Learta Pervizaj Oruqaj

Effect of pathophysiological stressors on the tissue expression of parathyroid hormone-related protein (PTHrP)

INAUGURAL-DISSERTATION
submitted for the Degree of
Doctor of Human Biology
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Justus Liebig University, Gießen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2016

1st Edition 2016

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

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by

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2016

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Date of disputation: 21.09.2016

This thesis is dedicated

to

the memory of my parents

to my husband, my lovely daughter and

my family

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1. Introduction - Parathyroid hormone related protein

Fuller Albright, in 1941 was first who reported a case with renal cell carcinoma, increased calcium and reduced phosphorus, in New England Journal of Medicine (Martin, Allan et al. 1989). The parathyroid cancer was excluded in this patient, so he thought that hypercalcemia and low phosphorus might be a result of a parathyroid hormone (PTH)-like factor, produced and secreted by the cells, which could not be from the parathyroid glands (Martin, Allan et al. 1989).

Later on using several anti-PTH sera, it was determined that PTH is not present in tumor samples from patients with hypercalcemic cancers. Thereby, it was deduced that a PTH-like substance causes the hypercalcemia derived from cancers, yielding the term humoral hypercalcemia of malignancy (HHM). This term indicates the hypercalcemic patients suffering from certain cancers without any metastasis in bone (Martin, Moseley et al. 1991).

During 1980-1990, its role in the syndrome named as HHM, the peptide identification and cloning of the cDNA were established. Further research revealed more information about the peptide regarding its expression and function. It was clarified that a factor, structurally related to PTH and named PTH-related protein (PTHrP) is expressed under normal circumstances, in a large number of cells and organs such as teeth, mammary glands, cardiovascular system, placenta, smooth muscle, respiratory epithelial cells, renal cells etc. and in some forms of tumors (Philbrick, Wysolmerski et al. 1996).

As indicated above, PTHrP firstly was discovered as a product of some solid tumors, nowadays it is considered to act in a paracrine, autocrine or intracrine manner under physiologic and pathologic conditions in numerous cells and tissues. Various biological effects have been attributed to its NH_2–terminal, midregion and carboxyl regions. PTHrP peptides play a pivotal role in development of many tissues and can modify the growth, differentiation, proliferation, function, and death of various cell types (Strewler 2000).

1.1. Structural properties of parathyroid family

PTHrP is a member of PTH family. This hormone family encompasses proteins with structural and functional similarities. The family members are parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP) and tuberoinfundibular peptide of 39 residues (TIP39) (Martin, Moseley et al. 1991).

A great homology was discovered between PTH and PTHrP, as 8 of the first 13 amino acids were identical. This similarity in the amino acid residues goes down in the region between 14-34 residues and thereafter there is no more resemblance. The structural similarities made it clear that this might have derived from gene duplication of a common ancestral gene, however it is still unknown which of them is the original gene (Suva, Winslow et al. 1987). PTHrP is encoded by PTHLH gene located on chromosome 12 (Suva, Mather et al. 1989), whereas PTH gene is located on chromosome 11 (Mannens, Slater et al. 1987). These two chromosomes are believed to derive from duplication process from a common ancestral chromosome (Comings 1972).

PTH comprises an 84 amino acid chain. It is produced by parathyroid glands and plays an important role in calcium homeostasis. Despite the fact that the structure of these two peptide hormones resemble to each other, they express their effects in the same way in classical target cells as such osteoblasts, osteoclasts, chondrocytes and tubule cells deriving from bone and kidney, respectively, but distinct effects on other cells (Schluter 1999).

PTHrP from rats is made up of 141 amino acids while in humans PTHrP family consists of three fragments (Schluter 1999) as such: PTHrP (-1–139), PTHrP (-1–141), and PTHrP (-1–173). Posttranslational modifications of these peptide fragments results in the generation of N-terminal and C-terminal PTHrP peptides. The N-terminal includes the region from 1-36 amino acids, which activates PTH1 receptor (Orloff, Reddy et al. (1994); Philbrick, Wysolmerski et al. (1996)). Mid-region includes peptides 38-94, 38-95, and 38-101 (Soifer, Dee et al. 1992) which is thought to modulate renal bicarbonate handling and stimulates the placental calcium transport needed for fetal skeletal development (Kovacs, Lanske et al. 1996). The C-terminal includes peptides 107-138 and 109-138, a region that inhibits osteoclast function and stimulates osteoblast proliferation. The actions regarding mid-region and the C-terminal of PTHrP are supposed to be mediated by other receptors than PTH1, receptors specific to these PTHrP regions, which are not yet known (Philbrick, Wysolmerski et al. 1996).

PTHrP displays its effects in an autocrine/paracrine fashion but also via a so-called intracrine pathway. The later one includes the translocation of the nascent protein into the nucleus. So that, PTHrP in its 88 ± 107 amino acid region carries a nuclear localization sequence (NLS), a sequence similar to the NLS in mammalian and viral transcription factors. This region of PTHrP regulates cell proliferation and apoptotic cell death (Clemens, Cormier et al. 2001).

Even though PTH and PTHrP bare some resemblance, they vary from each other as follows (Schluter 1999):

- the peptide chain of PTHrP is longer, being extended at the C-terminal
- PTHrP is expressed under physiological and malignant conditions
- PTHrP displays the effects in a paracrine and autocrine fashion

As regards TIP39, its structure slightly resembles the two other peptides of the parathyroid family. It has four amino acids similar to both PTH and PTHrP, and six amino acids identical with PTH (Usdin, Wang et al. 2000). However, high resolution NMR studies point out homology in three-dimensional structure between peptides (Piserchio, Usdin et al. 2000). TIP39 is predominantly expressed in two regions of the brain, and it is considered as a neuroendocrine hormone that regulates the stress response and body temperature (Dobolyi, Dimitrov et al. 2012).

1.2. Receptors of parathyroid family

The significant sequence homology between PTH and PTHrP gives the opportunity to bind and activate a common receptor, the so-called PTH-1R, or PTH/PTHrP receptor cloned in rat, mouse, and human. Actually, the N-terminal part (34 amino acids) of PTH and the amino-terminal peptides of PTHrP share the same receptor-binding domain. However, the midregion and osteostatin peptides bind other receptors, specific to these domains of PTHrP (Mannstadt, Juppner et al. 1999).

PTH-1R is a member of secretin family of G-protein coupled receptor (GPCR) with seven membrane helixes (Abou-Samra, Juppner et al. 1992). It is mainly expressed in kidney and skeletal tissues but it is present in various other tissues as well. In kidney and bone, it is important in the PTH-dependent regulation of mineral ion homeostasis. It mediates the autocrine/paracrine actions of PTHrP as endochondral bone formation (Juppner, Abou-Samra et al. 1991).

The type II receptor binds both PTH and TIP39, except PTHrP (Usdin, Gruber et al. 1995). PTH2 receptor is more located in brain and testes, and to a less extent in other tissues (Usdin, Bonner et al. 1996). TIP39 has a quite distinct structure than PTH and PTHrP and much more ability than PTH to activate selectively the type II receptor and consequently the adenylyl cyclase pathway (Clemens, Cormier et al. 2001).

1.3. Classical signal transduction pathways of PTHrP

As mentioned previously amino terminal fragments of PTHrP (1-36) and PTH (1-34) act through the same receptor, the PTH-1R. It is believed that the type I receptor may be an essential pharmacological target for curing some functional disorders like HHM, mineral ion homeostasis, and osteoporosis (Clemens, Cormier et al. 2001).

Binding and activating the PTH-1R by one of the abovementioned peptides leads to activation of two second messenger pathways: the adenylyl cyclase/protein kinase A signalling pathway and the phospholipase C/protein kinase C signalling pathway. As PTHrP binds to G-protein coupled receptor, via stimulatory G-alpha proteins ($G\alpha_s$), it activates adenylate cyclase (AC) and makes feasible the formation of cyclic 3`, 5`-adenosine monophosphate (cAMP). Afterward, cAMP activates protein kinase A (PKA) in these cell types: kidney cells, vascular smooth muscle cells, chondrocytes, and osteoblasts (Loveys, Gelb et al. (1993); Maeda, Wu et al. (1996)).

In the case when PTHrP signals via G-α proteins ($G\alpha_q$) (in other target cells like pancreatic islet cells and carcinoma cells), it activates phospholipase C (PLC) β, causing the diacylglycerol (DAG) formation and this leads to protein kinase C (PKC) activation and 1,4,5 inositol triphosphate (IP3) formation and consequently, the level of intracellular free Ca^{2+} is elevated. MCF-7 breast cancer cells and skin fibroblasts were suggested as the probable case where after binding of PTHrP to PTH-1R, the both pathways mentioned above can be activated (Fortino, Torricelli et al. (2002); Maioli, Fortino et al. (2002); Maioli and Fortino (2004)).

1.4. Physiological effects of PTHrP

In spite of the fact that PTHrP was discovered as a factor released from tumors, it acts locally in many tissues as a paracrine, autocrine or intracrine factor regulating a wide variety of physiological processes. There are some cases, that some PTHrP species, unknown till now, act as endocrine factor and are present in the circulation (Suva, Winslow et al. (1987); Lippuner, Zehnder et al. (1996); Hiremath and Wysolmerski (2013); Vanhouten and Wysolmerski (2013)) as:

- in humoral hypercalcemia of malignancy, where the peptide is released from the tumor
- during fetal development, in which case the peptide is produced by the parathyroid glands of the fetus and manages the placental calcium transport
- lactation where PTHrP is produced in the mother's breast and is present in the circulation

1.4.1. Cardiovascular system

Atrial and ventricular cardiomyocytes, smooth muscle cells, and pacemaker cells constitute the target cells for PTHrP in the cardiovascular system. The peptide is expressed in different cells as endothelial cells, atrial cardiomyocytes, and smooth muscle cells of the cardiovascular system. Cardiac myocytes and smooth muscle cells express PTHrP and the corresponding receptor PTH-1R thereby, activating PKA, a pathway, which leads to vasorelaxation (Schluter and Piper 1998). Moreover, PTHrP is believed to be involved in the proliferation of vascular smooth muscle cells, suggesting that the peptide may have an important role in vascular remodelling, specifically in the development of neointima after arterial injury (Pirola, Wang et al. (1993); Maeda, Wu et al. (1996); Massfelder, Fiaschi-Taesch et al. (1998); Song, Fiaschi-Taesch et al. (2009)). PTHrP knockout mice experiments demonstrate higher proliferation rate of vascular smooth muscle cells compared to the wild-type mice. This indicates that PTHrP may play a role as a physiological regulator of vascular smooth muscle cell proliferation (Massfelder, Dann et al. 1997).

Under circumstances like mechanical deformation of the arterial wall, release of a vasoconstrictor substance as angiotensin II, or arterial pressure increase, the production of PTHrP increases. In such cases, PTHrP acts through its receptor PTH-1R activating PKA and relaxing the vasculature. Vasoconstrictors that lead to the increase of PTHrP mRNA and protein levels are endothelin, angiotensin II, noradrenaline (Pirola, Wang et al. 1993).

PTHrP (1-34) exerts positive chronotropic and inotropic effects on isolated and perfused rat hearts (Nickols, Nana et al. 1989) but on cellular level, to be more specific in adult ventricular cardiomyocytes, it activates adenylate cyclase and stimulates their contractile response (Schluter, Weber et al. (1995); Schluter, Weber et al. (1997)).

1.4.2. The Skeleton

Researchers investigating the HHM syndrome found out the circulating PTHrP protein as a source of series events happening in this disorder. This discovery urged the scientists to study more and more the functions and effects of this peptide hormone (Suva, Winslow et al. 1987). As a result, it was detected that PTHrP shows a variety of actions including smooth muscle relaxation and regulation of cellular proliferation and differentiation (Strewler 2000).

The genesis of mesenchymal condensations indicates the starting of bone development. Groups of mesenchymal cells gather and initiate a genetic program. In some of the bones as in the flat bones of the skull, these collections then directly differentiate into osteoblasts. However, there are a great number of bones where mesenchymal cells differentiate into chondrocytes and an adjacent perichondrium. Endochondral bone formation is a process, which makes feasible the chondrocytes conduct the differentiation of perichondrial cells into osteoblasts (Kronenberg 2003).

PTHrP is released from chondrocytes and is very important for endochondral bone formation. The peptide is released firstly by immature chondrocytic cells in response to a molecule the so-called Indian Hedgehog (IHH) secreted by differentiating hypertrophic chondrocytes. Then PTHrP triggers the corresponding receptor the PTH-1R, found in proliferating and prehypertrophic cells, and carries on their proliferation and decelerates the differentiation into hypertrophic cells. This is the way, how these both molecules IHH and PTHrP cooperate in order to regulate the chondrocyte differentiation. It was proved that the amino terminal PTHrP interacts with the corresponding receptor the PTH-1R, in order to maintain the continuation of chondrocyte differentiation, and regulating the growth of long bones during development (Kronenberg 2006).

As it is shown in Fig.1, on chondrocytes PTHrP displays its actions via the PTH-1R, whereby it stimulates Gs, cAMP production and protein kinase A activity. Thereafter a chain of downstream

processes is thought to take place as the inhibition of p57 expression, Bcl-2 and cyclin D1 expression, the induction of Gli3, the phosphorylation of SOX9, and the phosphorylation and degradation of Runx2 and Runx3. The latest ones Runx2 and Runx3 are important transcription factors for chondrocyte differentiation (Hilton, Tu et al. (2005); Koziel, Wuelling et al. (2005); Kronenberg (2006)). Signalling pathways shown here and certainly multiple others, which support them are involved in the process of bone development.

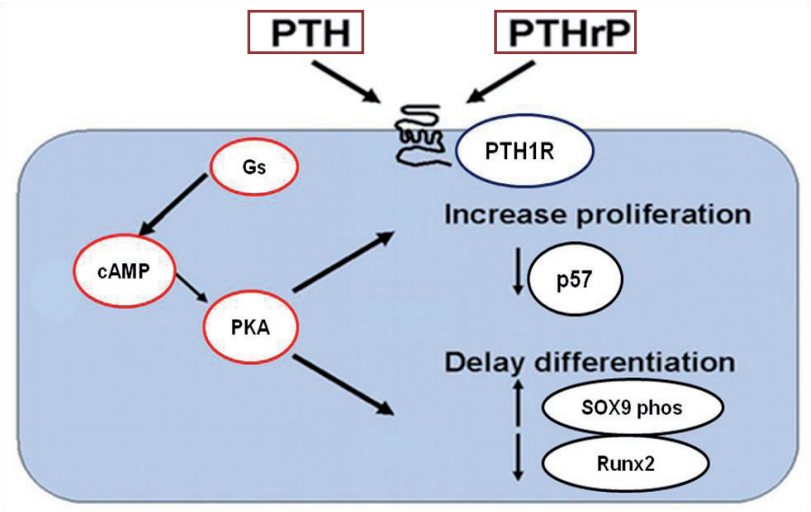

Figure 1. Intracellular signalling pathway of PTH/PTHrP receptor on chondrocytes (modified from Kronenberg 2006).

1.4.3. Mammary Gland

PTHrP is widely distributed in a variety of non-malignant tissues, and plays a pivotal role during fetal organogenesis and tissue differentiation (Ferrari, Rizzoli et al. 1994).

Mammary epithelial cells express PTHrP since initial phases of mammary gland development. In mice, at the stage when mammary bud formation starts, PTHrP is produced in epithelial cells. Furthermore, the peptide interacts with its corresponding receptor PTH-1R found on nearby mesenchymal cells. The loss of this signalling as in the case of PTHrP or PTH-1R knockout mice

causes a discontinuity of the interaction between epithelial and mesenchymal cells (Wysolmerski, Cormier et al. 2001).

During mammary gland development if the expression of PTH-1R is reduced it may influence the nipple formation and delivering of milk during lactation (Kuenzi and Sherwood 1995). According to this study, during mammary gland development, decreased PTH-1R signalling correlate with reduced smooth muscle. Smooth muscle cells are influenced from relaxin action in the nipple and in numerous other tissues (Kuenzi and Sherwood 1995). Relaxin is a peptide hormone that belongs to insulin family and in female is produced by placenta, during pregnancy, corpus luteum of the ovary and breast. Relaxin knockout mice model shows that, nipples in the absence of relaxin does not grow during pregnancy. The study suggests that failure of nipple growth during pregnancy is because small size of smooth muscle beds and absence of connective tissue remodeling. Finally, during mammary gland development, the main cause of this reduced receptor signalling is a mesenchyme, which later on is capable of producing enough smooth muscle in the nipple, the important factor for regulating processes during pregnancy and lactation (Zhao, Roche et al. 1999).

The PTHrP role except during breast development, it was found to be important during lactation, where it regulates the systemic calcium metabolism. In lactation large amounts of calcium are needed and maternal skeleton is considered as a supplier of that in order to produce milk (Kovacs 2001). During lactation, CaR is also expressed from the breast, which regulates PTHrP secretion in reply to calcium transport to the breast. In the case of increased calcium transport to the breast, it suppresses PTHrP secretion (VanHouten, Dann et al. 2004).

1.4.4. Placenta

Calcium transport is a crucial process during pregnancy, accomplished through placenta from mother to fetus. PTHrP seems to be very important in promoting calcium transport seeing that PTHrP deficient fetuses are hypocalcemic. Moreover, the calcium is transported against a gradient, so there is a higher calcium concentration in fetus than in the mother (Kovacs, Lanske et al. 1996).

PTHrP is essential for maintenance of normal fetal calcium concentration (Kovacs 2001). The peptide is secreted from placenta and this is regulated by CaSR (Kovacs, Ho-Pao et al. 1998). It was shown that responsible for placental calcium transport is particularly the midregion of PTHrP (Kovacs, Lanske et al. 1996).

1.4.5. Renal effects

The presence of PTHrP in the normal fetal and adult kidney was proved by a variety of studies. It binds to an abundant PTH1 receptor (Lee, Brown et al. 1996). In kidney, PTHrP and PTH-1R are expressed in the vascular smooth muscle cells where PTHrP displays vasorelaxant effects (Clemens, Cormier et al. 2001). Studies performed in transgenic mice show that overexpression of the peptide or the corresponding receptor in the arterial wall of the animals yield hypotensive effects, a case that is thought to be mediated by NO and cAMP (Qian, Lorenz et al. 1999).

Numerous cellular responses to PTHrP are mediated by cAMP. One of them, the so called phosphaturic response takes place via protein kinase A increase and protein kinase C-mediated internalization of the (Type II) Na/PO_4 co-transporter which lead to reduced phosphate reabsorption (Murer, Hernando et al. 2003).

It was suggested that local PTHrP influences the glomerular filtration rate in the glomerulus. Locally administered N-terminal portion PTHrP in hydronephrotic rat kidney model resulted in vasodilatation of preglomerular vascular segments and renal blood flow increase (Endlich, Massfelder et al. 1995). In addition to that, increase in glomerular filtration rate and urine flow was observed when infusion of N-terminal portion PTHrP was administered intrarenal into the left renal artery of anaesthetized rats (Massfelder, Parekh et al. 1996). These studies suggest that locally secreted PTHrP may be involved in the glomerular filtration rate and renal blood flow.

In renal tubular cells PTHrP enhances the reabsorption of calcium in the distal tubule, however inhibits the reabsorption of bicarbonate and phosphate and Na^+/H^+ exchange. In the renal proximal tubule, it induces the activity of 1α-hydroxylase enzyme (Maeda, Wu et al. 1998). However, numerous studies suggest the involvement of PTHrP in various kidney diseases.

