatography (TLC) was performed on lipid extracted from right ventricular myocardial tissue from BNP-infused and control animals. We observed a marked increase in myocardial triglyceride content in the BNP infused group (3.01 ± 1.10 nmol/ mg wet weight (ww)) compared with the control group (0.64 ± 0.23 nmol/ mg ww, $P = 0.03$) (figure 22). In addition, there was a strong correlation ($r = 0.88$, $P = 0.0007$, 95% confidence interval [0.59; 0.97]) between cardiac triglycerides in myocardial tissue from the right ventricle and free fatty acids in plasma (AUC). No difference was found in tissue cholesterol between groups (figure 22).

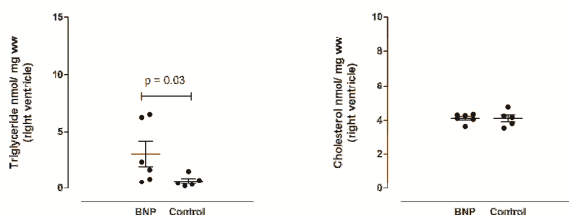


Figure 22
Cardiac lipid profile. Thin layer chromatography of cardiac tissue from the non-ischaemic part (the right ventricle) of the myocardium. Panel A shows the triglyceride contents (nmol/mg wet weight). Panel B shows the cholesterol contents (nmol/mg wet weight).

DISCUSSION

The results suggest that natriuretic peptides (both CD-NP and BNP) reduce myocardial injury, possibly partly via indirect mechanisms. Furthermore, BNP infusion specifically inverts the endogenous natriuretic peptide response as opposed to CD-NP and no infusion treatment. Finally, BNP infusion increases cardiac lipid accumulation, and both BNP and CD-NP increase plasma lipids.

THE CARDIOMYOCYTE PRESERVING EFFECTS

The first finding that natriuretic peptide infusion reduces cardiomyocyte injury was determined by a decrease in plasma TnT concentrations in the acute model. This finding was substantiated by RNA integrity measurements in the ischaemic tissue of the left ventricle that confirmed a better preservation of the myocardial tissue in the natriuretic peptide-infused animals. The results thus corroborate earlier findings of natriuretic peptide infusion/stimulation in different ischaemia/reperfusion injury models (111,112,115,139). The results were further supported by a 48-hour model determining whether TnT plasma concentrations in

this porcine model is single- or multi-phased. Cardiac TnT release to plasma was mono-phased, which strengthens the validity of TnT plasma concentrations measured in the acute model. However, TTC staining is considered the golden standard of evaluating myocardial damage. Regrettably, in our hands the method was not satisfactory. The main reason was probably difficulties in redefining AAR by Evans Blue in the closed chest model (for further discussion please refer to section 7.4, Limitations, subtitle: IS/AAAR). Nevertheless Troponin T is a cardiac specific biomarker and hence a very sensitive marker of myocardial damage (5). In the clinical setting TnT is both a diagnostic biomarker of AMI and also a strong prognostic marker of mortality and morbidity. Nevertheless, a disadvantage of TnT measurements in plasma is that both secretion as well as elimination is measured. Hence conditions where kidney perfusion is compromised TnT would be falsely increased.

We included a third group of animals in the acute model and subjected them to CD-NP infusion in order to determine whether the cardioprotective effects of BNP infusion was due to an indirect or direct effect on myocardial heart tissue. CD-NP had, at the time of the commencement of our studies, been reported to be less hypotensive than BNP (53,56). In the porcine model, however, we could not confirm this, and there was no difference in effect on mean arterial pressure compared with the BNP-infused animals. This finding raises the question of CD-NP receptor interaction and affinity in pigs. This has not been studied in the porcine model and is warranted if further investigation of CD-NP therapy in this model is to be pursued.

