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DISSERTATION

Prevention of cardiac remodeling after experimental myocardial infarction. Role of the angiotensin II type 2 receptor stimulation and modulation of MMP/TIMP axis

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List of Abbreviations

AIx	augmentations index
Akt	Protein kinase B (PKB)
Ang II	angiotensin II
AP	augmentations pressure
AP-1	activating-protein-1
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
A-wave	late atrial filling velocity
BK	bradykinin
C21	compound 21
CB1R	cannabinoid 1 receptor
CFs	cardiac fibroblasts
cGMP	cyclic guanosine monophosphate
CMs	cardiac myocytes
COX2	cyclooxygenase 2
Cy3	cyanine 3
DMEM	Dulbecco´s modified Eagle´s medium
dP/dt_{max}	maximal pressure time index
dP/dt_{min}	minimal pressure time index
ECM	extracellular matrix
EDT	diastolic duration time
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
E-wave	early passive ventricular filling velocity
FITC	fluorescein isothiocyanate
FS	fractional shortening

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gata-4	transcription factor GATA-4
HDACs	histone deacetylase
HF	heart failure
HKa	cleaved high molecular weight kininogen
HP	hydroxyproline
IL	interleukin
iNOS	nitric oxide synthase
IP	intraperitoneal
JAK	Janus kinase
LAD	left anterior descending artery
L-NAME	L-N^G-nitroarginine methyl ester
LV	left ventricle
LVEDP	left ventricular end diastolic pressure
LVIDd	left ventricular end-diastolic inner dimension
LVIDs	left ventricular end-systolic inner dimension
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinases
MEF2	myocyte enhancer factor-2
MI	myocardial infarction
MMP	matrix metalloproteinase
NaCl	sodium chloride
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nkx-2.5	Homeobox protein
NO	nitric oxide
Notch-2	Neurogenic locus notch homolog protein 2
PAGE	polyacrylamide gel electrophoresis

PP	pulse pressure
RAAS	renin-angiotensin-aldosterone system
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
STAT3	signal transducer and activator of transcription 3
TGF-β1	transforming growth factor beta 1
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumour necrosis factor alpha
TTE	transthoracic echocardiogram
VSCMs	vascular smooth muscle cells

Abstract

This work summarizes our observations on the prevention of cardiac remodelling after experimental myocardial infarction (MI).

MI remains a leading cause of morbidity and mortality worldwide and hence requires further therapeutic investigations. In the late phase of MI, adverse post-ischemic cardiac remodeling is characterized by fibrotic processes, collagen deposition and extracellular matrix degradation. The cardiac renin-angiotensin-aldosterone system (RAAS) plays an important role in post-ischemic pathological processes via AT1 receptor subtype (AT1R) activation. The AT2 receptor (AT2R) counteracts the AT1R. Therefore, we studied the effects of the AT2R stimulation by the direct non-peptide agonist C21 in the experimental model of MI in the rat 6 weeks after coronary artery ligation (P1).
Treatment with C21 prevented left ventricular dilatation and dysfunction as evidenced by an improvement of cardiac systolic and diastolic parameters. Moreover, the AT2R stimulation decreased interstitial fibrosis and collagen accumulation. The anti-fibrotic effect was associated with a down-regulation of the pro-fibrotic cytokine Transforming Growth Factor beta 1 (TGF-β1) in the heart. Increased matrix metalloproteinase 9 (MMP9) levels and decreased tissue inhibitor of metalloproteases 1 (TIMP1) protein evidenced the proteolytic dysbalance. This dysbalance was ameliorated by C21 by activating TIMP1 which, in turn, inhibited MMP9. Collectively, these findings provide evidence of cardioprotective effects of the AT2R stimulation in the late phase post MI.

The second study (P2) investigated the effects of the selective cannabinoid-1 receptor (CB1R) antagonist rimonabant in the rat model of MI and experimental metabolic syndrome. Blocking of the CB1R improved systolic and diastolic cardiac functions in the early and late stage after myocardial infarction, decreased pulse wave reflections and reduced cardiac remodelling. Importantly, CB1R blockade was also cardioprotective in rats with metabolic syndrome. Inhibition of proteolysis and decreased collagen accumulation due to reduced expression of TGF-β1 are the main mechanisms involved in the protective action of CB1 receptor blockade in the heart.
We have also investigated the role of the kallikrein-kininogen-kinin system in the regulation of MMPs and TIMPs (P3). The cleaved form of high molecular weight kininogen (HKa) regulated the expression of MMP9, MMP2 and TIMPs in vascular smooth muscle cells. HKa reduced the IL-1α-stimulated release of MMP9 and MMP2.