1.5. Role of PTHrP in Lung Development, Homeostasis and Repair

The lung is an organ, which enables the gas exchange. Lung development and maturation is crucial for survival especially during the transition to postnatal life (Jobe and Ikegami 2001). Branching morphogenesis and alveolization (Torday 2014) are two stages of mammalian embryonic lung development.

PTHrP is a stretch-regulated (Torday and Rehan 2002) gene implicated in lung development. It is essential for the formation of alveoli, structures necessary for adaptation to cyclic inspiration/expiration of oxygen and CO_2 (Torday and Rehan 2004). It was proved that lack of PTHrP gene leads to failure to form alveoli, which is lethal for the newborn due to pulmonary insufficiency (Torday and Rehan 2004).

As shown in Fig. 2, pulmonary PTHrP is expressed in alveolar type II cells, and PTH-1R localizes nearby, in the adepithelial mesenchyme. The expression pattern of both enables formation of a paracrine loop for epithelial mesodermal interactions, which is considered an important pathway regarding lung development, homeostasis, and repair (Doi, Lukosiute et al. 2010).

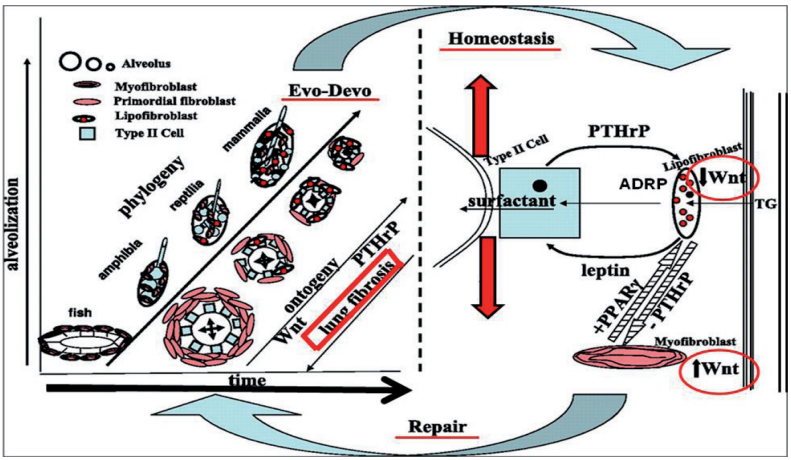

Figure 2. Model of lung evolution, development, homeostasis, and repair (modified from Torday and Rehan 2007).

The left side of the figure displays the comparison between lung evolution from the fish swim bladder to the mammalian lung and the development of the mammalian lung. It is evident that alveoli get smaller through phylogeny, alveolar myofibroblasts decrease and lipofibroblasts increase (Torday 2014).

The right part of the figure demonstrates the importance of the epithelial-mesenchymal cross-talk for lung homeostasis. PTHrP stimulates surfactant production through this paracrine feedback loop between lung endoderm and mesoderm. Binding of PTHrP to its receptor downregulates the myofibroblast Wnt signalling and upregulates the cyclic AMP-dependent Protein Kinase A (PKA) signalling pathway. Stimulation of this signalling pathway leads to upregulation of the interstitial lipofibroblast differentiation, PTH1R, and downstream molecules like ADRP, leptin, triglyceride uptake, by stimulating PPAR-γ expression (Torday, Torres et al. 2003). Leptin secreted from lipofibroblasts binds to the type II cell leptin receptor, and stimulates the differentiation of the epithelial type II cell (Torday, Sun et al. 2002), a process determined by epithelial-mesenchymal cell interactions (Rutter, Pictet et al. 1978). Leptin by the paracrine mechanism (from epithelium to mesenchyme and back to epithelium) stimulates the surfactant production and protein synthesis by epithelial type II cells (Kirwin, Bhandari et al. 2006).

The synthesis and secretion of surfactant increases late in gestation when the lungs of fetus are already matured (Kirwin, Bhandari et al. 2006), and is particularly essential for survival at the time of transition from intrauterine to extrauterine life. It reduces the surface tension at this period, thus preserving the stability of lung and preventing the collapse of alveoli (Kirwin, Bhandari et al. 2006). Pulmonary surfactant is composed of proteins, phospholipids, carbohydrate, and lipids. Phospholipids (Torday and Rehan 2002) are responsible for reducing the surface tension. ADRP is thought to be necessary for uptake, storage, and trafficking of neutral lipid from lipofibroblast to the alveolar type II cell for surfactant production (Schultz, Torres et al. 2002).

It was shown that any impairment to the epithelium caused by nicotine, infection, prematurity (Cerny, Torday et al. 2008) or lack of PTHrP (Torday, Torres et al. 2003) impair the cross-talk between epithelium and mesoderm. In this case, Wnt signalling increases, PTH1R and downstream molecules are downregulated, lipofibroblasts transdifferentiate to myofibroblasts, condition that may lead to loss of homeostasis and eventually to lung fibrosis (Torday, Torres et al. 2003) or other chronic lung diseases. The model of lung evolution, development, homeostasis, and repair (Torday and Rehan 2007) is a key point regarding novel diagnostic and therapeutic targets.

1.6. Role of PTHrP in Sepsis

Sepsis is a systemic immune response to infection. It can result in multiple organ dysfunction syndrome (earlier named as multiple organ failure) and death, which indicates severe sepsis. Septic shock implies a condition with inappropriate organ function due to insufficient blood supply, hypotension, leading to ischemia and particularly collapse of the cardiovascular system (Nemzek, Hugunin et al. 2008).

In research experiments, a cell wall component of gram-negative bacteria-lipopolysaccharide (LPS) is used to create a sepsis model to investigate the inflammatory response. Humans are considered more sensitive to LPS dose. Therefore, the dose administered to animal is generally 250 times higher than the dose causing sepsis in humans (Copeland, Warren et al. 2005).

Tumor necrosis factor (TNF) was firstly discovered by (Carswell, Old et al. 1975) as a pro-inflammatory cytokine, an important mediator of inflammation. Funk, Krul et al. (1993) found the role of PTHrP in an animal model of endotoxemia. When sublethal dose of lipopolysaccharide (LPS) was used, it increased markedly the PTHrP mRNA in the spleen. TNF was found to be an important mediator of the LPS induced splenic PTHrP expression. Thus, it was suggested that PTHrP could be a member of the cascade of proteins, which is induced during innate immunity response to a local injury. In this model PTHrP expression was elevated following increase of cytokines like TNF and interleukin-1 (IL-1). However, circulating PTHrP levels were not noticeable.

Later on, Dinarello (2000) proposed a pathway (Fig.3) relating to inflammatory cytokines, in particular TNF and IL-1 which by targeting the endothelium they initiate a cascade of inflammatory mediators in response to infection, ischemia, or toxins.

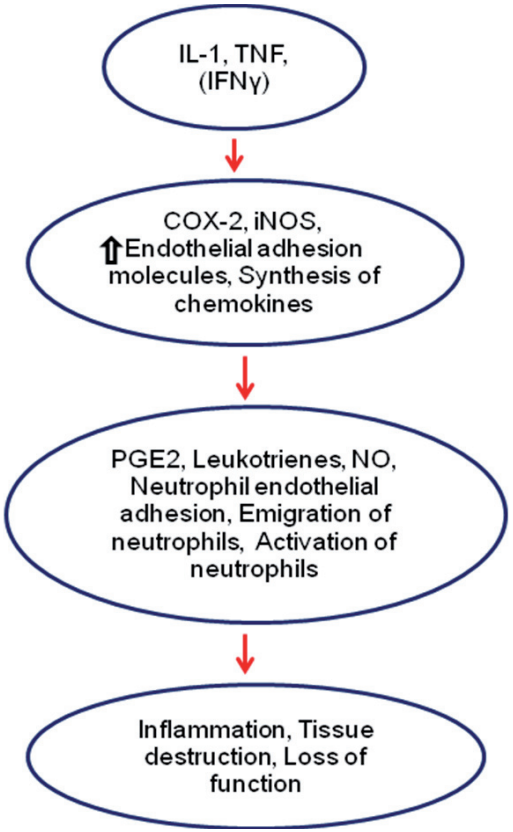

Figure 3. Inflammatory pathway initiated by TNF and IL-1 (modified from Dinarello 2000).

Studies performed by Funk, Moser et al. (1996) using near lethal dose of endotoxin to create an overwhelming sepsis model found that PTHrP mRNA expression was induced in heart, liver, kidney, lung and spleen. PTHrP serum levels were also increased, reaching the peak at two hours, just as mRNA levels in the investigated vital organs, suggesting that systemic levels increased as e result of increasing the local PTHrP levels. Therefore, Funk, Moser et al. (1996) illustrated the inflammatory pathway triggered by TNF and IL-1 adding PTHrP as an inflammatory mediator being induced following the increase of cytokines.

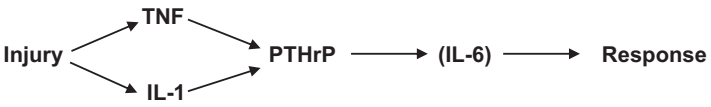

Figure 4. Pathway of cytokines and PTHrP in injury (Funk, Moser et al. 1996).

Hypotension is considered as an important factor among many unknown ones to cause the death from sepsis. PTHrP mRNA expression was found to be induced in smooth muscle cells in spleen after a sublethal dose of LPS administration (Funk, Krul et al. 1993). When a lethal dose of endotoxin was administered to the animal, PTHrP as a vasodilator (Takahashi, Inoue et al. (1995); Maeda, Sutliff et al. (1999)) extremely dilated the vessels, decreased the systemic vascular resistance, causing hypotension and multiple organ failure which lead to mortality (Funk, Moser et al. 1996).

1.7. Obesity Paradox

Accumulation of the excessive body fat is known as obesity (Childers and Allison 2010). It is a complex medical condition contributing to detrimental health effects. It is a risk factor for numerous chronic diseases like type 2 diabetes, heart diseases, etc (Vendrell, Broch et al. 2004).

Despite the fact that obesity impairs the health and reduces the lifespan, decreasing the calories in diet proved to increase the life span. There are three explanations of the phenomenon (Childers and Allison 2010):

- ✓ The U-shaped curve which describes the relation between the BMI and the risk of mortality. The finding suggests that people with relatively highest and lowest BMI are associated with increased mortality risk than people with intermediate BMI.
- ✓ The second one displays the nadir of the U-shaped curve that increases with age. It was found to be higher in older compared to younger people.
- ✓ Obesity paradox, the phenomenon firstly described in 1999, indicating better survival of obese hemodialysis patients (Schmidt and Salahudeen 2007). Later on was found that obesity-survival paradox is linked to other clinical settings as well, like congestive heart failure (Schmidt and Salahudeen 2007), myocardial infarction (Wang, Liu et al. 2016), acute coronary syndrome (Niedziela, Hudzik et al. 2014). There is evidence that patients with increased BMI experiencing a cardiac disease may have a better prognosis compared to their leaner counterparts (Poncelas, Inserte et al. 2015). However, the mechanisms of this clinical paradox remain to be elucidated.

Since Gruberg, Weissman et al. (2002) described the obesity paradox in cardiovascular disease, the phenomenon was analyzed by more researchers in this regard as well. The beneficial effect of it has been shown for a variety of cardiovascular diseases like myocardial infarction, atrial fibrillation, coronary bypass, hypertension, etc (Oga and Eseyin 2016).

According to Poncelas, Inserte et al. (2015), high-fat diet decreased the consequences of LAD occlusion by reducing the infarct size and improving the left ventricular function in mice, supporting the beneficial effects of the paradox in ischemia/reperfusion. Obesity contributes to a proinflammatory state (Vendrell, Broch et al. 2004), and there is a deficiency in regulation of pro- and anti-inflammatory factors. Poncelas, Inserte et al. (2015) proved a significant increase in plasma levels of TNF-α, resistin and leptin in the HFD mice fed for 6 months.

The cardioprotective effect of obesity might be associated with modifications in cytokines, leptin and insulin before the ischemic event. Moreover, the study suggests that dietary obesity with hyperinsulinemia and without hyperglycemia or hypertension reduces negative influences of ischemia/reperfusion injury by increasing insulin signalling and RISK activation.

Donner, Headrick et al. (2013) investigated the impact of dietary obesity in an insulin-insensitive rat model. The finding implies that obesity could be beneficial in case of insulin insensitivity, suggesting cardioprotection, which can happen via reperfusion injury salvage kinase (RISK) signalling. Data indicate that insulin resistance is a key factor that damages the resistance to ischemia/reperfusion.

However, obesity is a complex disorder linked to a number of co-morbidities like inflammation, dislipidaemia, hyperglycaemia, oxidative stress and overt diabetes (Donner, Headrick et al. 2013). The relation between these factors and obesity remain unclear. Despite the fact that weight loss alleviates the symptoms of the cardiovascular diseases and thereby improves the cardiac function, there is no official evidence regarding optimal body weight for patients suffering from a cardiovascular disease (Lavie, Sharma et al. 2016).

1.8. Cardiac ischemia/reperfusion

Myocardial ischemia is a case that occurs because of partial or complete occlusion of coronary arteries. Therefore, there is a deficiency in blood flow and oxygen to tissues, which leads to not sufficient oxygen-rich blood. Most importantly is the rearrangement of the blood flow to tissues as quickly as possible (Kalogeris, Baines et al. 2012).

Myocardial infarction, peripheral vascular disease and stroke are common disorders that worsen the disease and lead to death. Duration, size of ischemia and the injury caused by following reperfusion are crucial regarding tissue damage or death (Kalogeris, Baines et al. 2012). Cardiac injury induced by myocardial infarction leads to damage and loss of cardiomyocytes. This is followed by induction of innate immune pathways that activates the inflammatory response. The inflammatory cascade helps to heal and repair the myocardium by removing the dead cells and matrix fragments and finally formation of a scar (Frangogiannis 2008). With the onset of reperfusion the blood flow and oxygen reaches the tissues, however reperfusion has harmful consequences. The release of ROS and proinflammatory neutrophils that penetrate the ischemic tissue worsen the damage to the cells (Kalogeris, Baines et al. 2012).

Simultaneously, activation of macrophages and mast cells contribute to the release of inflammatory molecules, chemokines, and cytokines like TNFα, IL-1, IL-6, LTB4, etc. that participate in ischemia/reperfusion injury (Rodrigues and Granger 2010). In general, ischemia causes the hydrogen, intracellular sodium and calcium ions to be collected and induce tissue acidosis. On the other hand, reperfusion stimulates changes in ion flux, however, the swift restoration of blood flow and pH deteriorates the cell and tissue injury (Turer and Hill 2010).

Particularly, lung is the organ, which gets the whole stroke volume of the heart. Therefore, dysfunction of the left ventricle may cause impairment in the right ventricle and lung function (Torday, Torres et al. 2003). Usual complications after the myocardial infarction are pulmonary hypertension and lung remodeling (Jasmin, Calderone et al. (2003); Jasmin, Mercier et al. (2004)). A pivotal role in pulmonary remodelling and structure formation of the lung has PTHrP. As mentioned before, it binds to and activates the PTH1R, which leads to activation of adenylyl cyclase, thereafter, cAMP production and activation of PKA. Activation of this signalling pathway in lung increases the expression of ADRP, PPARγ leptin, contributing to surfactant synthesis and thereby inhibiting the transdifferentiation of lipofibroblasts into myofibroblasts (Rehan, Sakurai et al. 2007). Absence of interaction between PTHrP and its corresponding receptor causes the myofibroblast formation that leads to pulmonary stiffness and fibrosis (Torday, Torres et al. 2003).

1.7. Aims of the study

PTHrP is considered as a peptide hormone having a great impact on biological processes in various tissues. However, the role of PTHrP in pulmonary remodeling following the left ventricular ischemia/reperfusion, the effect of high fat diet on its pulmonary expression and the influence of sepsis is not yet elucidated. Therefore, our aims of investigation were as follows:

- To analyze the effect of high fat diet and ageing on PTHrP tissue expression under ischemia/reperfusion
- To identify a possible role for PTHrP in sepsis and the modification by hypothermia that allows protecting tissues from the consequences of sepsis.

In order to reach these aims, tissue samples from mice fed with either normal or high fat diet that underwent an established ischemia/reperfusion protocol; pigs that underwent an established protocol of simulated sepsis; isolated perfused rat hearts; and rat ventricular myocytes were used for analysis.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Table 1. Chemicals systematically used during the study

Chemical	Manufacturer
PeqGold TriFast™ (Trizol)	PEQLAB, Biotechnology GmbH, Germany
Ethanol	Roth, Karlsruhe, Germany
Chloroform	Roth, Karlsruhe, Germany
Isopropanol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Aqua bidest.	B. Braun Melsungen AG, Melsungen Germany
5xRT Buffer	Invitrogen, Karlsruhe, Germany
Oligo-dt	Roche, Mannheim, Germany
dNTP's	Invitrogen, Karlsruhe, Germany
Dithiotreitol	Invitrogen, Karlsruhe, Germany
RNAsin	Promega, Madison, USA
M-MLV Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
Absolute™ SYBR Green Fluorescein qPCR-Mix	Thermo Scientific, UK
Lysis Buffer	Cell Signalling Technology,
PMSF	Roth, Karlsruhe, Germany
Laemmli Buffer 2x concentrate	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
SDS-PAGE broad range protein ladder (molecular weight marker)	Thermo Scientific Inc., MA, USA
NuPAGE® MOPS SDS Running Buffer	Life Technologies, CA, USA
NuPAGE® Antioxidant	Life Technologies, CA, USA
Bovine Serum Albumin 5%	Roth, Carl Roth GmbH+Co.KG, Karlsruhe, Germany
10xTBS Buffer	KPL, Gaithersburg, USA
Tween20	AppliChem GmbH, Darmstadt, Germany
NuPAGE® Transfer Buffer	Life Technologies, CA, USA
Methanol	Roth, Karlsruhe, Germany

Peroxide solution (ECL reagent)	Life Technologies, CA,USA
Luminol Enhancer solution (ECL reagent)	Life Technologies, CA,USA
LPS from Escherichia coli O111:B4	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
RNase AWAY	Molecular BioProducts, San Diego, CA
Collagenase (crude collagenase from clostridium histolyticum) Type 2	Worthington Biochemical Corporation, USA
NaCl	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
Hepes	Roche, Mannheim, Germany
$CaCl_2$	Merck, Darmstadt, Germany
Glucose-Monohydrate	Merck, Darmstadt, Germany
M199	Biochrom, Berlin, Germany
Creatine	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
Carnitine	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
Taurine	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
Cytosin-Arabinofuranoside	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
FCS (4%)	PAA Laboratories, Austria
Penicillin	gibco by Life Technologies, USA
Streptomycin	gibco by Life Technologies, USA
PTHrP(1-34)	Bachem , Weil am Rhein, Germany
PTHrP(7-34)	Bachem , Weil am Rhein, Germany

2.1.2. Antibodies

Table 2. Primary antibodies

Antibody	Host	Concentration	Manufacturer
Anti-Actin	rabbit	1:1000	Sigma, Taufkirchen
Anti-PTHLP	rabbit	1:1000	Calbiochem, USA
Anti-PTH/PTHrP receptor	mouse	1:100	Sigma-Aldrich Inc., SaintLouis, Missouri, USA
Phospho-p42	rabbit	1:1000	Cell Signalling Technology, USA
p42 (ERK-2)	rabbit	1:1000	Santa Cruz Biotechnology, USA

Table 3. Secondary antibodies

Antibody	Concentration	Manufacturer
Sheep anti-rabbit IgG	1:1000	Affinity Biologicals, Ontario, Canada
Goat anti-mouse	1:1000	Affinity Biologicals, Ontario, Canada

2.1.3. Primers used in qPCR experiments

Primers used in the study were provided from Invitrogen GmbH, Karlsruhe, Germany.