To pursue a possible direct cardiomyocyte preserving effect of BNP an in vitro study of ischaemia/reperfusion was performed. This study could not confirm a direct cardiomyocyte preserving effect in HL-1 cells and these results underscores an earlier study (140). Several factors may, however, contribute to the negative result. Shizukuda et al reported that 5 minutes of perfusion of dog hearts with hypoxic blood was sufficient to induce cardioprotection (141). The "hypoxia" period that preceded the full "anoxia" period in the in vitro set up could very well have induced a hypoxic preconditioning effect. However, most importantly: the stimulation doses compared with the dose used in the porcine study were fairly high. Whereas the concentration of BNP and CD-NP in the in vivo studies corresponds well with a previous study in neonatal rat cardiomyocytes that report stimulation with ANP 1nM may act anti-apoptotic (142), the doses in the in vitro study does not. The doses were chosen due to the cGMP measurements that were performed in the cell model to validate signal transduction elicited by BNP stimulation. However, Kato et al. pointed out that augmentation of cell survival is sensitive to stimulation intensity with high-level induction by ANP leading to apoptotic cell death rather than enhancing resistance to apoptotic stimuli (142). In addition, Gorbe et al. reported a clear inverse concentration dependency of the cGMP analogue 8-Br-cGMP in infarct-limiting action (118). The use of an immortalized cell line could also influence the results. Previous studies in our group (Bisgaard et al, unpublished results) have disclosed low BNP mRNA in this cell line. Furthermore, comparison of NPR expression levels with NPR expression levels in extracts from mice hearts, the NPR-C level was much higher than NPR-A and NPR-B levels. Since NPR-C acts a clearance receptor this may interfere with effects of BNP stimulation. However, since BNP stimulation is shown to increase cGMP in the HL-1 cells, this is likely not a possible explanation. Several aspects in validating cardiomyocyte damage could influence the results (as thoroughly discussed in manuscript I). A study on BNP stimulation in a cell culture re-

ported phospholamban phosphorylation in combination with a PKA inhibitor could be used as an endpoint in determining cardioprotective effects and the relationship with intracellular signalling pathways (118). The ongoing disclosure of which signalling pathways the natriuretic peptides act through will be helpful when deciding which endpoints to use when determining direct effects of natriuretic peptide stimulation in vitro. Nevertheless, it is likely that the cardiomyocyte preserving effect of natriuretic peptides may be related to both a direct cardiomyocyte preserving effect (through intracellular signalling mechanisms) and indirect peripheral effects due to vasodilation and RAAS inhibition.

THE ENDOGENOUS NATRIURETIC PEPTIDE RESPONSE

The second finding that BNP infusion specifically inverts the endogenous natriuretic peptide response was determined by proANP plasma concentrations. This finding corresponds with those of a small clinical study in humans (122). Interestingly, CD-NP infusion did not elicit an inverted endogenous natriuretic peptide response. This suggests a major difference in natriuretic peptide receptor interaction between BNP and CD-NP in the heart itself, which results in a BNP specific feedback signalling mechanism. In this respect, natriuretic peptide infusions are often considered to elicit the same intracellular mechanisms and response, while the present data strongly suggest that this is not the case. Furthermore, natriuretic peptide treatment may affect long-term synthesis and secretion differently than in an acute setting. Thus, tachyphylaxia may be expected. Long-term treatment should therefore be tested in an animal model. Also, further studies should clarify differences in natriuretic peptide treated patients compared with not treated patients. If using natriuretic peptide infusion as therapeutic in cardiovascular disease per se proANP concentration in plasma as prognostic marker will depend on the infused natriuretic peptide.

LIPID METABOLISM

The plasma lipid analyses suggest that cardiac lipid accumulation is associated with increased peripheral lipolysis. This effect does not seem to be BNP specific. Lipolysis was determined by an increase in plasma free fatty acids and glycerol. BNP and CD-NP infusion revealed similar effects on peripheral lipolysis defined as an increase in plasma free fatty acids and glycerol. Furthermore, the effects on global cardiac lipid accumulation mediated by BNP were assessed as cardiac lipid accumulation (triglycerides) in the non-ischaemic cardiac tissue (right ventricle). The results corroborate the finding of a peripheral lipolysis by a correlation between plasma free fatty acids and cardiac triglyceride accumulation in BNP-infused pigs. Regrettably, cardiac tissue from the CD-NP infused pigs was not sufficient to perform later lipid analyses. Previous studies have reported that atrial HL-1 cells incubated with oleic acid increase intracellular lipid storage and decrease ventricular ANP mRNA (76). Taken together, the data suggest that cardiac triglyceride accumulation per se may be due to excess in plasma free fatty acids as well as a cardiac shift to glucose metabolism. Since natriuretic peptides act lipolytic in adipose tissue it seems reasonable to suggest that this lipolytic action may also be induced in other NP-A containing tissues, i.e. cardiac, renal, and adrenal tissue (143). Non-ischaemic cardiac tissue was used in our studies. However, a chamber-specific distribution of natriuretic peptide gene expression has earlier been described (26). Further studies should examine cardiac tissue from all four regions of the heart (as well as non-ischaemic compared to ischaemic cardiac tissue) to determine the chamber-specific differences