These results demonstrate that HKa affects the regulation of MMPs/TIMPs axis. Hence, HKa may also be a potential drug target for treatment of cardiovascular diseases.

In summary, modulating of cardiac MMPs/TIMPs axis and prevention of TGF-β1 associated fibrosis obviate left ventricular remodelling and promote functional improvement after MI.

Zusammenfassung

Diese Arbeit fasst unsere Beobachtungen über die Prävention von kardialem Remodeling nach experimentellem Myokardinfarkt zusammen.

Herzinfarkt stellt die Hauptursache für Morbidität und Mortalität weltweit dar und erfordert weitere therapeutische Untersuchungen. In der Spätphase des Herzinfarktes ist postischämisches kardiales Remodeling durch fibrotische Prozesse, Kollagenablagerung und extrazelluläre Matrixdegradation gekennzeichnet. Das kardiale Renin-Angiotensin-Aldosteron-System (RAAS) spielt eine wichtige Rolle in den postischämischen pathologischen Prozessen durch die AT1-Rezeptor-Subtyp (AT1R) Aktivierung. Der AT2-Rezeptor (AT2R) wirkt dem AT1R entgegen. Daher untersuchten wir die Effekte der Stimulation mit dem direkten nicht-peptidischen AT2R-Agonist C21 in einem experimentellen Infarkt-Modell in Ratten 6 Wochen nach Koronararterien-Ligatur (P1).

Die Therapie mit C21 hat die Dilatation des linken Ventrikels und die Dysfunktion durch eine Verbesserung der kardialen systolischen und diastolischen Parameter unterbunden. Außerdem verringerte die AT2R Stimulation die interstitielle Fibrose und Kollagenakkumulation. Die antifibrotische Wirkung war mit einer Hemmung des pro-fibrotischen Zytokins Transforming growth factor β1 (TGF-β1) im Herzgewebe assoziiert. Erhöhte Matrix-Metalloproteinase 9 (MMP9)-Niveaus und eine reduzierte Tissue inhibitor of metalloproteases 1 (TIMP1)-Expression auf Proteinebene belegten die proteolytische Dysbalance im postischämischen Herzgewebe. Dieses Ungleichgewicht wurde mit C21 durch TIMP1-Aktivierung, die wiederum MMP9 hemmte, verbessert.
Zusammenfassend weisen diese Ergebnisse auf kardioprotektive Wirkungen der AT2R Stimulation in der Spätphase des Myokardinfarkts hin.

Die zweite Studie (P2) untersuchte die Effekte des selektiven Cannabinoid-1-Rezeptor (CB1R) Antagonisten Rimonabant im Rattenmodell eines MI und eines experimentellen metabolischen Syndroms. Die Blockade des CB1R verbesserte die systolischen und diastolischen Herzfunktionen in der frühen und späten Phase nach MI, reduzierte die Pulswellenreflexionen und das kardiale Remodeling. Wichtig ist, dass die CB1R Blockade auch kardioprotektiv bei Ratten mit metabolischem Syndrom Wirkung zeigte.

Die Hemmung der Proteolyse und die verminderte Kollagenakkumulation durch reduzierte TGF-β1-Expression stellen die Hauptmechanismen der Schutzwirkung der CB1-Rezeptor-Blockade im Herz dar.

Die Rolle des Kallikrein-Kininogen-Kinin Systems in der Regulation von MMPs und TIMPs wurde auch untersucht (P3). Die gespaltene Form von hochmolekularem Kininogen (HKa) regulierte die Genexpression des MMP9, MMP2 und TIMPs in glatten Gefäßmuskelzellen. HKa verminderte die IL-1α-stimulierte MMP9 und MMP2-Freisetzung. Diese Ergebnisse zeigten, dass HKa die MMPs/TIMPs-Achse moduliert. Daher kann HKa auch als mögliches Zielmolekül für die Behandlung von kardiovaskulären Krankheiten dienen.
Zusammenfassend eliminiert die Modulation der kardialen MMPs/TIMPs-Achse und die Prävention von TGF-β1- assoziierter Fibrose das linksventrikuläre kardiale Remodeling und fördert die Funktionsverbesserung nach einem Myokardinfarkt.

1 Introduction

1.1. Left Ventricular Remodeling

Ischemic heart disease remains the major source of morbidity and mortality worldwide despite significant advances in therapies developed for this disease [1].