Table 4. Mouse primers

Gene	Annealing temp (°C)	Sequence	
		Forward	Reverse
B2M	60	GCT ATC CAG AAA ACC CCT CAA	CAT GTC TCG ATC CCA GTA GAC GGT
HPRT	59	CCA GCG TCG TGA TTA GCG AT	CAA GTC TTT CAG TCC TGT CC
PTHrP	60	GAG ATC CAC ACA GCC GAA AT	CGT CTC CAC CTT GTT GGT TT
PTH-1R	66	TTG CCT CCC TCA CCG TGG CT	CGG CGC GCA GCA TAA ACG AC
ADRP	60	CCC GCA ACC TGA CCC AGC AG	CGC CTG CCA TCA CCC CCA AG
PPAR-γ	61	GCC TTG CTG TGG GGA TGT	TCA GCG GGA AGG ACT TTA TGT
Elastin	60	CTG CTG CTA AGG CTG CTA AG	CCA CCA ACA CCA GGA ATG C

Table 5. Pig primers

Gene	Annealing temp (°C)	Sequence	
		Forward	Reverse
GAPDH	64	GATTTGGCCGCATCGGGCG	CGCCTTGACTGTGCCGTGGA
PTHrP	65	GCCACTGAAGACACCGGGCA	CCCACTCCCGACCATGCTGC
PTH-1R	65	GGTAGCAGGCACCAAGGGCG	CCCAGCTGCCATTGCGGTCA
ADRP	64	ACGCCCCTCAACTGGCTGGT	TCACCTGACTGGCTGCCCCA
Bcl-2	65	TGAACCGGCACCTGCACACC	GCTCCCACCAGGGCCAGACT
MCP-1	66	CAGCCACCTTCTGCACCCAGG	CACAGATCTCCTTGCCCGCGA
TGF-β1	63	TTCACGGCATGAACCGGCCC	TGCCGCACGCAGCAGTTCTT
Collagen-1	66	CCTCCTGACGCACGGCCAAG	CTGGCAGGGCACGGGTTTCC

Table 6. Rat primers

Gene	Annealing temp (°C)	Sequence	
		Forward	Reverse
HPRT	63	CCAGCGTCGTGATTAGTGAT	CAAGTCTTTCAGTCCTGTCC
PTHrP	55	AGCTACTCCGTGCCCTCCCG	AGGAAGAAACGGCGGCGCAA
PTH-1R	64	GGCTGCACTGCACGCGCAA	TTGCGCTTGAAGTCCAACGC
MCP-1	63	TCACGCTTCTGGGCCTGTTGT	TCCAGCCGACTCATTGGGATCA

2.1.4. Materials

Table 7. Materials used consistently in experiments

Material	Manufacturer
Pipettes	Eppendorf – Netheler – Hinz, Hamburg, Germany
Pipette tips	Sarstedt AG&Co., Nümbrecht, Germany
SafeSeal Tubes 0.5 ml; 1.5 ml; 2.0 ml	Sarstedt AG&Co., Nümbrecht, Germany
Precellys® ceramic beads	PEQLAB, Biotechnology GMBH,Erlangen, Germany
Scalpel	Feather®, Japan
0.2 ml 8-tube with domed cap, PCR strips	Thermo Scientific, UK
Latex balloon size 5	Harvard Apparatus, Qbiogene GmbH, Heidelberg, Germany
Operation surgical thread Nr. 40	Gruschwitz GmbH Tech-Twists, Neu – Ulm, Germany
NuPAGE® mini-gels	Life technologies, CA, USA
Nitrocellulose transfer membrane	Whatman GmbH, Dassel, Germany
Glassware	Schott, Mainz, Germany
Filter paper	Biotec-Fischer GmbH , Reiskirchen, Germany
Hamilton syringe (50 µl)	Hamilton, Switzerland
Tweezers	Aesculap, Heidelberg, Germany
Syringe	B. Braun Melsungen AG, Melsungen, Germany
Glass plate	Biometra, Göttingen, Germany
pH-Meter	WTW, Weilheim, Germany
200 µm Nylon mesh	neoLab, Heidelberg, Germany
Parafilm	Bemis Company, USA

2.1.5. Instruments

Table 8. Instruments utilized during the study

Instrument	Manufacturer
Laboratory scale	Kern & Sohn GmbH, Balingen
Precellys24 Homogenizer	PEQLAB, Biotechnology GMBH, Germany
Centrifuge	Beckmann Coulter®, Krefeld, Germany
UV equipment	Stratagene®, CA, USA
Vortex Minishaker	IKA® Works, Inc., Wilmington, NC
Thermal cycler	Techne LTD, Cambridge, UK
iCycler	Bio-Rad, Munich, Germany
NanoDrop® ND-1000 Spectrophotometer	PEQLAB, Biotechnology GMBH, Germany
Ultrasound apparatus (Sonoplus GM70)	Bandelin, Berlin, Germany
Block heater (Thermoblock)	Labtech International, UK
Langendorff apparatus	Institute of Physiology, Giessen
XCell SureLock™ Mini-Cell Electrophoresis System	Invitrogen™ by Life Technologies,CA, USA
Shaker	Biometra, Göttingen, Germany
ECL imager	PEQLAB, Biotechnology GMBH, Germany
Mcllwain Tissue chopper	Gala Gabler Laborbedarf, Bad Schwalbach, Germany

2.1.6. Software

Table 9. Software programs used in the study

Software	Manufacturer
iCycler™ iQ Optical System Software	BioRad, München, Germany
NanoDrop 1000 V3.5.1	Coleman, München, Germany
ChemiCapt 5000	Vilber Lourmat, France
QuantityOne	BioRad Laboratories, München, Germany
Microsoft Office®	Microsoft Deutschland, Unterschleißheim

2.2. Methods

2.2.1. High fat diet, ageing, and ischemia/reperfusion in mice

Tissue samples from animals undergoing ischemia-reperfusion and those administered to normal or high fat diet were analyzed in this study. Lung tissue samples were sent from Barcelona to Germany on dry ice. The following paragraph summarizes the experimental protocol.
The animal handling and experimental studies were approved by Research Commission on Ethics of the Hospital Vall d'Hebron. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto1201/2005.

The experimental sets were arranged in the way that half of mice from C57BL/6 model were fed with the appointed diet for 6 months and half of them for 12 months before a sham surgery or ischemia/reperfusion procedure was performed.
6-week-old C57BL/6 mice were classified in two groups according to their diet. The first group was fed for 6 months with a standard diet containing 24% kcal from protein, 18% kcal from fat and 58% kcal from carbohydrate provided by Harlan Iberica (Barcelona). The other group was fed for 6 months with a high fat diet containing 20% kcal from protein, 60% kcal from fat and 20% kcal from carbohydrate provided by Research Diets (New Brunswick, NJ). The type of high fat diet and other ingredients of diet induced obesity formula are shown in Table 10.

After 6 months the mice were anesthetized by 2% isoflurane inhalation, orally intubated and mechanically ventilated (Inspira ASV, Harvard Apparatus). Afterwards sham surgery was applied to a group of animals (half of them fed with standard diet and half with high fat diet) and the other group of mice (half of them fed with standard diet and half with high fat diet) was subjected to ischemia where the left anterior descending coronary artery was occluded for 45 minutes. Reperfusion was the following procedure applied to the latter group for four weeks. In the study 6-month-old mice were defined as 7 months and 12-month-old mice as 13 months because they were subjected to reperfusion for 4 weeks and after this period, the mice were sacrificed. Lungs were excised and frozen in liquid nitrogen up to further analysis.

Table 10. The diet induced obesity (DIO) formula

Ingredient	gm %	Kcal %
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0

2.2.2. Experimental endotoxemia *in vivo* in pigs

Tissue samples from pigs undergoing a model of simulated sepsis were analyzed in this study. Lung, left ventricle of the heart, liver, and aorta tissues from septic pigs were sent from Austria to Germany on dry ice. The following paragraph summarizes the experimental protocol.
The experimental protocol was approved by the Austrian Bioethics Commission (BMWF-66.010/0099-II/3b/2010) in Vienna and complies with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

13 overnight-fasted landrace pigs (64±1 kg) were put under sedation with 0.5 mg/kg midazolam and 20 mg/kg ketamine. Pigs were anesthetized by 1mg/kg propofol and tracheotomy was performed using an endotracheal tube. Then sheaths were placed in both common carotid arteries (9F and 10F), both internal jugular veins (6F and 8F) and the left-sided femoral vein (14F). Throughout the procedure drugs like 1 % sevoflurane, 35 µg/kg/h fentanyl, 1.25 mg/kg/h midazolam, and 0.2 mg/kg/h pancouronium were used to maintain the anesthesia.

The respirator (volume-controlled mode) was set to 5 mmHg positive end-expiratory pressure, 10 ml/kg tidal volume, I:E-ratio of 1:2 and 50% oxygen. The end-expiratory carbon dioxide partial pressure was retained between 35 and 40 mmHg setting the respiration rate.
With the aid of sheaths, a Swan-Ganz catheter (Edwards Lifesciences CCO and Vigilance I, Edwards Lifesciences, Irvine, CA, USA) and a pacing probe were placed in the pulmonary artery and the right atrium of pigs, respectively.

A catheter (5F, 12 electrodes, 7 mm spacing, MPVS Ultra, Millar Instruments, Houston, Texas, USA) for pressure conductance was inserted in the left ventricle and linked to a signal processing unit (MPVS Ultra, Millar Instruments, Houston, Texas, USA). In the descending aorta was inserted a valvuloplasty catheter (20 ml, Osypka, Rheinfelden, Germany), and an intravascular cooling catheter (Accutrol™ Catheter 14F, Philips Healthcare, Vienna, Austria) connected to a cooling device (InnerCool RTx Endovascular System, Philips Healthcare, Vienna, Austria) was put in the inferior vena cava. Using this intravascular cooling device the body temperature was kept constant at 38°C.

Heparin bolus dose of 150 IU/kg and thereafter a continuous infusion of 75 IU/kg/h were given to prevent venous thrombosis. During the whole instrumentation period, a balanced crystalloid infusion (Elo-Mel Isoton, Fresenius) was given at a fixed rate of 10 ml/kg/h at room temperature.
After the instrumentation procedure, pigs were let for 30 min to stabilize.

2.2.2.1. Administration of LPS

LPS infusion (E. coli serotype O111:B4, Sigma-Aldrich Inc., SaintLouis, Missouri, USA) in pilot experiments was administered at a rate of 10 µg/kg/h in 4 normothermic pig. 3-4 hours after the onset of LPS infusion 3 of 4 pigs died. Consequently, in the other set of experiments, the LPS dose was reduced to 0.5 µg/kg/h for 1 hour and 1.0 µg/kg/h for further 3 hours. So, LPS was given for 4 hours, and the pigs were observed for further 4 hours, overall a follow-up of 8 hours.
Since the onset of LPS infusion, pigs were assigned consecutively 1:1 to either mild hypothermia (MH, n=8, 33°C) or normothermia (NT, n=8, 38°C). In MH group of animals, cooling was initiated at the same time with LPS administration by the intravascular device and infusion of ice-cold (4 °C). Usually 3-4 hours after the onset of LPS infusion, the mean aortic pressure decreased lower than 55 mmHg. In this case, 4 boli of 500 ml crystalloid infusion were given. 1 µg/kg epinephrine was added in particular to the third and the forth bolus. In case where arterial oxygen saturation decreased below 90%, FiO2 was increased to 1.0, and the I:E-ratio was switched to 1:1.

Finally, the heart was checked and after verifying no coronary damage, the animals were sacrificed by a bolus of 80 mmol potassium chloride.

2.2.3. Experimental endotoxemia in vitro in rats

2.2.3.1. Isolated perfused rat heart assay - Principle

Oscar Langendorff developed such a perfusion system for mammalian heart in 1895. Langendorff preparation is a method based on the perfusion of hearts and inspection of the cardiovascular parameters without having to take into consideration the problems or obstacles coming from the living animal.

As shown in Fig.5 the Langendorff apparatus is coated with warm circulating water in order to keep the temperature of the heart and the fluid at 37°C. The heart removed from the animal is perfused by retrograde flow from aorta. This is named as retrograde perfusion because the fluid flows down into the aorta. In contrary, blood in situ, flows out of the left ventricle through the aorta.
An oxygenated buffer is delivered to the heart via a cannula inserted into the ascending aorta. The buffer feeds the myocardium with the needed nutrients and oxygen, in order to function for hours after its excision from the animal. The reverse pressure of the buffer closes the leaflets of the aortic valve, as a consequence it does not allow the perfusate to enter the left ventricle but it permits it into the coronary arteries (naturally, they provide blood to the heart). Then, via coronary sinus the buffer gets into the right atrium and in the end it effuses via the right ventricle and pulmonary artery. The perfusion flow rate is kept constant during the experiment by either a constant hydrostatic pressure or constant flow rate. Systolic pressure under physiological conditions or in vivo experiments ranges from 70-90 mmHg. For the isolated heart it is recommended to be between 60-70 mmHg. Values higher than this range may cause incompetence of the aortic valve.

Langendorff perfusion system is a useful tool in terms of estimating cardiac inotropic, chronotropic and vascular effects, malfunction, and effects of different pharmacological drugs on the cardiovascular system regarding metabolic activity, contractility, etc.

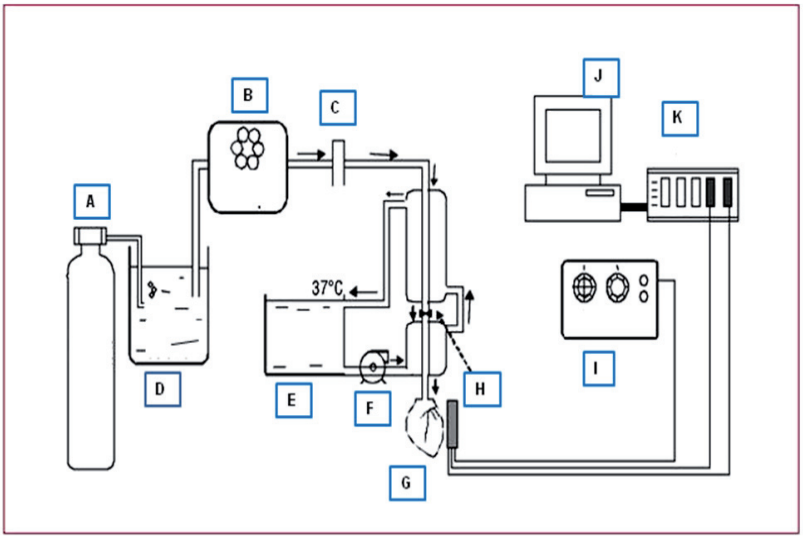

Figure 5. Langendorff heart perfusion model (modified from Chorro, Such-Belenguer et al. 2009). A. Oxygenation 95% O_2, 5% CO_2; B. Peristaltic pump; C. Filter; D. Tyrode solution; E. Heat sink; F. Pump; G. Heart; H. Flow/Pressure regulator; I. Stimulator;; J. PC; K. Data acquisition.

2.2.3.2. Preparation and perfusion of the rat hearts

The use of rats was registered at the JLU Giessen (472_M). Rat hearts were used for Langendorff experiments.

Rats were bred and raised in the animal facility of the Institute of Physiology, Justus Liebig University. Animal nutrition (standard diet: Altromin®) and water were given ad libitum.

At the start, 3-month-old female Wistar-Hannover rats with a weight of 240 g ± 20 g were put into a Plexiglas chamber and shortly thereafter were anesthetized by isoflurane. Muscle tone and corneal reflex were checked and after verifying that the reflex activity was lost, they were sacrificed by a cervical dislocation. Afterwards, the abdomen was opened, the heart together with the attached organs were excised from the animal's body and immediately were immersed in a petri dish filled with 4°C cold sodium chloride. Cold sodium chloride is used to prevent any ischemic injury during the time interval between removal of the heart from the animal and perfusion in the Langendorff system.

Using tweezers the surplus organs like esophagus, trachea, and thymus were cut away from the heart. Perfusate was permitted to slowly drop from the cannula before cannulation for the reason that this aids to remove any air bubble during the insertion of the heart to cannula.

Then, through the opened aortic root, heart was held with forceps to the cannula (perfusion cannula is made of stainless steel with inner diameter 1.8mm and the external diameter 3.0mm) in the chamber of the Langendorff apparatus and finally it was fixed with a surgical thread Nr. 40. The cannula is connected to the reservoir of nutrient rich, oxygenated solution named Krebs-Henseleit buffer (Table 11). It is steadily gassed with Carbogen® gas mixture containing 5% CO_2 and 95% O_2. The perfusion fluid was allowed to flow thoroughly since the heart was securely attached to the cannula. Finally, an intraventricular latex balloon (size 5) was inserted to assess the contractile activity of the heart. The deflated balloon was linked to a rigid catheter and pressure transducer. It was inflated with water until the diastolic pressure ranged from 5-8 mmHg. As soon as the balloon was fixed, parameters like left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), and heart rate (HR) could be measured and documented. Having these parameters recorded helped to calculate the left ventricular developed pressure (LVDP) and coronary vascular resistance (CVR).

Table 11. Krebs-Henseleit Buffer (pH 7.4)

Substance	Molarity (mM)	Concentration (g/L)
NaCl	140	8.18
KCl	2.7	0.20
NaH_2PO_4 x H_2O	0.4	0.055
$MgCl_2$ x $6H_2O$	1.0	0.2
Glucose	5.0	0.99
$CaCl_2$ x $2H_2O$	1.8	0.26
$NaHCO_3$	24	2.02

Experiments run at the constant flow. In the first 20 minutes, a stabilization phase took place before treatment, where the flow rate was held constant between 7-10 ml/min. Aortic pressure was arranged from 41-50 mmHg and the diastolic pressure 10-12 mmHg. After this period, mean LVDP was 98±7 mmHg (37°C) and 124±5 mmHg (32°C; p=0.007 vs. 37°C). For 20 min stabilization period the cardiac functions were re-established, then the perfusion phase followed for 120 min. Every 15 min the heart rate was recorded, the values ranged between 170-250 beats/min.

Experiments were divided into two sections, each of them performed using Krebs-Henseleit Buffer (pH 7.4) but at different temperatures. A set of rat hearts was perfused at 37°C and the other one at 32°C. The experiments were carried out in the chamber of the Langendorff system where two rat hearts were hanged, one of them being perfused with Krebs-Henseleit Buffer mixed with LPS (1 mg/ml), and the other one with Krebs-Henseleit Buffer only, for two hours. At the end of perfusion phase PTHrP (1-34) (100 nmol/L) was added to the perfusion buffer for 10 min as seen in Fig. 6. The same protocol was applied for the perfusion of rat hearts in the Langendorff system, except temperature change for two sets of experiments, namely 37°C and 32°C. LVDP was recorded after 120 min LPS administration (1 mg/ml). Whereas, PTHrP responsiveness was determined according to the changes observed in LVDP after administration of PTHrP(1-34) (100 nmol/L) to the perfusate for 10 min.

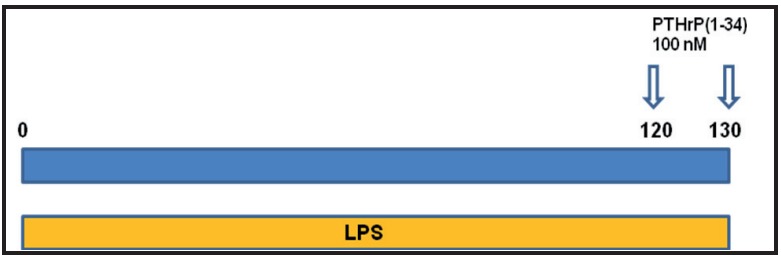

Figure 6. Perfusion flow of rat hearts in the Langendorff system

2.2.3.3. Isolation of rat ventricular myocytes

As mentioned before the use of rats was registered at the JLU Giessen (472_M). Animals were fed with standard diet Altromin® and water. Both were given ad libitum. They were bred and raised in the animal facility of the Institute of Physiology, Justus Liebig University.