in cardiac triglyceride accumulation and metabolism. Finally, cardiac lipid accumulation is associated with cardiac myocyte apoptosis, endoplasmic reticulum stress and contractile dysfunction (144-146). Although storage of intracellular triglycerides is relatively inert, other products formed during hydrolysis of triglycerides (such as diglycerides and fatty acids) and ceramides formed from fatty acids are toxic. In this respect, it is interesting that the direct cardioprotective action of cGMP-dependent PKG is reduced in hyperlipidaemic rats (85). This should be considered when evaluating natriuretic peptides as a putative therapy in cardiovascular disease.

LIMITATIONS

The experimental model

In general the use of experimental models can be discussed. In our model, Copeptin, the C-terminal part of the vasopressin pro-hormone was already dramatically increased at the beginning of the experiment (unpublished results). This was further corroborated by relatively high concentrations of Chromogranin A, a neuroendocrine secretory hormone (Frydland, Kousholt et al, manuscript in preparation). This suggests that, in spite of choosing a closed chest model, it is a difficult task to avoid neuroendocrine stress in the experimental setting.

Species, gender and age

We studied juvenile, healthy female pigs with induced regional cardiac ischaemia. However, human acute coronary syndromes mainly affect middle-aged to old people and is caused by a complex pathophysiology characterized by atherosclerotic lesions and development of collateral blood supply and eventually coronary vessel obstruction. Ideally, an animal model of a disease must closely resemble the disorder in humans with respect to both structural and functional characteristics, also with respect to pathophysiology. Investigating a primarily atherosclerotic induced disease such as AMI should be performed in an atherosclerotic animal model. When using a larger animal model, the pig has proven prone to atherosclerosis and therefore is useful as an atherosclerotic model. In the animal research facility at Aarhus University steps are taken to create such a model. However, the model poses several difficulties. It is a very time-consuming and expensive model. The animals have to be fed a high-cholesterol diet over several months and for this reason become extremely overweight which poses difficulties in care as well as challenges animal welfare ethics (147). It is highly relevant to study BNP infusion in a larger atherosclerotic animal model since hyperlipidaemia has shown to deteriorate the cGMP-dependent cardioprotection induced by BNP stimulation in rats (85). The influence of gender was minimized by using sexually immature animals. However, the use of young animals may pose other challenges to our results than mentioned above, as senescence per se is believed to influence the natriuretic peptide system.

Anaesthesia and medication

The animals were of course anaesthetised during the intervention and amiodarone and potassium was administered to avoid arrhythmias. Both anaesthesia and anti-arrhythmias could act cardioprotective per se and influence the outcome. However, this bias was minimised by medicating all animals equally. The advantage should be to measure a whole population and not just the "strongest" animals which must be considered a severe bias.

IS/AAR

Infarct size over area at risk is considered golden standard for evaluation of myocardial damage and salvage between intervention groups in experimental cardiology (87). The manual delineation by an observer can be performed with good precision, if the myocardial slices are stained brightly by Evans Blue dye and TTC, and have a high contrast to the surrounding tissue. However, no common standard for the intensity of colours seem to exist and the observer individually defines the delineation of the different areas. This fact makes it prone to bias, and both inter- and intra-observer variation is liable to occur if the tissue staining is insufficient for a clear delineation of the areas. Interestingly, inter- and intra-observer variation is often not reported. Unfortunately, the method in our hands was not satisfactory. This might be due to the difficulties in delineating the precise area at risk when choosing a closed chest model. The model was chosen to minimize the neuroendocrine response to major surgery. This leaves, however, the occlusion site to be visualized by angiography and the exact occlusion site can be difficult to re-occlude. For secure delineating of the area at risk, it might be advisable to use an open chest model where no bias is introduced when re-ligating the vessel. Furthermore, due to the fact that blood perfuses the heart as opposed to a non-coloured buffer (Krebs-Henseleit solution) used in the Langendorff preparation it can be difficult to visually delineate the area at risk in the TTC stained tissue slides. We therefore delineated AAR at once in the Evans Blue stained tissue slides and IS in the TTC stained slides. In this respect, designing and validating an operator independent computer image analysis might also further add objectivity to the method. This has been reported in a rat model of cerebral ischaemia (148).