Pathological left ventricular (LV) remodeling after myocardial infarction (MI) strongly contributes to the development of heart failure (HF) and has been associated with clinical outcomes in numerous HF trials [2].

LV remodeling refers to alterations in size, shape, and histological heart structure, such as pathological cardiomyocyte (CMs) hypertrophy, myocyte apoptosis, fibroblast proliferation, and interstitial fibrosis [1]. These changes lead to progressive LV dilatation and dysfunction and could be associated with the risk of ventricular arrhythmias [3].

LV remodeling is closely linked to activation of renin-angiotensin-aldosterone system (RAAS), the adrenergic nervous system, endothelin, oxidative stress and increased production of proinflammatory cytokines [4].

Although ACE inhibition [6], AT1-receptor blocker therapy [7], and β-blocker therapy [8] exhibit cardioprotection in patients with HF and LV dilation, and aldosterone inhibition leads to clinical improvement, LV remodeling is still observed in many patients and is related to an adverse prognosis [9]. Thus, pathological LV remodeling represents an important therapeutic target in patients after MI or with HF.

In acute phase post MI treatment aims to prevent cell death and inflammatory response, whereas in the late phase post MI the treatment is focused on processes advancing cardiac remodeling such as fibrosis, collagen deposition and extracellular matrix (ECM) degradation.

Based on recent insights into the mechanisms leading to LV remodeling several novel therapeutic targets have been proposed. They include modulators of NO activity and signaling pathways, cyclic guanylyl cyclase activators, anti-oxidant strategies, modulators of inflammation and pro-inflammatory cytokines, modulators of MEF2 or HDACs, pro-angiogenetic factors and/or cell transfer, antagomirs, metalloproteinase inhibitors and others (reviewed by Landmesser, 2009 [10]).

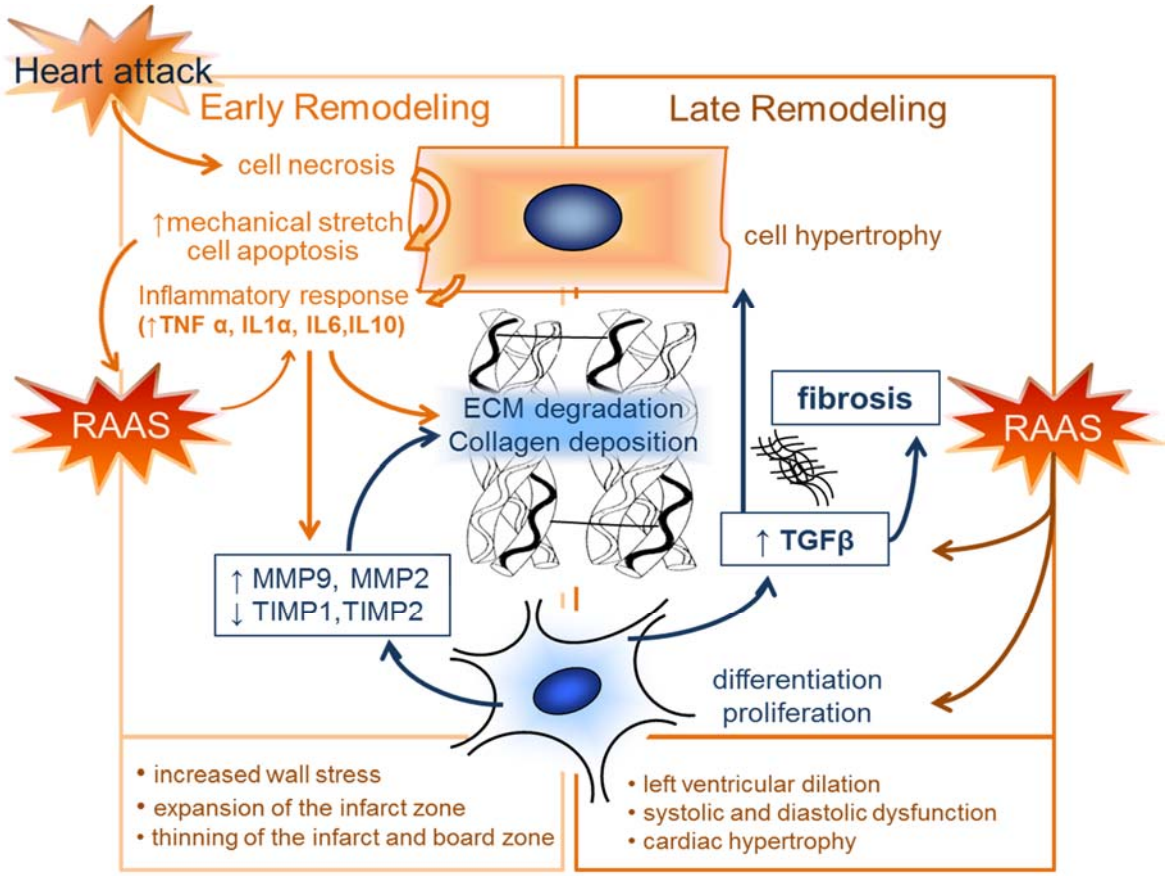

Figure 1: Pathological factors leading to early and late cardiac remodelling after MI