Ventricular cardiomyocytes were isolated from 3-4-month-old male Wistar rats with a weight of 300-400 g. Initially, the animal was put into a Plexiglas chamber and anesthetized by isoflurane. After it lost the reflex activity, the thorax was opened by cervical dislocation and the heart was removed from the animal. It was immediately put into the ice-cold saline (9 g/l NaCl) in the Petri dish. Remnant tissue organs adjacent to the heart were removed and finally the heart was

mounted on the Langendorff perfusion system. Firstly, for 3-5 min at 10 ml/min the heart was perfused with perfusion buffer (Table 12) to get rid of blood on the organ.

Table 12. Perfusion Buffer (Powell Medium) (pH 7.4, gassed with 95% O2/ 5% CO2)

Perfusion Buffer	**Molarity (mM)**
NaCl	110
KCl	2.5
KH_2PO_4	1.2
$MgSO_4$ x $7H_2O$	1.2
Hepes	25
Glucose	11

Then collagenase was added to the buffer and perfusion was continued for further 25 min. Thereafter, the heart was disconnected from the Langendorff perfusion system, ventricles were cut from the rest of the heart and minced. The cell suspension was incubated for 5 min in a beaker filled with collagenase buffer. Carbogen (5% CO_2/ 95% O_2) was added to the cell suspension for 5 min and the solution was mixed through a pipette, which facilitated the release of cells from tissue pieces, namely the digestion. Using a 200 µm nylon mesh the cell suspension was filtered and then centrifuged shortly for 3 min at 25 *g*. The pellet containing cardiomyocytes was resuspended in buffer solution, and at this point 0.2% (v/v) calcium stock solution (100 mmol/L $CaCl_2$ in H_2O) was added to the medium (final concentration 200 µmol/L). Finally, 2-3 ml of the cell suspension was added to 12 ml bovine serum albumin gradient (BSA gradient contains 4% BSA (w/v), 1% calcium stock solution in perfusion buffer) and solution was centrifuged for 1 min at 15 *g*. Afterwards, the pellet with rod shaped cardiac myocytes was suspended in 25 ml CCT medium.

To culture cardiomyocytes firstly the media required for plating and washing the cells were prepared a day before starting the cell isolation procedure. Culture dishes were prepared by adding 1 ml of plating medium to each cell culture dish and stored in the incubator at 37°C. The washing medium was stored in the 4°C fridge overnight. However, hours before use it was placed in the water bath at 37°C.

Table 13. Cell culture media

Plating medium	Washing Medium	CCT Medium
Basal culture medium (CCT)	Basal culture medium (CCT)	Medium 199
FCS 4% (vol/vol)	Penicillin/Streptomycin 2%	Creatine 5 mmol/l; carnitine 2 mmol/l; taurine 5 mmol/l; cytosine-β-arabinofuranoside 10 µg/ml; penicillin 100 IU/ml; streptomycin 100 µg/ml
Penicillin/Streptomycin 2%		

After isolation of cardiomyocytes, the cell culture dishes stored in the incubator overnight were taken out and the preincubation solution (plating medium) was removed. 1 ml of the cell suspension was plated to each culture dish and stored in the incubator at 37°C for 1-2 hours. Afterwards, cell culture dishes were washed with washing medium (see Table 13) in order to remove round and nonattached cells. LPS 10 µg/ml and PTHrP (7-34) 10 µM were added to particular dishes and all together were stored in the incubator at either 37°C or 32°C for the following cell contraction procedure.

2.2.3.4. Measurement of cell shortening

Ventricular myocytes represent the cardiac contractile unit. The technique used to assess the ventricular contractile function is the edge-detection system. This method provides information about changes in myocyte length.

Rod-shaped myocytes with regular contraction and clear edges were used for assessment. The cell being analyzed is shown via a line-camera on the computer monitor. Cell stimulation was applied with 50 V, 5 msec duration using two AgCl electrodes with biphasic electrical stimuli made up of a pair of wires placed in an opposite way. Cells were exposed to LPS (1 mg/ml) at 32ºC or 37ºC for 2 h and paced at a frequency of 2 Hz in the presence or absence of the receptor antagonist PTHrP (7-34) (100 nmol/l).. The contraction of an individual cell was measured every 15 sec four times, the median was calculated and this was used as the average of cell shortening of the particular cell. Nine cells were analyzed pro culture dish, for a particular condition, three plates were prepared. In total two sets of preparations were analyzed. Data are expressed as percentage of systolic cell length relative to diastolic cell length (dl l^{-1} (%)).

2.2.4. Quantitative real time PCR

2.2.4.1. RNA isolation

Tissue samples were put in the tubes with bulk beads, 1ml PeqGold Trifast™ reagent (Trizol) was added to each tube, and thereafter samples were homogenized in Precellys® 24 Bead Mill Homogenizer using the program 3 with these features: 6000 rpm, 2 times 20 seconds. Then, 100 µl chloroform was added to each tube and mixed in vortex till the mixture was milky. Subsequently tissue samples were centrifuged for 15 min, at 4°C, at 12.500 rpm and this led to formation of three phases in the tube. The upper phase contains the RNA which was taken with pipette and put into 1.5 ml tubes. The amount of RNA in the tube was mixed in 1:1 proportion with isopropanol. The tubes with bulk beads containing the lower two phases were thrown away in P/C waste.

Tubes containing the RNA and isopropanol were mixed in vortex and thereafter stored in the fridge at -20°C for 1 hour or overnight. After that tubes were centrifuged for 15 min, at 4°C, at 12.500 rpm. The supernatant was taken out with pipette and discarded in P/C waste. Whereas the pellet wax mixed with 1ml ethanol 70%, vortexed and put in the centrifuge for further 15 min, at 4°C, at 12.500 rpm. Ethanol was discarded after centrifugation and the tubes with RNA were left for one hour to dry. And finally, an amount of water was added to the RNA containing tubes, according to the tissue sample 20 µl for lung and 50 µl for left ventricle tissue. Tubes were shortly vortexed and stored on ice in the fridge (4°C) for one hour and thereafter for long-term storing in the -80°C fridge.

2.2.4.2. Determination of RNA concentration

NanoDrop® ND-1000 Spectrophotometer was used to determine the concentration of RNA. Samples were kept on ice during the whole process. Initially the blank sample containing only aqua bidestilata was measured, continuing with other tissue samples. 1.5 µl tissue sample was quickly given to the sensor and measured. The value coming out from the measurement should be between 200-2500 ng/µl. If the sample was more concentrated, then a.b. was used to dilute the sample until its concentration became between the values aforementioned. In case the sample was lung or left ventricle 20 µl or 50 µl respectively was added to the previously measured sample. The amount of RNA was calculated in following way:

(1000/Value after dilution)*1 = X (RNA amount taken from the sample) →5 µl - X = Y (a.b.)

The amount of RNA (X) and a.d. (Y) were mixed in a tube, vortexed, and proceeded with cDNA synthesis.

2.2.4.3. cDNA synthesis

Before starting the procedure the bench-tops, pipettes, plasticware and everything needed for cDNA synthesis were cleaned with RNase AWAY™ in order to eliminate the RNase and DNA from the laboratory surfaces.

Tissue samples were kept on ice, vortexed and thereafter put into the thermocycler. Using Program 1 samples remained for 15 minutes at 60°C in the thermocycler for denaturation of the single stranded mRNA.

In the meantime, the reaction mix was prepared as follows:

5xRT-Buffer (2 µl/sample)

Oligo dt (1 µl/sample)

dNTP's (1 µl/sample)→ Deoxynucleoside triphosphates (dGTP, dTTP, dCTP, dATP).

DTT (0.5 µl/sample)

RNAsin (0.2 µl/sample)

Every component was added in a 2 ml tube, mixed and finally M-MLV-RT (0.3 µl/sample) was added but not vortexed any longer.

After 15 min. tubes containing mRNA and a.d. were taken out of thermocycler and the reaction mix was added in 1:1 proportion. Mixture was vortexed and tubes were put in the thermocycler. Using the program 3, incubation carried out for 60 min at 37°C. Subsequently it proceeded with inactivation of the reverse transcriptase for 5min at 95°C. The obtained cDNA was diluted with a.b. in 1:10 proportion and stored in -20°C fridge for qPCR experiments.

2.2.4.4. Quantitative real time PCR (qPCR)

cDNA samples diluted in proportion 1:10 were used for qPCR. 3 µl of sample was put in PCR-tubes. Each sample was applied in duplicate.

Thereafter the PCR-Mix for one tube was prepared as follows:

10 µl SYBR Green Mix

0.6 µl Primer (forward + reverse)

6.4 µl Aqua Bidest.

Forward and reverse primers were mixed in 1:1 proportion and this mixture was diluted with a.b. in 1:10 proportion.

Plate was initially designed according to the samples.

3 µl cDNA sample and 17 µl PCR-Mix were put into the PCR tubes. 3 µl of a.b. and Mix were put in the first two tubes and named as „Mix". Prepared samples were put in the iCycler, exactly in the same positions as appointed in the plate. Primer temperature was set, and PCR was run.
The procedure of PCR in the iCycler comprises 20-50 cycles. These are temperature changes repeating throughout the process:

1. Cycle (1x): Initialization for 15 minutes at 95°C (enzyme activation step)
2. Cycle (45x): Denaturation for 30 seconds at 95°C (this step implicates the disruption of the DNA template and formation of single-stranded DNA molecules)
   Annealing for 30 seconds at x°C (annealing of the forward and reverse primers to the single-stranded DNA molecules)
   Elongation for 30 seconds at 72°C (at the extension/elongation step a new DNA strand complimentary to the DNA template is created. This is done by DNA polymerase which adds dNTP's in 5' to 3' direction)
3. Cycle (100x): Melting curve for 10 seconds at 50°C

An invariant endogenous control (reference gene), a so-called housekeeping gene was used to normalize the data obtained in quantitative RT-PCR experiments. Data regarding mouse and rat samples were normalized to Hypoxanthine Phosphoribosyltransferase (HPRT) expression. β2 microglobulin (B2M) was used as an alternative housekeeping gene, but it showed high variability between samples. Therefore, results are represented according to HPRT normalization. While pig samples were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The procedure includes dividing of the measured relative abundance value for each target gene sample by the amount of the housekeeping gene in the corresponding sample. After normalization, different sample groups can be compared.

2.2.4.5. Statistics

Data obtained from quantitative qPCR are presented as means ± S.D. or box plots. The results between groups were compared by two-side ANOVA and Student-Newmann-Keuls post hoc analysis. To assess the normal distribution of the samples Levene's test was performed. A p-value of 0.05 implied a statistically significant result.

2.2.5. Western Blotting

2.2.5.1. Preparation of lysate from tissue

The tissue samples to run on a gel firstly need to be lysed in order to release the protein of interest. The lysis buffer enables the cell membrane to disrupt and solubilizes the intracellular proteins. Thus, proteins can move separately through the gel. The following table includes the components of the lysis buffer used for tissue sample preparation.

Table 14. 10X Lysis Buffer

Reagents	
Tris-HCl (pH 7,5)	20 mM
NaCl	150 mM
Na_2EDTA	1 mM
EGTA	1 mM
Triton	1%
Sodium Phosphate	2.5 mM
β- glycerophosphate	1 mM
Na_3VO_4	1 mM
Leupeptin	1 μg/ml

10X lysis buffer were then diluted in 1:10 proportion with a.b. and afterwards 200 μl of a serine protease inhibitor 0.1 M PMSF (phenylmethylsulfonyl fluoride) was added.

The frozen tissue samples were left to thaw in room temperature and then 0.1 g from each sample was mixed with 500 μl lysis buffer in tubes with bulk beads. Subsequently the samples were homogenized in Precellys24 for 2x20 seconds at 6000 rpm.

After homogenization, samples were kept on ice for 5-10 minutes. Then ultrasonic treatment was applied to each sample for 30-60 seconds in order to unfold more the proteins and reduce the intermolecular interactions.

These sample suspensions were centrifuged for 10 min. at 4°C, at 14.000 rcf. The supernatant was taken into 1.5 ml tubes. 20 μl of sample from each tube was taken into new tubes and diluted with 380 μl a.b. (1:20 proportion). Finally, 100 μl from the latter tubes were mixed with 40-μl Laemmli buffer (Table 15) and boiled for 5 min at 95°C in thermoblock.

Table 15. Sample Buffer, Laemmli 2x Concentrate (pH 6.8)

Components	
SDS	0.2 g
Dithiotreitol	0.154 g
Glycerin (100%)	2.0 ml
BPB	0,2 ml
Tris	0.5 M
Aqua Bidestillata	1.8 ml

The SDS detergent is used to denature the proteins. The bromophenol blue is a dye, which moves before proteins and enables to see the sample throughout loading. The glycerol is used to increase the sample density that facilitates the sample to stay in the sample well.

2.2.5.2. Loading the samples and running the gel

The XCell *SureLock*™ Mini-Cell system was used to carry out the SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). 10% NuPAGE® Bis-Tris mini-gels were used to separate small to medium-sized proteins during gel electrophoresis. 1x MOPS SDS running buffer was prepared (50 ml 20x MOPS running buffer + 950 ml a.b.) for use in the next steps. 2 gel cassettes initially were washed with a.b., the lower part of the cassette with 1x MOPS running buffer and then inserted into the lower buffer chamber. The upper buffer chamber (cathode) was filled with 200 ml 1x MOPS running buffer and 500 µl antioxidant. The sample wells were washed with 1x MOPS running buffer using a syringe and a cannula.

Samples were boiled once more for 5 min at 95°C in the thermoblock, shortly centrifuged and vortexed. 20 µl of sample was used for gels with 10 wells, whereas 10-15 µl sample for gels with 15 wells depending on the number of samples.

The Hamilton syringe was used to underlay the samples into the gel wells. The molecular weight marker was put via syringe into the first well, tissue samples in other wells.

The lower buffer chamber (anode) was filled with 600 µl 1x MOPS running buffer and afterwards the lid was properly closed and electrode cords were connected to the power supply so that the red one to + jack and the black one to – jack. The power was turned on and the run was conducted for 50 min. at 200 V.

2.2.5.3. Wet electroblotting (Wet transfer)

At the time, while electrophoresis of the gels was in progress the materials and the buffer needed for transfer were prepared. For blotting NuPAGE® gels, the following buffer was prepared:

Table 16. Transfer Buffer

Reagent	Amount
NuPAGE® Transfer Buffer (20x)	25 ml
NuPAGE® Antioxidant	500 µl
Methanol 20%	100 ml
Deionized Water	375 ml

4 filter paper (9 x 7.5 cm), 2 membranes (nitrocellulose), and 5 sponges were used for transferring the protein from the gel to membrane.
40 ml of buffer was used for transfer in the mini-cell unit. 10 ml was used to rinse the membranes before preparing the sandwich. 10 ml to soak the filter papers briefly before using and the rest was used for the sponges.
The gel/membrane sandwich was prepared according to the manufacturer's instructions (sponge-filter paper-gel-transfer membrane-filter paper-sponge). This gel sandwich was placed in the mini-cell unit and the tank filled with 40ml transfer buffer (until the sandwich is covered with buffer). The run was carried out at 30 V for 1 hour.

2.2.5.4. Antibody incubation

After the electrotransfer the nitrocellulose membrane was washed 2-3 times with distilled water and thereafter the membrane was blocked for 1 hour at room temperature on a shaker using 5% BSA in 1xTBS buffer.

Table 17. 10xTBS Buffer (pH 7.4)

Reagent	Molarity
Tris/HCl	10 mM
NaCl	150 mM

First antibody was added in 5% BSA and TBS buffer solution and the membrane incubated overnight at 4°C on a shaker. In the case where the membrane was incubated with the loading control (actin), 0.1% Tween20 was added to the incubating solution. Afterwards the membrane was washed in TBST buffer (1xTBS buffer + 0.1% Tween20) 2-3 times, 10 min each washing on the shaker at room temperature.

The enzyme conjugated (horseradish peroxidase) secondary antibody was prepared in 5% BSA and TBST solution in appropriate proportion and added to the membrane, incubated at room temperature for 1 hour on the shaker.

After one hour the membrane was washed 2 times with TBST solution and once with 1xTBS buffer, 10 min each washing. The last washing was applied with TBS buffer without Tween20 to reduce the high background, a problem caused by Tween20 interfering with the ECL solution, used in the following step.

2.2.5.5. Enhanced chemiluminescence (ECL) Detection

ECL is a method used to acquire image of the membrane following the western blot protocol. In the darkroom, firstly, the chemiluminescence reagents namely peroxide and enhancer solutions were mixed in a dish, 4 ml each. This solution was poured into a dish with the membrane and incubated at room temperature for 2 min.

Then membrane was placed on a glass plate inside the ECL device and the result visualized according to the exposure time recommendations given by the instrument.

Quantity One is the program used to accomplish the evaluation of western blot analysis. Actin was used as loading control in performed western blot experiments.

3. Results

3.1. Pulmonary expression of PTHrP in mice

3.1.1. Effect of high fat diet on pulmonary expression of PTHrP in adult mice

Mice fed with HFD in comparison with those fed with ND gain weight from 35.8 ± 0.9 g to 46.7 ± 0.8 g and plasma leptin levels elevated from 4.95 ± 0.83 ng/ml to 15.91 ± 2.41 ng/ml (each $p<0.001$, n=6-8). As shown in Fig. 7A, PTHrP mRNA expression in the lung of control mice fed with HFD increased significantly. 45 min ischemia and four weeks reperfusion elevated the pulmonary mRNA expression of PTHrP in adult mice independently of diet. HFD seemed not to affect significantly the PTHrP expression in the lung when mice experience ischemia/reperfusion (Fig 7A).

Fig. 7B displays the pulmonary mRNA expression of PTH-1R. It is obvious in the figure that it increased in the sham HFD mice whereas in mice subjected to ischemia/reperfusion was stable, no matter if they were fed with normal or high fat diet.
ADRP and PPARγ expression was analyzed as PTHrP downstream targets in the lung to analyze receptor activity. As shown in Fig. 7C and 7D, HFD increased their mRNA expression in sham mice. PTHrP and ADRP mRNA expression seemed to be strongly correlated. Pulmonary mRNA expression of PPARγ increased significantly in ischemia/reperfusion HFD mice (Fig. 7D). The data suggest that leptin could play a pivotal role in regulating the pulmonary expression of PTHrP system under basal conditions.

Therefore, leptin deficient mice (ob mice) were used to prove the significance of leptin in this process (data not shown). Ob-mice increased body weight (50.5 ± 3.8 g vs. 26.0 ± 3.2 g, n = 4, $p < 0.001$). Experiments showed a significant decrease in mRNA expression of PTHrP in this mice model. The decrease in pulmonary expression of PTHrP did not cause the subsequent decrease in the mRNA expression of PTH-1R or the downstream targets ADRP and PPARγ. Therefore, the data indicate that leptin induces the pulmonary PTHrP expression and the downstream targets in mice fed with HFD only in case it is activated.