Surrogate markers of cardiomyocyte damage

Instead of planimetry to define IS/AAR, we based our conclusions of myocardial damage on measurements of the cardiac specific biomarker TnT. TnT is a cardiac specific marker of cardiomyocyte damage. However, the troponins have previously been questioned as being cardiac-specific (149). Nevertheless, our assay utilizes an epitope which is similar in human, mouse, rat, and pig and the young pigs are not prone to the diseases listed by Djalili et al as bias. In a canine model of myocardial infarction the decrease in tissue TnT levels in left ventricle ischaemic tissue correlated well with infarct size three weeks after infarction (150). As mentioned in the discussion (section 7.1) a disadvantage of TnT measurements in plasma is that both secretion and elimination is measured. This will affect the outcome in case of a decrease in kidney perfusion and TnT values will be falsely high. In addition, to our acute studies we performed a 48-hour study to disclose whether there was a single- or multiphase TnT release was seen. Furthermore, since biological variation in coronary anatomy must exist we substantiated our TnT measurements in the acute model with mRNA analysis to validate the integrity of the ischaemic myocardial tissue (RIN).

The challenge of translating experimentally obtained results in to the clinical setting

In the experimental setting, the ischaemia/reperfusion set-up is very strict e.g. control of temperature, duration of ischaemia and reperfusion etc. Certainly, this is not comparable with the clinical setting where patients arrive at the emergency room on different time points in relation to onset of ischaemia and often have comorbidities such as diabetes and hypertension which may alter the natriuretic peptide receptor expression as well as release and modification of natriuretic peptides. The intercellular interaction

of natriuretic peptides is reported to change (85) or be supplemented in chronic heart disease patients (121). Indeed this will change the responsiveness of natriuretic peptide therapy in patients (6). The translation of different treatment strategies from the experimental setting to the clinic has been discussed as a major challenge by Hausenloy and colleagues (151).

Natriuretic peptide blocker

Two different natriuretic peptides versus a control group were examined. No specific NPR-A blocker has been validated in pigs and could not be incorporated in the study. Retrospectively, a positive control group e.g. ischaemic preconditioning could have validated the results further (152). In theory, a blood pressure lowering pharmaceutical devoid of any other actions, could clarify whether the tissue-preserving effects of natriuretic peptide infusion were solely due to cardiac unloading or due to, at least in part, other physiological actions.

Dose

Natriuretic peptides bind their receptors in a concentration-dependent manner and their physiologic effects are dose-dependent (112). We chose a physiological dose but could have considered a group receiving 10-fold less and 10-fold more to further evaluate the response and substantiate the results.

Endogenous response

RT-PCR is a quantitative measure of mRNA. It is an indirect measure of the protein synthesised and thereby a transcriptional marker of gene expression. ANP peptide release was quantitated by plasma proANP measurement. This was not done for BNP, since the method is much more cumbersome and generally not well validated in pigs. The measurement of proBNP and proCNP would have been desirable to elucidate any differences in endogenous natriuretic peptide response.

Cardiac lipid accumulation

The results indicate a peripheral lipolytic action but needs to be elaborated further by peripheral tissue measurements, and relevant protein analysis (such as phosphorylated HSL) and lipid metabolising genes in cardiac tissue to determine the exact metabolic effects of natriuretic peptides in the ischaemic challenged heart tissue.

CONCLUSIONS

- BNP and CD-NP infusion both reduce infarct size measured as decreased plasma Troponin T release and better preserved RNA integrity in the ischaemic tissue in a porcine model of regional cardiac ischaemia and reperfusion.
- A direct effect on cardiomyocytes could not be determined.
- Troponin T measurements in a 48-hour porcine model of regional cardiac ischaemia and reperfusion revealed a single-phased TnT release during the first few hours after onset of reperfusion.
- BNP - and not CD-NP - inverses the endogenous natriuretic peptide (proANP) secretion in a porcine model of regional cardiac ischaemia and reperfusion.
- BNP infusion increases cardiac lipid accumulation in a porcine model of regional cardiac ischaemia and reperfusion.

The three substudies in this thesis highlight some of the diverse actions natriuretic peptides possess and furthermore express the

complexity of natriuretic peptide hormone interaction during peptide infusion in a porcine model of regional cardiac ischaemia.