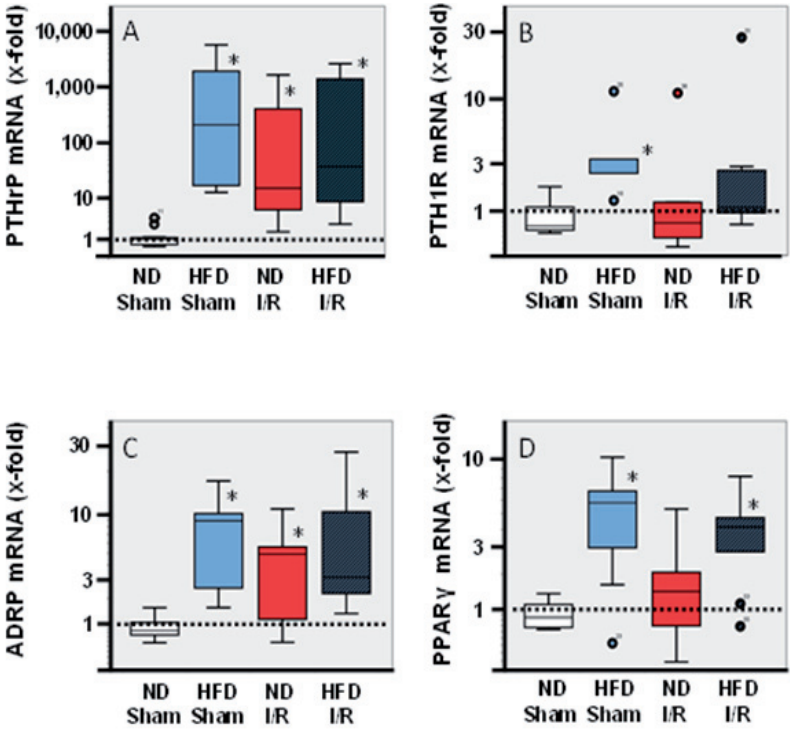

Figure 7. Pulmonary mRNA expression of PTHrP, PTH-1R, ADRP, and PPARγ in seven months old mice fed with either normal diet or high fat diet and subjected to either sham surgery or ischemia/reperfusion. The first bar (white) shows the group of mice fed with normal diet and subjected to sham surgery (ND-Sham, n=12); the second bar (blue) shows mice fed with high fat diet which underwent sham surgery (HFD-Sham, n=7); the third bar (red) indicates the group of mice fed with normal diet which underwent ischemia/reperfusion (ND-I/R, n=9); and the fourth bar (black) indicates the mice fed with high fat diet which underwent ischemia/reperfusion (HFD-I/R, n=10). Data are expressed as box plots. The dashed line indicates the mean expression level of ND-Sham. *, $p<0.05$ vs. ND-Sham.

3.1.2. Effect of high fat diet on pulmonary expression of PTHrP in older mice

The impact of ageing, high fat diet, and ischemia/reperfusion on the expression of PTHrP and its downstream targets was analyzed in the lungs of 13-month-old mice.

Fig. 8 shows no differences between the groups compared, except for PPARγ. PTHrP mRNA expression was higher in older mice (Fig. 8A, white bar) compared to adult ones when mice were fed with ND and experienced no treatment (Fig. 7A). However, no significant differences were observed, neither in the case when mice were fed with HFD nor when they underwent ischemia/reperfusion protocol. mRNA expression of PTH-1R and ADRP showed similar results with no significant differences (Fig. 8B and 8C, respectively). Pulmonary expression of PPARγ displayed in Fig. 8D indicates a significant increase in HFD group of mice subjected to ischemia/reperfusion compared to sham group fed with ND.

Finally, the observed results suggest that pulmonary expression of PTHrP is age-dependent being up regulated in older mice. However, the induction of PTHrP expression in the lungs of older mice does not activate the entire PTHrP system. HFD and ischemia/reperfusion did not have a remarkable effect in these mice.

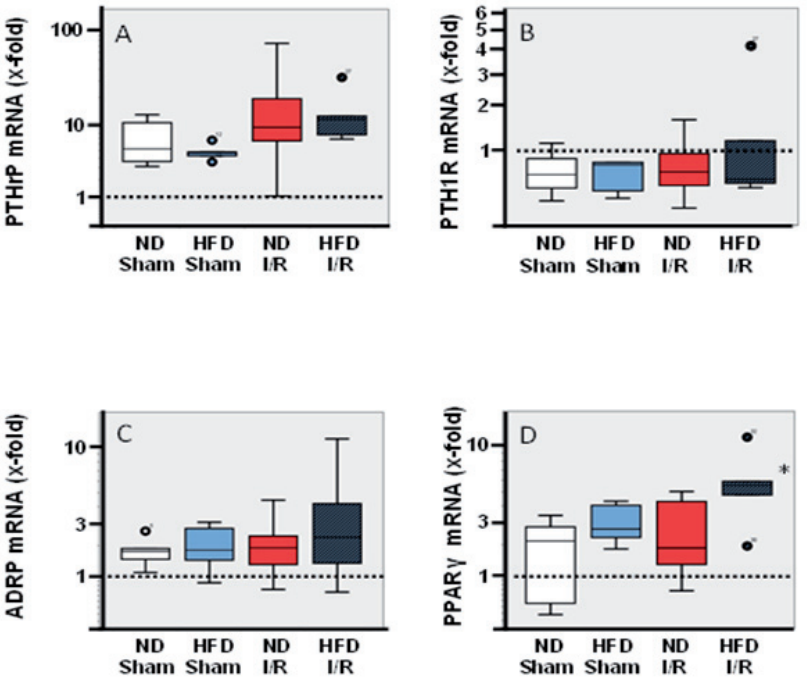

Figure 8. Pulmonary mRNA expression of PTHrP, PTH-1R, ADRP, and PPARγ in thirteen-month-old mice fed with either normal diet or high fat diet and subjected to either sham surgery or ischemia/reperfusion. The first bar (white) displays the group of mice fed with normal diet and subjected to sham surgery (ND-Sham, n=6); the second bar (blue) shows mice fed with high fat diet which underwent sham surgery (HFD-Sham, n=5); the third bar (red) indicates the group of mice fed with normal diet which underwent ischemia/reperfusion (ND-I/R, n=8); and the fourth bar (black) indicates the mice fed with high fat diet which underwent ischemia/reperfusion (HFD-I/R, n=6). Data are expressed as box plots. The dashed line indicated the mean expression level of ND-Sham. *, $p<0.05$ vs. ND-Sham.

3.1.3. Effect of HFD, ageing, and ischemia/reperfusion on PTHrP and PTH-1R expression

Ageing, ischemia/reperfusion, and HFD are conditions which animals were subjected to. The data observed by now recommended that these conditions have an influence on PTHrP expression. A high induction of PTHrP was detected under all aforementioned conditions. Further experiments were performed in order to verify if there is the same impact on protein expression of PTHrP and PTH-1R.

Fig. 9A and 9B display the protein expression of PTHrP where it is obvious a high increase of pulmonary PTHrP protein in adult mice undergoing ischemia/reperfusion. HFD seemed to have no additive impact on PTHrP protein. Apparently, expression of PTHrP protein was not significantly elevated in the steady state, which implies a high pulmonary PTHrP turnover under stress conditions.

As for the protein expression of PTH-1R, it was significantly increased only in mice fed with HFD and thereafter subjected to ischemia/reperfusion (Fig. 9A and 9C).

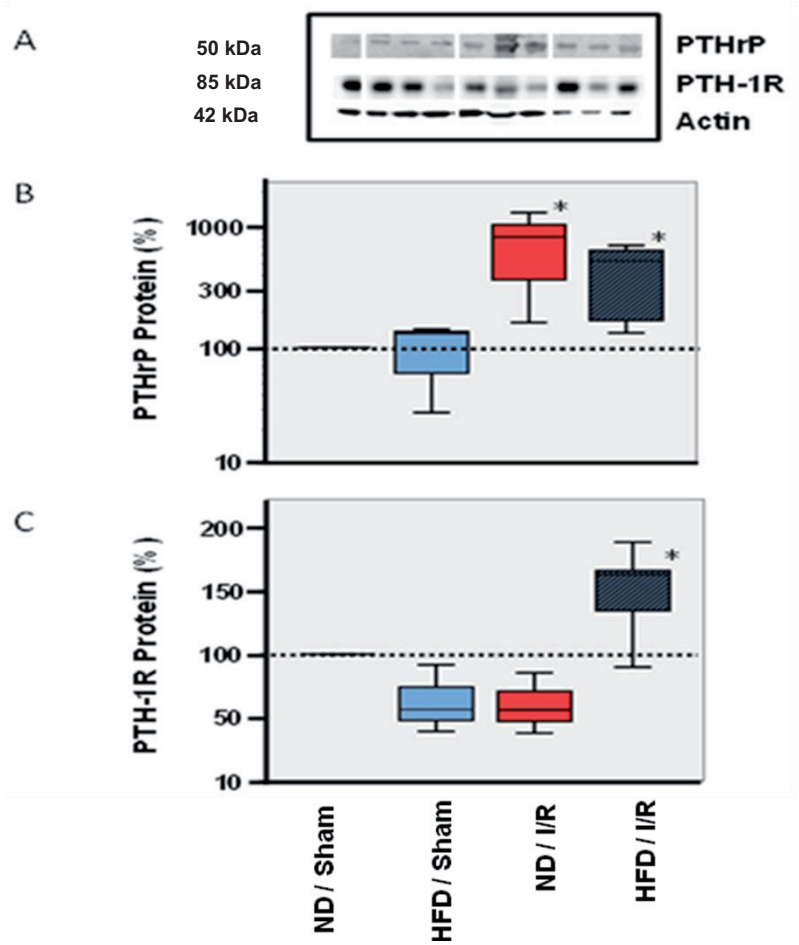

Figure 9. Pulmonary protein expression of PTHrP and the corresponding PTH-1R in seven months old mice. A) Representative western blot; B) Mean protein expression of PTHrP; C) Mean protein expression of PTH-1R. Data are expressed as box plots. The dashed line indicates the mean expression level of ND-Sham. *, $p<0.05$ vs. ND-Sham.

Experiments carried out in old mice (Fig. 10) revealed no significant differences between the groups compared.

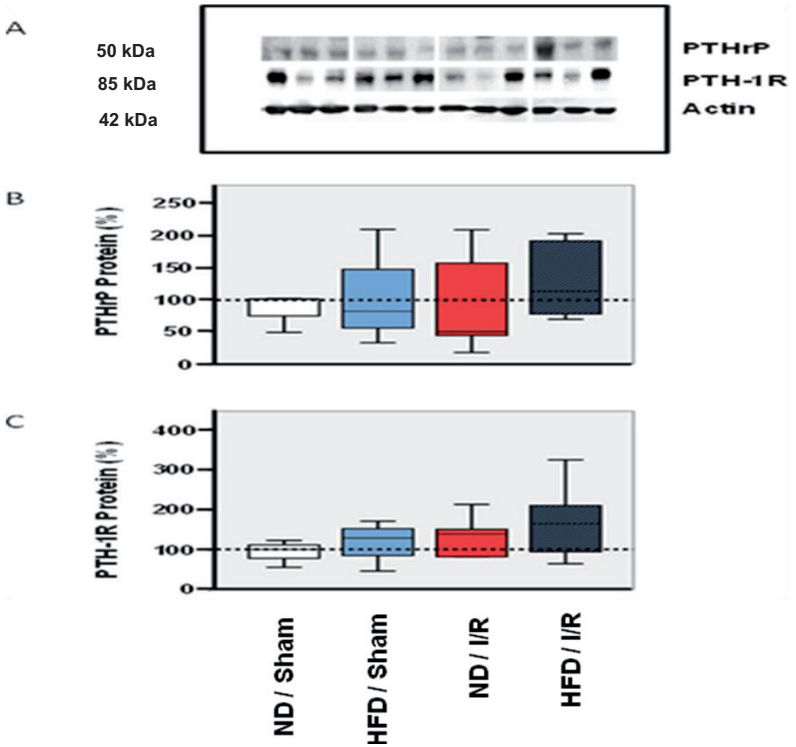

Figure 10. Pulmonary protein expression of PTHrP and the PTH-1R in thirteen months old mice. A) Representative western blot; B) Mean protein expression of PTHrP; C) Mean protein expression of PTH-1R. Data are expressed as box plots. The dashed line indicates the mean expression level of ND-Sham.

3.1.4. Effect of high fat diet on pulmonary elastin expression

The mRNA expression of PTHrP and the downstream targets ADRP and PPARγ was upregulated in mice fed with high fat diet. This indicates an activation of the paracrine pathway under high fat diet conditions. Activation of this paracrine loop implies the stimulation of surfactant production and lung function. However, pulmonary function depends on lung elasticity as well, which is determined by extracellular matrix proteins. Therefore, pulmonary elastin expression was checked in order to analyze this special feature.

Experiments conducted in adult mice revealed no significant differences between groups (Fig. 11A). Whereas in older mice (Fig. 11B) the mean elastin expression was reduced significantly in sham mice fed with standard diet.

Results indicate that the induction of surfactant production by PTHrP paracrine pathway is selectively activated by high fat diet.

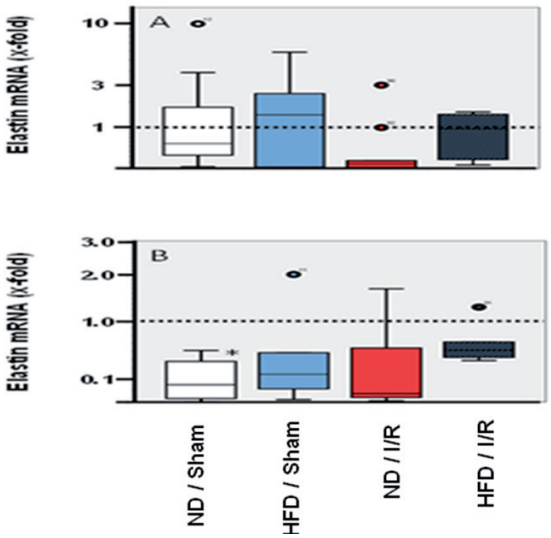

Figure 11. Pulmonary mRNA expression of elastin in seven (A) or thirteen (B) months old mice. Data are expressed as box plots. The dashed line indicates the mean expression level of ND-Sham.
*, $p<0.05$ vs. ND-Sham.

3.2. Effect of HT and LPS on PTHrP receptor responsiveness *in vivo*

3.2.1. Role of inflammatory cytokines in LPS induced sepsis

As mentioned before, chemokines, cytokines, and other inflammatory regulators are released following the feedback of the innate immune response toward pathogenic microorganisms or toxins invaded into the blood circulation as a result of sepsis, we analyzed one of pro-inflammatory cytokines, down-stream target of NFκB termed monocyte chemoattractant protein-1 (MCP-1).

The expression of pro-inflammatory cytokine MCP-1 was analyzed in the lung, left ventricle of the heart, liver, and aorta under both temperature conditions. As shown in Fig.12. LPS administration increased the mRNA expression of MCP-1 in all tissues under investigation. Liver (Fig.12C) is an exception where LPS did increase the mRNA expression of MCP-1 under both temperature conditions in comparison with sham group. Nevertheless, decreasing the temperature did not have any significant impact on the mRNA of MCP-1 compared to other tissues. In lung (Fig.12A), left ventricle (Fig.12B), and aorta (Fig.12D) tissues, decrease in temperature (33°C) elevated more the MCP-1 mRNA compared to normothermic pigs. However, there is an increase in both cases independently of temperature compared to the control group, which neither received LPS nor was assigned to temperature change. Results corroborate no impact of temperature on proinflammatory effects of LPS.

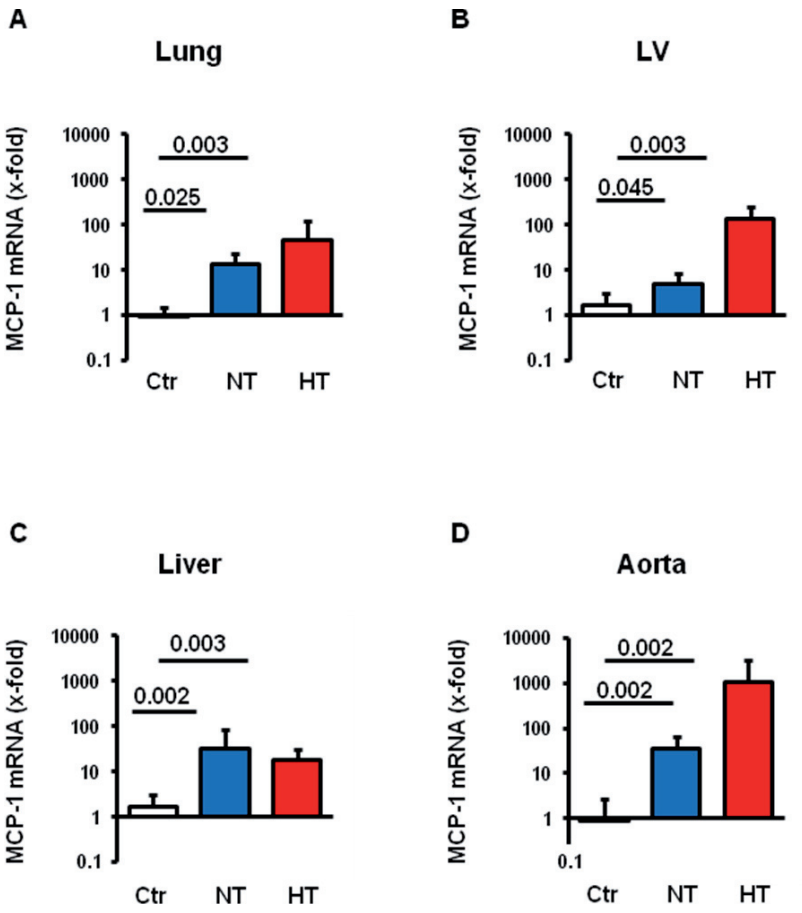

Figure 12. mRNA expression of MCP-1 in different tissues after LPS-treatment. (A) lung, (B) left ventricle, (C) liver, and (D) aorta. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). Data are expressed as means ± S.E.M. from n=6-7 samples. $p<0.05$ vs. Sham.

3.2.2. Role of hypothermia in PTHrP expression in the lungs of septic pigs

PTHrP plays an important role in lung development and function. The aim of investigation was to find out changes in mRNA and protein expression of PTHrP and downstream targets under sepsis conditions and temperature alteration.

Fig. 13A displays the PTHrP mRNA expression in lung, where the white bar shows the PTHrP mRNA (control) being not influenced neither from bacterial endotoxin, nor from temperature decrease. Pigs given LPS showed significant increase in PTHrP mRNA (marked with blue bar) compared to control. Red bar shows an increase in mRNA expression of PTHrP in the group of pigs that received LPS and the temperature was reduced to 33°C. Endotoxin elevated the PTHrP expression in the lung but temperature did not have any significant impact.

PTH-1R mRNA shown in Fig. 13B decreased after the LPS administration independently of temperature.

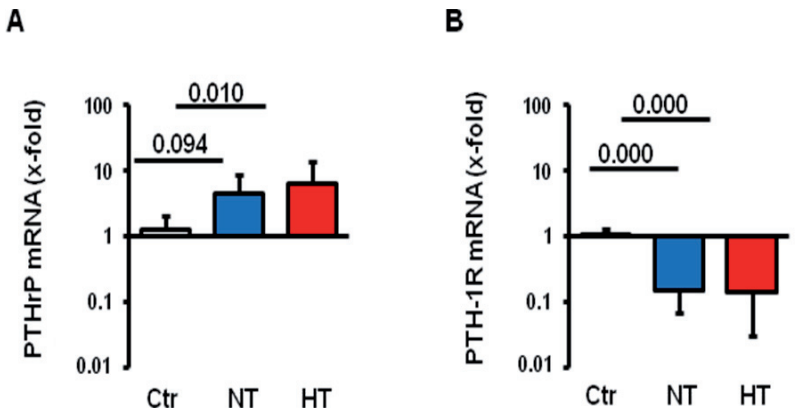

Figure 13. Pulmonary mRNA expression of PTHrP (A) and PTH-1R (B) in the lungs of septic pigs. White bar indicates sham animals. Blue bar marked indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT =33°C). Data are expressed as means ± S.E.M. from n=6-7 samples. $p<0.05$ vs. Sham.

As for protein expression of PTHrP shown in Fig. 14A and 14C there was an obvious increase after the LPS administration compared to sham animals, indicating that induction of PTHrP protein is caused by transcriptional activation. However, no significant difference was observed, neither in case when temperature was kept normal (38°C) nor decreased to 33°C.

In contrast, protein expression of PTH-1R remained stable independently of temperature and LPS administration (Fig. 14B and 14D). The inverse correlation between mRNA and protein expression of PTH-1R indicates that the decrease in mRNA expression of the receptor might not have impact on its protein expression because the half-life of the receptor may be too long.

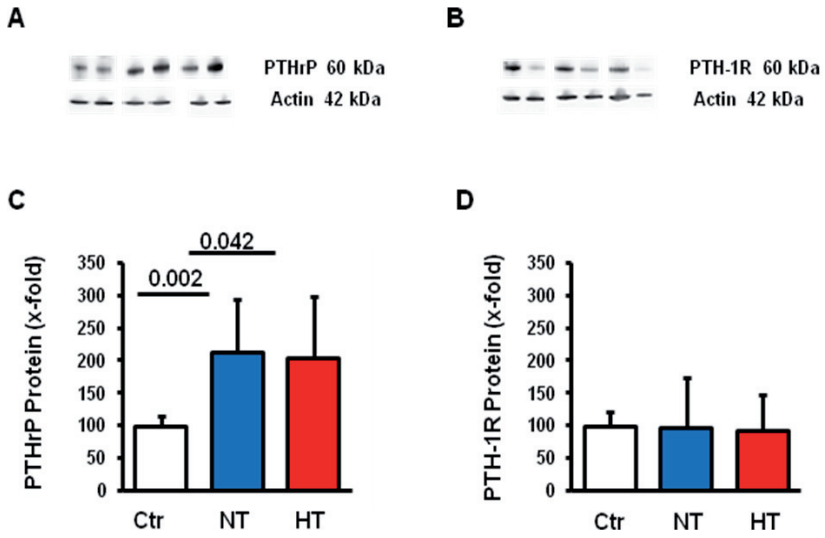

Figure 14. Effect of LPS on protein expression of PTHrP and PTH-1R in the lung. Representative western blots (A) and (B), respectively; mean protein expression (C) and (D), respectively. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

One of pulmonary PTHrP downstream targets expressed in lung lipofibroblasts and presumably involved in lipid storage and transfer, namely ADRP was analyzed (Fig 15A). ADRP mRNA increased noticeably in the lungs of septic pigs, indicating an activation of the PTHrP system in the lung. Mild hypothermia seemed to decrease the ADRP mRNA expression compared to normothermia.

The mRNA expression of an anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) was investigated, which, is believed to be important for survival and function of lymphocytes. Apparently, endotoxin did not alter significantly the mRNA expression of Bcl-2 (Fig. 15B). Keeping the temperature constant or decrease had not impact on the expression of Bcl-2.

Finally, data regarding pulmonary expression of PTHrP and downstream genes suggest an induction in the PTHrP system under septic conditions but hypothermia did not have any significant impact on analyzed pulmonary parameters.

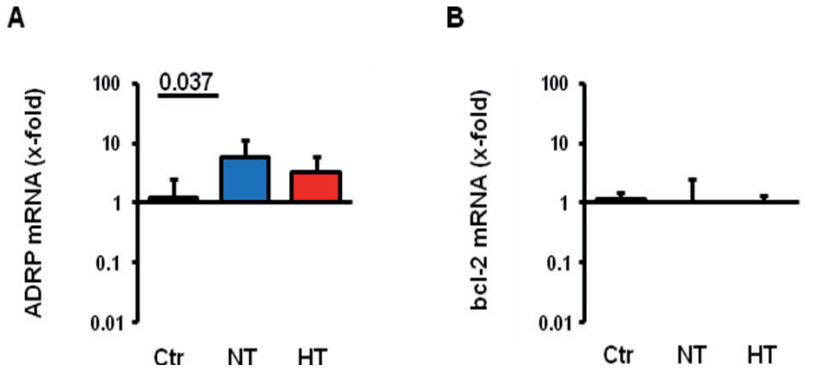

Figure 15. Effect of LPS on pulmonary mRNA expression of PTHrP downstream targets, ADRP (A) and Bcl-2 (B). Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

3.2.3. Effect of hypothermia and endotoxin on PTHrP system in the liver

Liver is one of vital organs known to fail as a consequence of septic shock. PTHrP is known to be induced in vital organs under septic conditions. The aim was to analyse if temperature change could modify the situation. Administration of LPS elevated considerably the PTHrP mRNA levels (Fig. 16A). Mild hypothermia displayed with red bar in the figure, increased the PTHrP expression towards normothermia. Thereby, endotoxin and even temperature change did affect the PTHrP levels in the liver.

Regarding PTH-1R shown in Fig. 16B, as found in the lung tissue, LPS decreased its mRNA expression in a temperature dependent way.

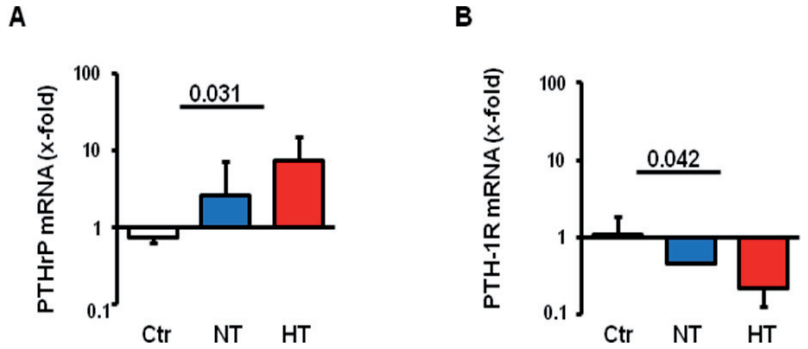

Figure 16. mRNA expression of PTHrP (A) and PTH-1R (B) in the liver of septic pigs. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

Protein expression of PTHrP in liver (Fig. 17A and 17C) is higher in control animals than after giving the LPS. As shown with blue and red bars, temperature did not influence significantly the protein expression.

On the other hand, protein expression of PTH-1R is as high as PTHrP protein and neither LPS administration nor temperature change did alter the expression of the receptor.

Fig. 17B shows no difference between the loading control β-Actin and PTH-1R, in the same way like bars in Fig. 17D.

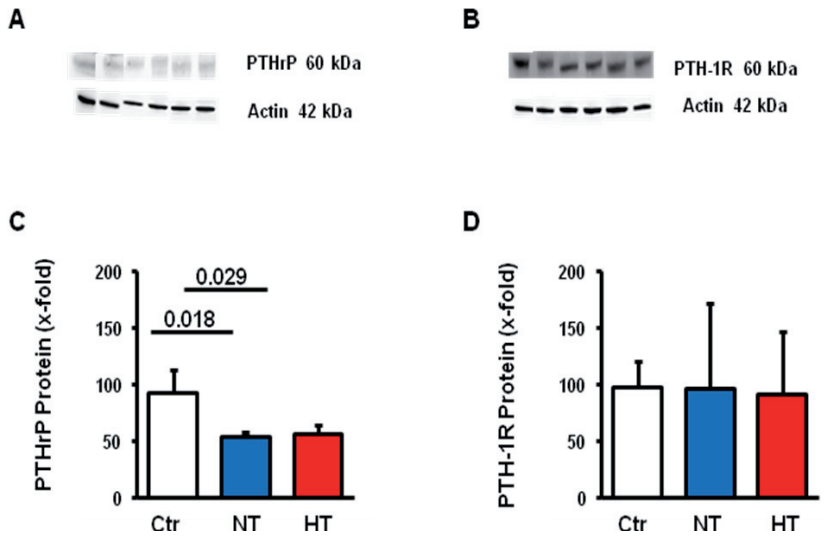

Figure 17. Effect of LPS on protein expression of PTHrP and PTH-1R in the liver. Representative western blots (A) and (B), respectively; mean protein expression (C) and (D), respectively. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

Liver response to LPS and most importantly the role of temperature change were checked through the expression pattern of an important regulator of the immune system and profibrotic protein, the cytokine transforming growth factor ß1 (TGF-ß1). Sepsis, as expected elevated the cytokine levels under normothermic conditions, as shown in Fig. 18A, blue bar. Hypothermia reduced the TGF-ß1 mRNA levels compared to normothermia.

The mRNA expression of profibrotic protein Collagen-1 was quantified in the liver of septic pigs and during the temperature alteration (Fig 18B). When temperature was kept normal (38°C), LPS administration up regulated the expression of COL1A1, whereas hypothermia decreased its mRNA expression. As shown in the figure below there is a correlation between the mRNA expression of TGF-ß1 and alpha-1 type I collagen (COL1A1) in the liver. However, the results revealed no significant effect of endotoxin and hypothermia on the expression of these profibrotic proteins. Therefore, data reveal a little proof that PTHrP system in the liver might have any functional relevant activation.

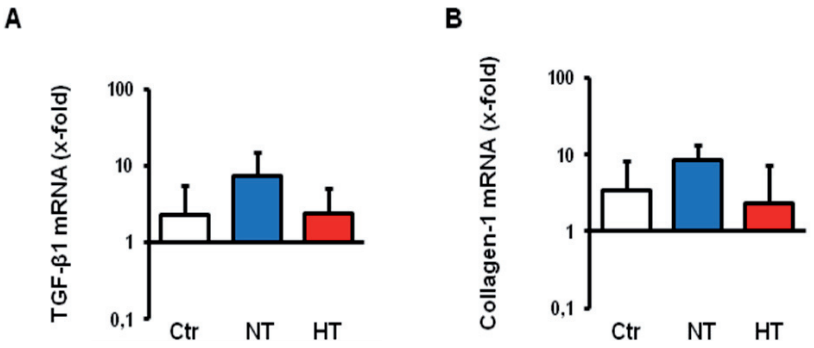

Figure 18. Effect of LPS on mRNA expression of PTHrP downstream targets, TGF-ß1 (A) and Collagen-1 (B) in the liver. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

3.2.4. Effect of LPS and hypothermia on PTHrP system in the left ventricle

Left ventricle of the heart was used to investigate the role of LPS and hypothermia in the cardiac expression of PTHrP. LPS appeared to downregulate the mRNA expression of PTHrP and PTH-1R in a temperature dependent way, as shown in Fig. 19A and 19B respectively.
Findings in the left ventricle are the opposite to other tissue organs investigated so far, where LPS steadily increased the mRNA expression of PTHrP.

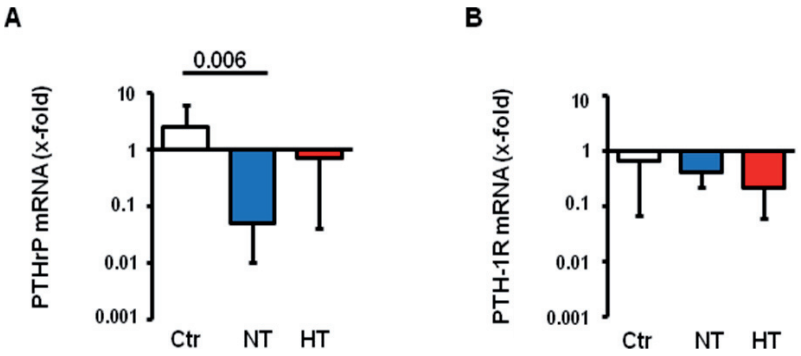

Figure 19. Effect of LPS on mRNA expression of PTHrP (A) and PTH-1R (B) in the left ventricle. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

On the other hand, protein expression of PTHrP system was analyzed as well, as shown in Fig.20. Endotoxin administration at 38°C did not alter significantly the PTHrP protein expression but when temperature was decreased to 33°C, it reduced the protein expression (Fig. 20A and 20C). LPS decreased the protein expression of PTH-1R (Fig. 20B and 20D) in a temperature dependent way. However, there were no significant changes observed regarding protein expression of PTHrP and its corresponding receptor. LPS and mild hypothermia seemed to affect differently the expression of PTHrP system in the cardiovascular system.

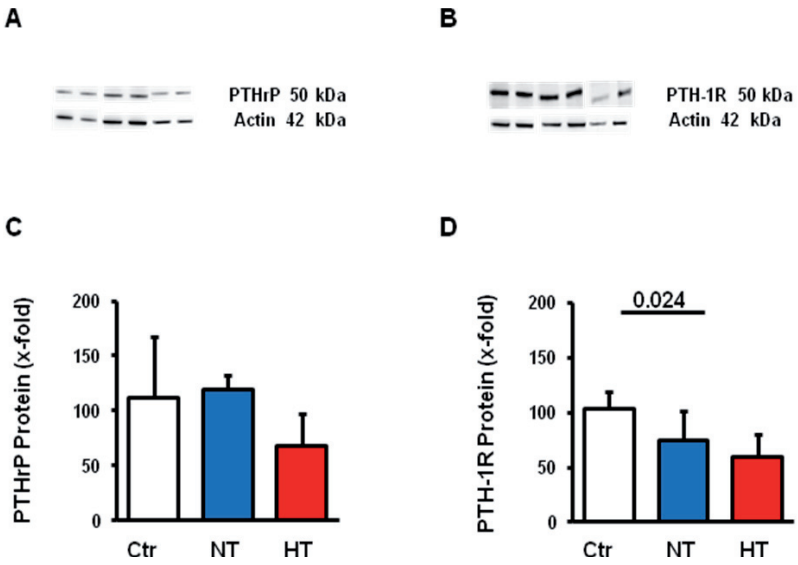

Figure 20. Effect of LPS on protein expression of PTHrP and PTH-1R in the left ventricle. Representative western blots (A) and (B), respectively; mean protein expression (C) and (D), respectively. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

TGF-ß1 mRNA expression elevated under sepsis conditions compared to control group as seen in Fig. 21A. Hypothermia increased its expression towards normothermia, in contrast to liver (Fig. 18A) where temperature decrease, reduced the cytokine expression bringing to constitutive levels in the liver.

As displayed in Fig. 21B, there was a difference in the collagen type I expression in liver and heart. Endotoxin administration at constant temperature did reduce its expression but on the other hand, hypothermia increased the mRNA of COL1A1. It suggests that increased expression of TGF-ß1 during mild hypothermia may influence the increase of ECM protein collagen type I in the heart.

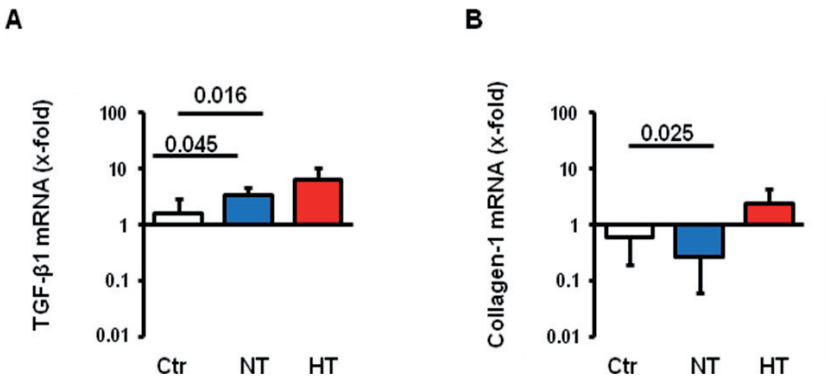

Figure 21. Effect of endotoxin on mRNA expression of PTHrP potential downstream targets, TGF-ß1 (A) and Collagen-1 (B) in left ventricle. Data are expressed as means ± S.E.M. from n=6-7 samples. White bar indicates sham animals. Blue bar indicates normothermic animals (NT=38°C) that received LPS. Red bar indicates animals that received LPS and assigned to mild hypothermia (HT=33°C). $p<0.05$ vs. Sham.

Hypothermia influenced the left ventricle of the heart differently compared to lung and liver. PTHrP mRNA expression was stronger, protein expression of PTH-1R was reduced and COL1A1 mRNA was increased in the left ventricle of hypothermic pigs. Therefore, this suggests that hypothermia affects differentially the PTHrP system in the heart compared to other tissue organs investigated under endotoxemia.

Furthermore, Langendorff perfused rat hearts were used to find out if this cardiovascular specific effect of temperature on the expression of PTHrP in LPS-treated pigs indicates a participation of PTHrP in the protective effect of moderate hypothermia and whether LPS modifies receptor responsiveness, the role of temperature alteration in this case.

3.3. Effects of hypothermia and LPS on PTHrP expression *in vitro*

3.3.1. Effects of endotoxin and temperature on MCP-1 in Langendorff perfused hearts

The observed results in pigs so far revealed not similar effects of LPS and hypothermia on organs under investigation. Endotoxin increased the mRNA expression of the pro-inflammatory cytokine MCP-1 in liver, left ventricle, aorta and lung. Mild hypothermia did not show significant variation in comparison with normothermia.

The previous results in pigs concerning the effect of temperature on pro-inflammatory cytokine MCP-1 and cardiac expression of PTHrP in a model of endotoxemia caused by LPS could be reproduced in the Langendorff rat heart system.

As described earlier, hearts in the Langendorff system were perfused with LPS for 2 hours and lastly PTHrP (1-34) 100 mM was added for 10 minutes. Experiments were carried out at different temperatures, namely 37°C and 32°C in order to find out this cardiovascular specific effect of temperature on the PTHrP expression in experimental endotoxemia and to investigate the functional response to PTHrP.

Fig.22 shows the mRNA expression of the pro-inflammatory cytokine MCP-1 where it is obvious that LPS significantly induced cytokine mRNA levels regardless of temperature. The same results were obtained from experiments performed in different tissue organs like liver lung, aorta, and left ventricle of the pigs indicating a sepsis condition after the endotoxin administration.

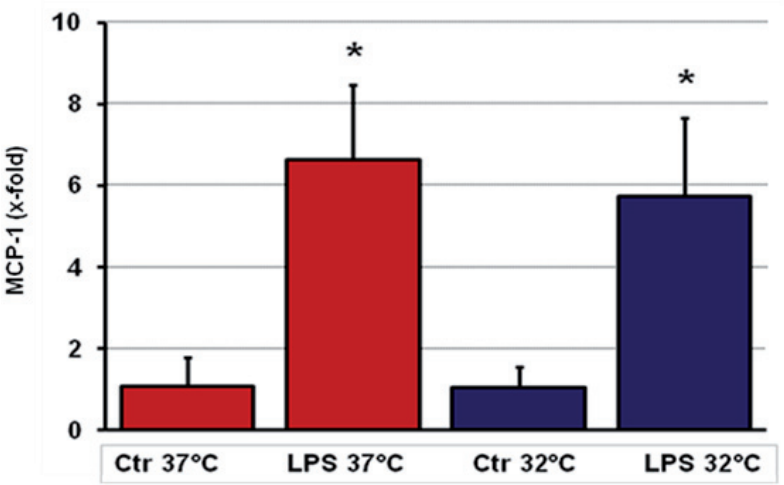

Figure 22. mRNA expression of MCP-1 in the left ventricle of Langendorff perfused hearts after LPS treatment at different temperatures. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. *; $p \leq 0.05$

3.3.2. Effects of endotoxin and temperature on PTHrP expression in Langendorff perfused hearts

As for PTHrP, LPS did elevate its expression in the lung and liver of septic pigs and temperature change did not have significant effect, whereas, cardiovascular system under endotoxin effect influenced the PTHrP system, downregulating its expression in a temperature dependent way.
Hearts perfused in the Langendorff apparatus showed that LPS differentially changed the expression of PTHrP (Fig.23) in a temperature dependent way. PTHrP mRNA was higher in hearts subjected to hypothermia than to normothermia-treated ones under experimental endotoxemia.
mRNA expression of PTH-1R displayed no significant changes (data not shown).