PERSPECTIVES

When de Bold and colleagues discovered the first natriuretic peptide they must have sensed that this was a pivotal scientific discovery. However, they could hardly predict the massive contribution they had just made to the elucidation of cardiovascular physiology and treatment strategies. In the United States of America and Japan, natriuretic peptide analogues (Nesiritide and Carperitide) have been approved for the treatment of acute congestive heart failure. Different studies and researchers either support or discourage the use of natriuretic peptide therapy in severe heart failure (153-155). As adjunctive cardioprotective therapy in cardiac ischaemia, the peptides appear promising. Nevertheless, it seems too early to bring natriuretic peptides into the clinical setting as adjunctive postconditional therapy for cardiac ischaemia and reperfusion. This is due to the multi-physiological actions that the peptides possess in the mammalian organism. Several aspects must be further explored. First, further investigation is needed to determine the extent of the direct effect on cardiomyocytes as well as the indirect peripheral actions with regard to cardioprotection. Also, the different natriuretic peptides should be evaluated against each other to find the most suitable peptide to be used in the treatment strategy. Furthermore, elucidation of the effect of natriuretic peptide infusion on the reliability of the endogenous natriuretic peptides as prognostic biomarkers in cardiovascular disease should be pursued. Finally, metabolic interactions of a natriuretic peptide infusion in cardiac tissue challenged by ischaemia should be elaborated to clarify the increase in lipid accumulation versus altered lipolysis. A recent study in a porcine model disclosed both feasibility and safety of fluoroscopy guided intra-myocardial gene injection (156). This opens possibilities for local myocardial delivery of natriuretic peptides

Complicating matters 2011

A paper recently published in JAMA by Canto and colleagues complicates matters further regarding cardioprotection in the clinical setting (157). Canto and colleagues show that in-hospital mortality is inversely correlated to common risk factors for myocardial infarction including diabetes, hypertension, smoking, dyslipidaemia and a family history of coronary heart disease. Previous studies have convincingly established that these risk factors predispose to atherosclerosis and myocardial infarction. However, these new data suggest that in patients with first time acute myocardial infarction, the prognosis for survival is slightly improved in patients with risk factors compared with patients without. Several explanations may be relevant here. One could speculate whether the myocardium in these patients in fact already have been pre-conditioned (partly by the endocrine heart itself?) with angiogenesis to withstand the impact of coronary artery occlusion. A different explanation could be that patients with established risk factors are better, i.e. more intensely, treated and monitored. As of now, cardioprotection per definition covers all means for reducing cardiac damage, and some factors may even be ones that caused the problem in the first place.

SUMMARY

Natriuretic peptides elicit vasodilation, increased sodium excretion and concomitant diuresis, and counteract the RAAS. In the heart itself, natriuretic peptides may also act anti-inflammatory

and antifibrotic. This has led to the pursuit of natriuretic peptides and chemically modified peptides as adjunctive therapy in myocardial ischaemia. However, natriuretic peptide infusion may also influence the endogenous natriuretic peptide response and lipid accumulation. We hypothesised that a) natriuretic peptide infusion (BNP and CD-NP) is cardiomyocyte protective, b) affects the endogenous response, and c) facilitate cardiac lipid accumulation. We examined these effects in a minimally invasive porcine model of regional cardiac ischaemia and reperfusion. The studies were supplemented by a 48-hour porcine model of ischemia and reperfusion as well as an in vitro study of BNP administered in a HL-1 cell model of "ischaemia/reperfusion". Infarct size was determined by TTC staining, plasma troponin T release, and total RNA integrity in cardiac tissue samples. The endogenous response was assessed by a processing-independent proANP immunoassay and mRNA quantitation. Lipids in plasma and myocardial tissue were determined by TLC. The studies show that natriuretic peptides decrease cardiomyocyte damage, possibly partly through indirect mechanisms. Furthermore, BNP infusion completely inverts the endogenous response, whereas CD-NP infusion does not. Finally, both natriuretic peptides increase plasma free fatty acids, which is associated with an increased cardiac lipid accumulation in non-ischaemic myocardium. In conclusion, the studies suggest that natriuretic peptides are beneficial in terms of reduced cardiac injury. In addition, the endogenous natriuretic peptide response is inverted. The results advocate for pursuing natriuretic peptide treatment in ischaemia/reperfusion damage. However, the metabolic consequences in a cardiac tissue challenged by ischaemia should be pursued before testing the peptides in patients.

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