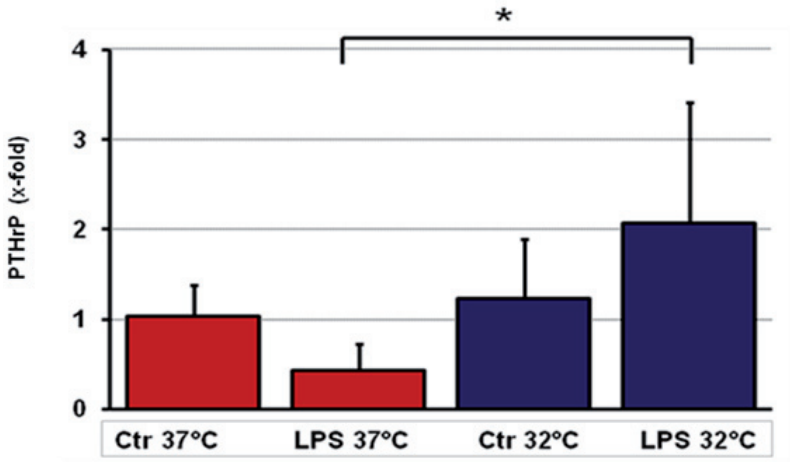

Figure 23. mRNA expression of PTHrP in the left ventricle of Langendorff perfused hearts after LPS treatment at different temperatures. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. *; $p<0.05$

3.3.3. Effects of endotoxin and temperature on cardiovascular functional parameters

Experiments on Langendorff rat heart system revealed similar findings as those on pigs regarding the effect of temperature on cardiac expression of PTHrP. Furthermore, functional parameters of heart like left ventricular developed pressure (LVDP) and heart rate could be recorded in the heart during the period of LPS perfusion and after adding the PTHrP (1-34) 100 mM to understand potential changes in cardiac function as a result of temperature modification. A typical measure of cardiac function, the LVDP is shown in Fig.24. Endotoxin administration for 2 hours did not change significantly the LVDP. Keeping the temperature at 37°C under LPS effect increased moderately the LVDP while decreasing the temperature to 32°C reduced the LVDP. However, in both cases the effect of LPS on LVDP was not significant during 2 hours, indicating that cardiac contractility was not affected significantly by endotoxin and temperature. The LPS concentration was lower than required to display direct cardio-depressive effects.

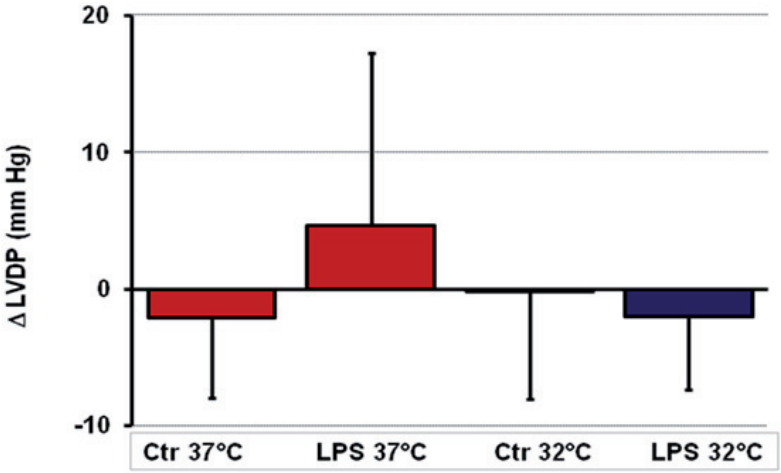

Figure 24. Left ventricular developed pressure (LVDP) 2 hours after LPS perfusion of rat hearts. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. $p<0.05$ vs. Ctr.

Similar effect of LPS was found on heart rate of the rats. Adding the LPS at 37°C reduced the HR compared to control (Fig. 25) while LPS addition at 32°C increased the HR. Still the endotoxin effect was not significant during 2 hours perfusion.

Both cardiac parameters, LVDP and HR were not influenced significantly from the LPS administration and mild hypothermia.

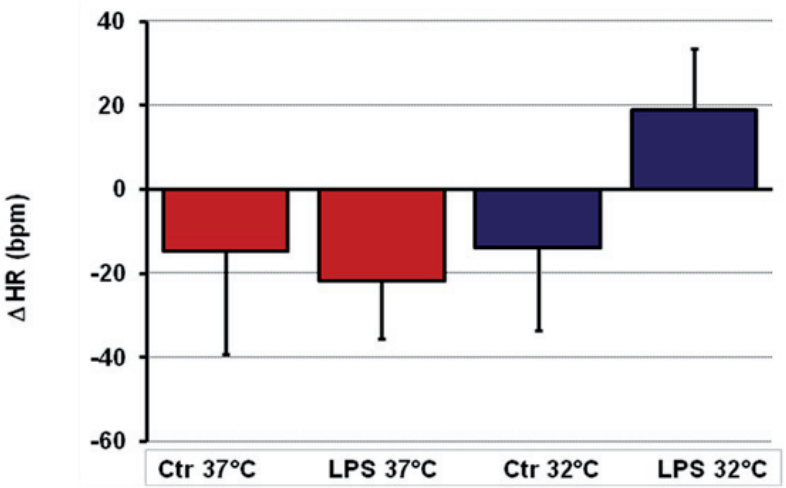

Figure 25. Heart rate (HR) 2 hours after LPS perfusion of rat hearts. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. $p<0.05$ vs. Ctr.

3.3.4. Effects of PTHrP and temperature on cardiovascular functional parameters

The contraction force of the heart, namely the LVDP was analysed not only under LPS conditions but also after adding the PTHrP agonist (PTHrP (1-34)) at different temperatures. Hearts receiving endotoxin at 37°C for 2 hours displayed a drop in left ventricular developed pressure following 10 min PTHrP stimulation. However, the agonist did not have significant effect at 37°C (Fig.26). In hearts treated at 32°C, the agonist increased LVDP, a positive contractile effect was seen, indicating that PTHrP responsiveness is improved in LPS treated hearts under hypothermic conditions. The data suggest a different receptor responsiveness of PTH receptor under hypothermia and normothermia.

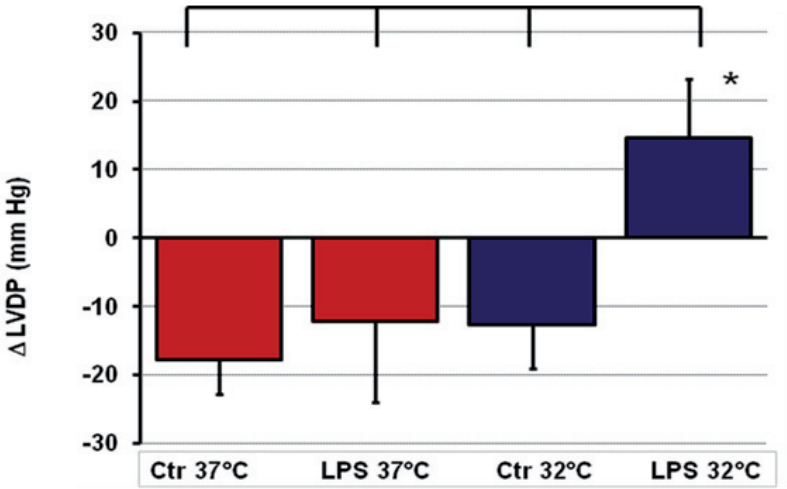

Figure 26. Left ventricular developed pressure (LVDP) ten min after PTHrP (1-34) (100 mM) administration in LPS-treated rat hearts. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. *; $p \leq 0.05$ vs. pre-application of PTHrP

Short-term PTHrP stimulation decreased the heart rate of LPS-treated rat heart under normothermia, as shown in Fig.27 with red bars. However, the decrease was more pronounced in hearts treated under hypothermic conditions, suggesting repression in PTHrP responsiveness in mild hypothermia (Fig.27 blue bars).

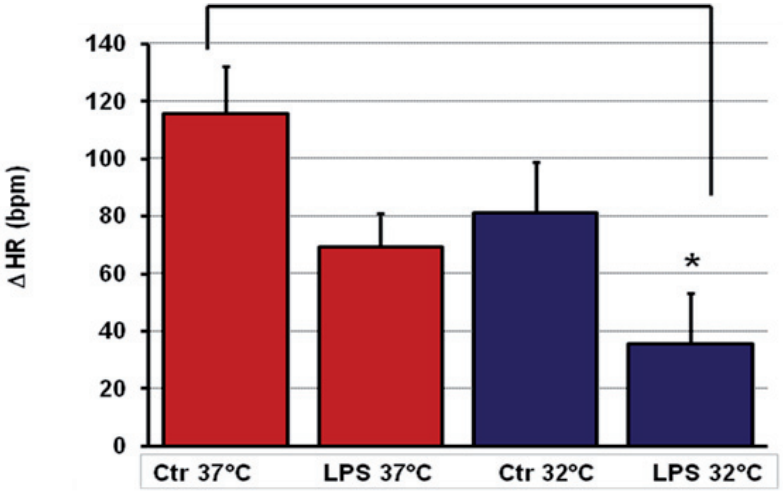

Figure 27. Heart rate (HR) ten min after PTHrP (1-34) (100 mM) administration in LPS-treated rat hearts. Data are expressed as means ± S.E.M. from n=6-7 samples. Red bars indicate rat hearts, either control or those that received LPS at 37°C. Blue bars indicate rat hearts, either control or those that received LPS at 32°C. *; $p<0.05$ vs. pre-application of PTHrP

3.3.5. Effect of PTHrP on MAPK activation

The effect of temperature and PTHrP on activation of ERK2 (also known as p42 or MAPK1, a member of extracellular-signal-regulated kinase family) as a downstream target of PTH receptor stimulation, in LPS treated hearts was investigated by western blot analysis. As displayed in Fig. 28A, LPS treatment at different temperatures or administration of short-term PTHrP did not affect the total p42 protein levels, used as control value. PTHrP administration increased the phosphorylated ERK2 expression in LPS treated hearts at 32°C compared to normothermic rat hearts (Fig. 28A and 28B). Results indicate an induction in PTHrP responsiveness (ERK activation) in hearts treated with LPS under hypothermic conditions.

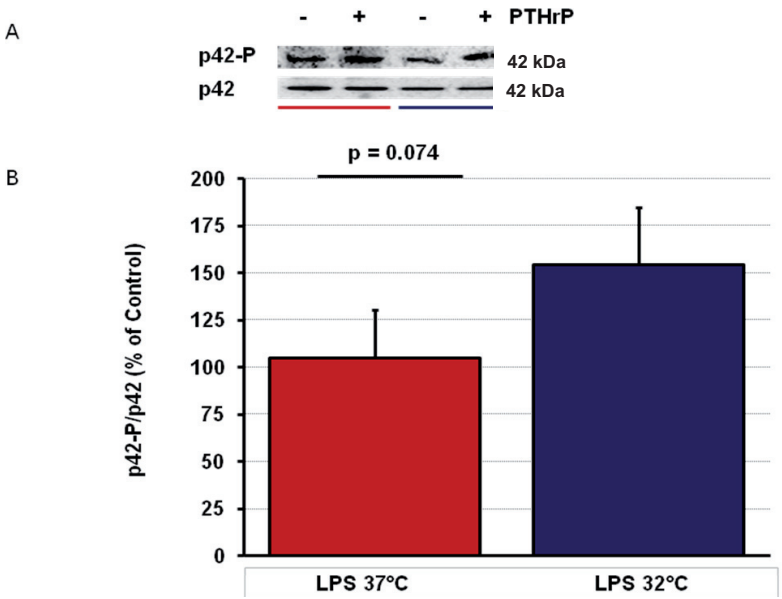

Figure 28. Effect of PTHrP on protein expression of ERK2 in LPS treated hearts. Representative western blot of p42 (loading control) and p42 phospho (A); mean protein expression (B). Data are expressed as means ± S.E.M. from n=6 samples. Red bar indicates rat hearts that received LPS at 37°C. Blue bar indicates rat hearts that received LPS at 32°C. *, $p \leq 0.05$ vs. 37°C

3.3.6. Effect of LPS and PTHrP receptor antagonism on cell shortening

Mechanical properties of isolated adult rat ventricular myocytes were monitored by cell-edge detection method. Using this system, the cell shortening was determined in cardiomyocytes treated with endotoxin in normothermic and hypothermic conditions and after administration of PTH/PTHrP receptor antagonist, PTHrP (7-34) 100 nM. Keeping the stimulation frequency 2Hz the contractility of the cell was measured and presented as a percentage of the cell length while resting. So far, results revealed an induction in PTHrP responsiveness in LPS treated hearts, therefore blocking the receptor under normo- and- hypothermia was the next aim of investigation.

As shown in Fig. 29, the shortening of the LPS treated cells was decreased after antagonist administration under normothermic conditions. Cardiac myocytes treated with endotoxin under hypothermia showed slight increase in cell shortening compared to normothermia. When the receptor antagonist was added to cardiomyocytes treated under hypothermic conditions, it increased the cell shortening. Thus, in both cases a slight change in cell shortening was monitored, however it was not significant. Therefore, it could be concluded that LPS treatment and receptor-antagonist responsiveness is different in LPS treated isolated rat ventricular myocytes under temperature alteration. However, blocking the receptor under hypothermic conditions increased more the contraction amplitude than normothermia. This temperature-dependent increase in contraction amplitude of the cells indicates that PTHrP (7-34) responsiveness is higher under hypothermic conditions.

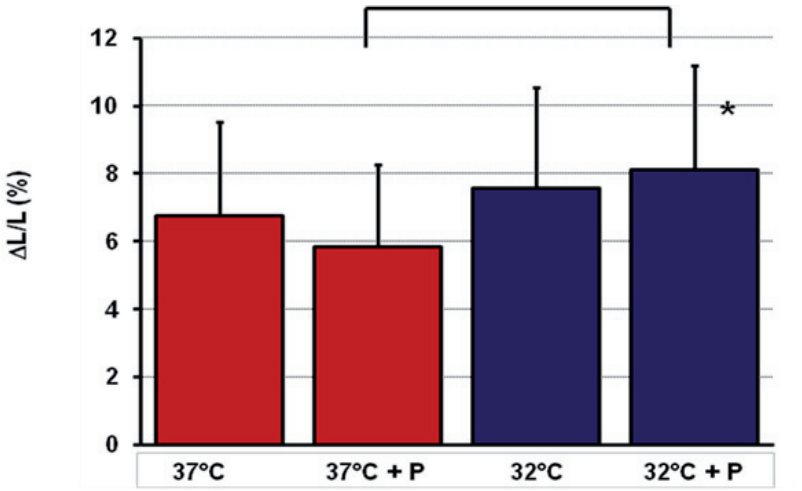

Figure 29. Effect of PTHrP (7-34) on cell shortening in LPS-treated isolated cardiomyocytes at different temperatures. Data are expressed as means ± S.E.M. from n=27 cells. Red bars indicate cardiac myocytes, either control that received only LPS or those that received LPS and PTHrP (7-34) at 37°C. Blue bars indicate cardiac myocytes, either control that received only LPS or those that received LPS and PTHrP (7-34) at 32°C. *, $p<0.05$ vs. 37°C

4. Discussion

4.1. Effects of high fat diet, ageing and ischemia/reperfusion on pulmonary PTHrP system in mice

The study reveals the role of different stressors in PTHrP expression in the lung. As regards high fat diet, it increased the steady state pulmonary mRNA expression of PTHrP *in vivo* under basal conditions. ADRP and PPARγ, the downstream targets of PTHrP were upregulated by high fat diet, as well. Elevated PTHrP expression may be associated with increased leptin levels, as Torday and Rehan (2007) elucidated the interaction between leptin and PTHrP expression. Experiments performed in leptin deficient mice (data not shown) showed downregulation of PTHrP mRNA expression in the lung. However, the reduced mRNA expression of pulmonary PTHrP in B6.V-Lep^{ob}/J mice did not lead to alterations in the mRNA expression of PTHrP dependent genes.
High fat diet, cardiac ischemia/reperfusion, and ageing elevated the mRNA levels of PTHrP and the downstream targets in adult mice; however, they had no significant effect in the pulmonary mRNA expression of PTHrP in older mice.

PTHrP is considered pivotal in epithelial-mesenchymal paracrine cross talk, being involved in surfactant production. Torday and Rehan (2007) elaborated this process in detail, demonstrating the communication between epithelial cells with alveolar type II cells via release of leptin (Torday and Rehan 2002). Exactly this positive feedback of the communication between two cell types has prompted us to investigate the role of high fat diet in the pulmonary expression of PTHrP, whether increase in leptin levels due to high fat diet could modify the PTHrP expression. This fact was proved by experiments. High fat diet elevated the leptin levels and this led to increase in mRNA expression of PTHrP in the lung. The expression of the corresponding receptor, PTH-1R, was not affected by high fat diet. PTHrP protein level was not induced under these conditions, indicating that there is a higher turnover of PTHrP. Therefore, our data suggest that high turnover of PTHrP is indicative for an activation of the PTHrP system in the lung, owing to the fact that PTHrP downstream target genes like ADRP and PPARγ appeared to be upregulated as well.

Induction of the PTHrP-driven epithelial-mesenchymal cross talk was assessed by numerous studies as a protective mechanism which provides better oxygenation of the pulmonary blood (Torday and Rehan (2007); Stern, Bernard et al. (2002)). PTHrP is known to contribute in developing and stabilizing the lung function through several mechanisms including the regulation of branching morphogenesis and type II cell maturation, the process of surfactant production which improves oxygen uptake, vasodilatation that improves perfusion, inhibition of lipofibroblast transdifferentiation into myofibroblasts (Torday and Rehan (2007); Hastings (2004)). Results obtained from our investigation regarding elastin revealed no alteration in its mRNA expression under any of the conditions, which complies with findings that increase in elastin expression may be a characteristic of interstitial pulmonary fibrosis that leads to altered lung mechanics (Cantor, Keller et al. (1987); Lucey, Ngo et al. (1996); Kuang, Zhang et al. (2007)).

Several conditions are known to alter the pulmonary development and function by inhibiting the protective role of PTHrP like barotrauma, oxytrauma, prematurity, infection and nicotine (Rehan, Wang et al. (2005); Rehan, Sakurai et al. (2007); Doi, Lukosiute et al. (2010); Hastings, Ryan et al. (2002)). Our findings could prove that myocardial infarction and ageing do not belong to these kinds of inhibitory factors.

On the other hand, the obesity paradox is a condition, which describes the fact that obese people tolerate ischemic events better than people of normal weight do. We hypothesized that such a situation is accompanied by better PTHrP-driven epithelial-mesenchymal paracrine cross talk. Experiments performed on mice fed with high fat diet but not subjected to myocardial infarction showed an increase in pulmonary expression of PTHrP.

Mice fed with high fat diet compared to those fed with normal diet, when both underwent ischemia/reperfusion, displayed a significant improvement in PTHrP and PTH-1R protein expression. This upregulation may indicate a more appropriate coupling of PTHrP and PTH-1R system in the high fat diet group, suggesting that PTHrP under these conditions could stimulate its corresponding receptor turning on a self-stimulation mechanism. High fat diet may be beneficial for lung function; however, this cannot be concluded from this study.

According to Vinten-Johansen and Shi (2011) ageing leads to reduction or even loss of protective mechanisms. In this study, the effect of high fat diet on ageing was analyzed. Seven-month-old mice were compared to thirteen-month old ones. In older mice, PTHrP mRNA expression was increased in contrast to elastin levels, which were low. The mRNA of PTHrP in older mice was higher than in young ones in the case when mice were fed with standard diet

and experienced only sham surgery. However, high fat diet had no significant influence on older mice.

The result obtained from experiments was expected knowing the assumption as how PTHrP attenuates epithelial-mesenchymal transition to fibrosis. High PTHrP levels lead to elevation of downstream molecules like ADRP and PPARγ and reduce the endothelial transdifferentiation into fibroblasts. Our experiments showed that high fat diet has no significant effects on pulmonary expression of PTHrP and PTHrP-dependent regulated targets in older mice. Its effect was more pronounced in younger mice than in older ones. PPARγ expression in lungs of thirteen-month-old mice was significantly elevated though. In general, PPARγ mRNA expression in the lung was more closely linked to high fat diet than that of PTHrP, PTH-1R or ADRP. The overall data show that high fat diet can modify the pulmonary mRNA expression of PTHrP in young mice.

Finally, this study reveals a pivotal effect of leptin on the steady state level of PTHrP mRNA *in vivo*. Ischemia/reperfusion and ageing elevated the PTHrP mRNA expression but not the entire PTHrP system was induced. The high fat diet effect on mRNA expression of PTHrP in lungs of young mice may contribute the observations that are generally named the obesity paradox.

4.2. Effects of hypothermia on PTHrP system in endotoxemic pigs and rats

As shown by other studies, it could be confirmed by our experiments as well that endotoxemia induces the PTHrP system in multiple tissues. The target of this investigation was to find out the role of hypothermia in increased expression of PTHrP in endotoxemic animals. Therefore, the study reveals that PTHrP-responsiveness in the heart is altered under hypothermic conditions, which ameliorates the cardiac function. This led us to conclude that hypothermia has beneficial effects on sepsis.

Earlier findings by Funk, Krul et al. (1993) regarding endotoxin, namely LPS have demonstrated increase in the PTHrP mRNA expression in the spleen of mice. Later on studies found elevation of PTHrP mRNA expression in different organs after administration of a near lethal dose of LPS, while inhibition of PTHrP led to delay in mice mortality (Funk, Moser et al. (1996); Funk, Moser et al. (1997)). In accordance with these studies, we could confirm the effects of LPS on PTHrP system in various tissue organs of a large animal model with a different experimental endotoxemia model. Sublethal dose of LPS was given to the animal for four hours and an increase in PTHrP mRNA in lung and liver was seen 8 hours after the LPS administration.

Concerning PTH-1R, it was found that LPS downregulates the receptor mRNA expression in rodents (Funk, Moser et al. 1997). Using PTHrP antagonist downregulated the mRNA expression but it was still a functional receptor (Funk, Krul et al. 1993). Our experiments showed that hypothermia downregulated and desensitized the PTH-1R mRNA expression in liver, heart, and lung. However, the protein expression was not significantly downregulated in liver and lung. The data suggest a causal role for PTH-1R downregulation in the beneficial effects of hypothermia. The disruption of PTH-1R system may be enhanced under mild hypothermia and contribute to better outcome.

Torday and Rehan (2007) proved that PTHrP in the lung is an essential factor for maintaining the epithelial-mesenchymal cross talk by regulating the expression of some downstream molecules. Therefore, we analyzed ADRP, as one of these PTHrP-dependent molecules, and observed an increase in pulmonary ADRP expression under normothermia in septic pigs. Pulmonary PTHrP mRNA and protein expression was increased. The obtained results indicate that LPS activates the PTHrP system in the lung. In the liver PTHrP protein expression was reduced which lets us to understand that there is a high turnover of PTHrP. However, this does not mean that PTHrP system is not important for liver function. Li, Seitz et al. (1996) elucidated the role of PTHrP in the liver as an autocrine or paracrine growth factor contributing in the regulation of hepatocyte proliferation.

When it comes to hypothermia, it was used as a method to make a better prognosis in the case of experimental endotoxemia. Data indicate that hypothermia decreases the mRNA expression of ADRP, therefore leading to a disruption of PTHrP-ADRP axis in the lung and this may contribute to the better functional recovery of the pigs. As described by Schwarzl, Seiler et al. (2013), hypothermia improves the heart contraction and respiratory function. In our model of experimental endotoxemia, hypothermia had an impact on the response of the PTHrP system particularly in the heart. In other words, hypothermia elevated the mRNA expression of PTHrP in the pig and rat model. When the effect of normothermia was compared to hypothermia in endotoxemic hearts, the different receptor responsiveness was remarkable. It was demonstrated that cardiac stress could acutely modify the PTH-1R responsiveness (Jansen, Gres et al. 2003) as the dose of PTHrP (1-34) needed to cause maximum vasodilatation in stunned myocardium was reduced remarkably, indicating a change in the density or to the endothelium-mediated effects of the receptor.

According to our data in isolated perfused rat hearts, PTHrP increased LVDP in hearts subjected to endotoxin under hypothermic but not normothermic conditions. Subsequently a significant activation of the protein kinase ERK was observed. ERK activation and functional improvement in rat hearts under hypothermia might be coupled; however, this has not been mechanistically investigated in the study because the aim was to find out the potential hypothermia-dependent changes in PTHrP responsiveness.

Measuring the cell shortening of isolated cardiomyocytes, we could show that PTHrP can improve the cardiac contractility in endotoxemia under hypothermic but not normothermic conditions. We could say that hypothermia effectively couples PTHrP receptors to positive contractility and that under hypothermic conditions the ventricular PTHrP system is activated. Data suggest that activation of the PTHrP signalling cascade contributes to the cardiovascular stabilizing effect of hypothermia in endotoxemia. Hypothermia improves cardiac effectiveness by increasing inotropy but decreasing chronotropy allowing the heart to maintain cardiac output at lower energy costs. This effect is caused by Gαq-coupled PTHrP receptors (in vitro in isolated rat hearts and isolated cardiomyocytes) as shown in Fig. 30. The aforementioned effects may contribute to the beneficial acute effect of moderate hypothermia in critically ill septic patients.

Data add evidence to an essential role of PTHrP system in proinflammatory signal transduction during endotoxemia and further demonstrate that mild hypothermia beneficially influences this pathway, suggesting that PTHrP may be involved in the protective effects of hypothermia under septic conditions. Temperature influences the effect of LPS on cardiac expression of PTHrP and receptor responsiveness independent of its effect on pro-inflammatory cytokines (in vivo in pigs; in vitro in isolated rat hearts). As described by Schwarzl, Seiler et al. (2013), in the same pig model LPS administration led to increased expression of classical proinflammatory cytokines (tumor necrosis factor-α, and interleukin 6 and 8) independently of temperature. In accordance with these data, our experiments showed steadily elevation in NFκB-target MCP-1 expression in all tissues under investigation, independently of temperature. This emphasizes the role and significance of PTHrP in temperature dependent alterations and especially under hypothermic conditions in experimental endotoxemia.

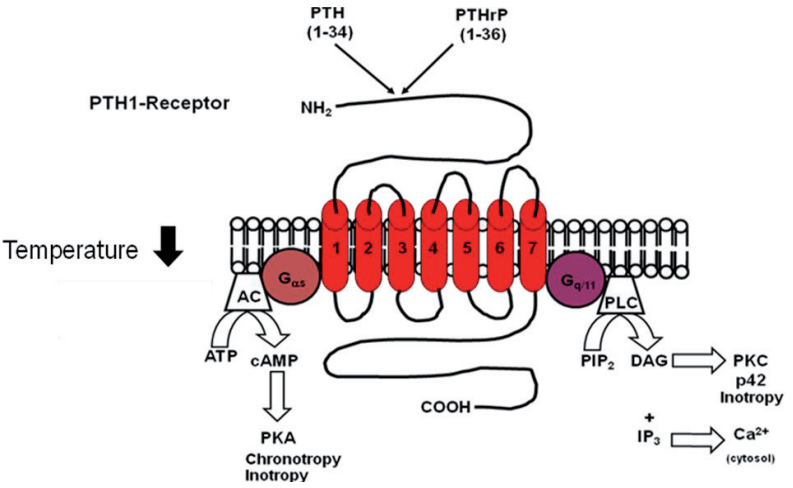

Figure 30. Proposed mechanism in this study: Hypothermia shifts PTHrP receptor responsiveness from Gαs to Gαq coupling. Hypothermia improves cardiac function in models of endotoxemic stress mimicking a sepsis related condition by modifying the expression and function of cardiac PTHrP receptors.

Clinical perspective of this study seems to be substantial. HFD by increasing the interaction between Leptin-PTHrP in the lung may contribute to lung remodeling and favor the adult obese patients who experience infarction, attenuating the complications following myocardial infarction, thereby improving the post-infarct outcome. However, HFD may not favor the obese old patients. On the other side, mild hypothermia may attenuate and stabilize the cardiac dysfunction during experimental sepsis, suggesting improvement in the outcome of patients with sepsis.

5. Summary

Myocardial infarction and sepsis are considered as devastating diseases in the world. Myocardial ischemia occurs when blockage of one or more coronary arteries reduces the blood flow to the heart. Thus, the amount of oxygen that myocardium receives decreases. Sepsis is a systemic immune response to the infection caused mostly by bacteria.
Therefore, the current study aimed to discover the role of high fat diet and ageing in PTHrP system under ischemia/reperfusion in order to explain the so-called obesity paradox, and the role of hypothermia in vital organs, in particular the cardiac expression of PTHrP under endotoxemia.

Results suggest an age-dependent expression of PTHrP, being highly elevated in older mice rather than in adult mice. High fat diet affected the expression of PTHrP, its corresponding receptor and the downstream molecules ADRP and PPARγ. Ischemia/reperfusion increased the mRNA expression of PTHrP and ADRP in the lung but it had no significant effect on PTH-1R and PPARγ. Results corroborate the activation of the pulmonary paracrine pathway under stress conditions, especially under HFD.
Concerning LPS, it induces the PTHrP system in the lung and liver but temperature alteration did not have any significant impact. Left ventricle revealed opposite results compared to other vital organs as LPS downregulated the mRNA of PTHrP and PTH-1R. To verify the downregulation of the PTHrP system in the heart, in vitro experiments performed in the Langendorff apparatus, and in vivo experiments in pigs showed that hypothermia improved mRNA expression of PTHrP in the heart. Hypothermia improved the PTHrP responsiveness in septic hearts as one of the functional parameters of the heart, the LVDP that represents the cardiac contractility, cell shortening of isolated rat heart cells and phosphorylation of ERK (ERK2) were increased under septic conditions.

Therefore, we hypothesize that high fat diet may increase the expression of PTHrP system in the lung under ischemia/reperfusion and thereby may improve the epithelial-mesenchymal paracrine cross talk in the lung, the paracrine loop important for pulmonary function. Increase of leptin and other PTHrP downstream targets may stabilize the pulmonary remodeling after ischemia/reperfusion, which describes the obesity paradox. The current data indicate that hypothermia influences the cardiac expression of PTHrP under septic conditions, which suggests that PTHrP participates in improving the cardiac function in this situation.

6. Zusammenfassung

Der Myokardinfarkt und septische Ereignisse gehören zu den schwerwiegensten Erkrankungen weltweit. Ein Moykardinfark entsteht, wenn eine oder mehrere Koronargefäße eingeengt oder verschlossen sind. In der Folge wird das Myokard sauerstoffarm. Eine Sepsis ist eine systemische Immunantwort auf eine generalisierte bakterielle Infektion.
Aus diesem Grunde wurde in die vorliegenden Studie näher untersucht, welchen Einfluss ein Myokardinfarkt und eine sterile Infektion mit LPS auf die gewebespezifische Expression von PTHrP hat, und ob dies durch fettreiche Diät zur Erzeugung eines „obesity paradox" oder durch Hypothermie zum besseren Schutz der betroffenen Organe beeinflusst wird.

Die Ergebnisse suggerieren eine altersabhängige Expression von PTHrP, die im Alter zunimmt. Eine fetthaltige Diät beeinflusst die Expression von PTHrP, seines Rezeptors und seiner Targetgene ADRP und PPARγ. In der Folge einer Reperfusion nach myokardialer Ischämie steigt die pulmonale Expression von PTHrP und ADRP aber nicht die des Rezeptors und des PPARγ. Die Ergebnisse suggerieren eine Aktivierung des pulmonalen PTHrP Systems bei fettreicher Ernährung.

In Bezug auf LPS konnte gezeigt werden, dass LPS die pulmonale und hepatische Expression steigert ohne dass eine Hypothermie darauf Einfluss nimmt. Im Gegensatz dazu wurde im linken Ventrikel des Herzens PTHrP herabreguliert. Diese Befunde konnte in vitro und in vivo verifiziert werden und im Herzen kam es unter Hypothermie zu einer Normalisierung der Expression von PTHrP und einer funktionellen Verbesserung. In ähnlicher Weise reagierten isolierte Herzmuskelzellen auf LPS und Hypothermie (Verbesserte lastfreie Zellverkürzung, ERK2-Aktivierung).

Aus diesen Befunden lässt sich suggerieren, dass eine fettreiche Ernährung zu einer verbesserten Stimulation des PTHrP-Systems in der Lunge nach Infarktereignissen beiträgt und dass dies zumindest partiell zum „obesity paradox" beitragen könnte. Ausserdem zeigen die Befunde, dass Hypothermie die kardiale Expression von PTHrP normalisiert und dadurch zu einer verbesserten kardialen Funktion hypotherm exponierten Sepsis-Patienten beiträgt.

7. List of Abbreviations

AC	Adenylyl Cyclase
ADRP	Adipocyte differentiation-related protein
B2M	β_2-microglobulin
Bcl-2	B-cell lymphoma 2 protein
BPB	Bromophenol blue
BSA	Bovine serum albumin
cAMP	3',5'- cyclic adenosine monophosphate
CaSR	Calcium-sensing receptor
cDNA	Complementary DNA
COL1A1	Collagen, type I, alpha 1
CVR	Coronary vascular resistance
DAG	Diacylglycerol
dNTP's	Nucleotide triphosphates containing deoxyribose
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence Detection
ERK-2	Extracellular signal-regulated kinase 2
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gli-3	Zinc finger protein
HFD	High fat diet
HHM	Humoral hypercalcemia of malignancy
HPRT	Hypoxanthine phosphoribosyltransferase
HT	Hypothermia
I/R	Ischemia/Reperfusion
IHH	Indian Hedgehog protein
IL-1	Interleukin 1
IP3	1,4,5 inositol triphosphate
KCl	Potassium chloride
KHB	Krebs-Henseleit Buffer
LPS	Lipopolysaccharide
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
LVSP	Left ventricular systolic pressure
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemoattractant protein-1
M-MLV-RT	Reverse transcriptase from Moloney murine leukemia virus
MOPS buffer	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
NaCl	Sodium chloride
ND	Normal Diet
NO	Nitric oxide
NT	Normothermia
Oligo dt	A short sequence of deoxy-thymine nucleotides
p57	Cyclin-dependent kinase inhibitor 1C
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTH-1R	Parathyroid hormone receptor type I
PTH-2R	Parathyroid hormone receptor type II
PTHrP	Parathyroid hormone related protein
qRT-PCR	Quantitative real-time-polymerase chase reaction
RNAsin	Ribonuclease inhibitor
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOX9	SRY (sex determining region Y)-box 9
TBS buffer	Tris-buffered saline buffer
TBST buffer	Tris-buffered saline and Tween 20
TGF-β1	Transforming growth factor beta 1
TIP39	Tuberoinfundibular peptide of 39 residues
TNF-α	Tumor necrosis factor alpha
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride

8. List of Figures and Tables

9. List of References

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10. List of Publications

Oruqaj L, Forst S, Schreckenberg R, Inserte J, Poncelas M, Bañeras J, Garcia-Dorado D, Rohrbach S, Schlüter KD. Effect of high fat diet on pulmonary expression of parathyroid hormone-related protein and its downstream targets. Heliyon. 2016 Oct 27;2(10):e00182. eCollection 2016.

In preparation:
Learta Oruqaj, Rolf Schreckenberg, Heiner Post, Michael Schwarzl, Burkert Mathias Pieske, Klaus-Dieter Schlüter. Effect of mild hypothermia on regional expression of parathyroid hormone-related peptide (PTHrP) in experimental endotoxemia

11. Declaration of Original Work

I, Learta Pervizaj Oruqaj declare that the work presented in this thesis exhibits the results of my original research work.

The thesis entitled 'Effect of pathophysiological stressors on the tissue expression of parathyroid hormone-related protein (PTHrP)' was performed in the period between February 2012 until 2015 at the Institute of Physiology, Faculty of Medicine, Justus-Liebig University, Gießen.

I corroborate that, for the literature I consulted, the references are given, and I acknowledged all people, which contributed to accomplish this thesis work.

Gießen, 04.04. 2016 Learta Pervizaj Oruqaj

12. Acknowledgements

I would like to express my special appreciation to my supervisor Prof. Dr. Klaus-Dieter Schlüter. Thanks for your endless support, guidance through these three years and your efforts to encourage and allow me to fulfill my desire to become a research scientist.

Prof. Schlüter is a person you will immediately love and always have fun when you talk to him. I consider him as one of the most intelligent people I have ever met. I hope that I could see myself in the future as active, energetic, patient, lovely, and polite as my supervisor, and be able to lead a research group likewise. He is my role model.

Many thanks, to Dr. Rolf Schreckenberg for his valuable suggestions and for sharing his scientific knowledge with me. Thanks a lot for good advice.

I am very grateful to Prof. David Garcia-Dorado and his research group for cardiovascular diseases, Institute de Recerca, Hospital Universitari Barcelona, Spain for their proper collaboration.

Special thanks to Dr. Heiner Post, Medical University of Graz, Austria for collaboration and keen interest to accomplish this work successfully.

I acknowledge with gratitude to Prof. Dr. Klaus Preissner for giving me the chance to be member of “PROMISE” (Protecting the Heart from Ischemia), International Research Training Group 1566 Giessen – Barcelona. Our interactive talks on Fridays together with all “PROMISE” members in Gießen, as well as conferences held together with other research groups from Germany and Barcelona had an immense importance for our research.

Sincere thanks to the technical staff, especially to Nadine Woitasky and Peter Volk whose enormous and excellent work made my progress feasible throughout these years.

I particularly want to thank my fellow doctoral students at the Institute of Physiology. Besides sharing the same working environment, we became good friends also. Thanks for your kindness and cooperation. The event of „Plätzchen backen“, and celebration the Christmas all together, will be memorable for me.

I especially appreciate my family for their unlimited love and support constantly. Confidently, I promise you that I am going to spend more time with you all hereafter. Being abroad since I was 18 years old, provided me awesome experience in all respects but also gave me more maturity. I

hope that I will have the opportunity to contribute in our homeland and build a better future for people there.

I owe my deepest gratitude to my husband, who helped me overcome every trouble I have had, and made me extraordinarily happy. I am truly blessed to have you in my life.

Finally, I would like to acknowledge the financial and technical support of the Institute of Physiology, Justus-Liebig University, and “PROMISE” IRTG Gießen-Barcelona (implemented by Deutsche Forschungsgemeinschaft).

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

édition scientifique
VVB LAUFERSWEILER VERLAG

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STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-6519-5