Hypoxia and the lack of essential nutrients secondary to the heart attack cause cardiac myocytes death and increased wall stresses. The early phase of remodeling is characterised by expansion of the infarct zone, RAAS activation, inflammatory response and MMPs activation which leads to the ECM degradation. Late remodeling is characterized by activated inflammation, TGF-β1 synthesis, fibrosis and cardiac myocyte hypertrophy. These processes lead to the left ventricular dilation and contractility dysfunction.

1.2. Extracellular Matrix Degradation, Matrix Metalloproteinases

Extracellular matrix degradation is an important mechanism that may contribute to LV wall-thinning in the remote region after MI [11]. Cardiac ECM components include fibrillar collagens I (80%) and III (10%), with smaller amounts of collagens IV, V, VI, elastin, laminin, proteoglycans, glycosaminoglycans, and others [12].

In the early period after MI (within first 72 hours), migration of macrophages, monocytes and neutrophils in infarct zone initiates intracellular signaling cascades which mediate inflammatory response [3] (Figure1). Pro-inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-6, and IL-10) activate neutrophils and cardiac fibroblasts which release Zn^{2+} - containing endopeptidases - matrix metalloproteinases (MMPs).

The gelatinases MMP2 and MMP9 exhibit proteolytic activity against denatured fibrillar collagen, elastin and proteoglycans [11]. Thus, these MMPs are directly involved in the heart remodelling [11]. Activation of MMPs underlies myocyte slippage, ventricular wall thinning, and chamber dilation post-MI (Figure 1). Further chronic MMPs activation mediates deposition of collagen in ECM, fibrotic changes, left ventricular remodeling and progression to HF [13].

MMP production is regulated transcriptionally; posttranscriptionally by the activation of the proenzyme to the active form; and posttranslationally by endogenous antagonists, the tissue inhibitors of metalloproteinases (TIMPs) [11].

Concentration ratios of serum MMP/TIMP correlate with functional values of LV volume and ejection fraction and predict clinical outcome of myocardial infarction [14]. Selective MMP inhibition reduces LV remodeling after MI in experimental models [15].
Summarising, left ventricular remodeling and HF can be treated by modulating ECM turnover, especially via the regulation of MMP/TIMP axis.

1.3. Cardiac Fibrosis

The late phase post MI is characterized by cardiac fibrosis which is associated with altered myocardial mechanical performance and arrhythmogenesis [3]. Diffuse ECM deposition results in exaggerated mechanical stiffness and contributes to diastolic dysfunction. Moreover, increased collagen content disrupts electrotonic connectivity between cardiac myocytes and provides an electrical substrate for reentrant arrhythmogenesis. Perivascular fibrosis of coronary vessels further increases cardiac myocyte ischemia [16,17].

Cardiac fibroblasts play a critical role in the fibrotic process: As a source of inflammatory cytokines, growth factors, MMPs and their native tissue inhibitors TIMPs, fibroblasts synthesize and remodel newly created extracellular matrix [17].
Post-MI ECM turnover in myocardium is closely correlated with TGF-β1, which regulates collagen synthesis; induces hypertrophy of cardiac myocytes and proliferation of fibroblasts [13].

Ang II- induced TGF-β1 expression correlates with fibrosis and cardiac hypertrophy [13]. Through the activation of p38 MAP kinase Ang II induces the early response gene c-fos, a part of the activating-protein-1 (AP-1) binding complex which increases expression of TGF-β1. TGF-β1, in turn, via Smad proteins mediates expression of collagen type I, fibronectin and proteoglycans increasing the extracellular matrix (ECM) deposition [4]. ACE inhibitors and AT1R antagonists both attenuate effects of Ang II including enhanced TGF-β1 [18].

Thus, the key role of the fibrosis regulation belongs to TGF-β1 which acts as a signal-transducer-effector to collagen synthesis and, therefore, fibrosis [19]. Modulation of TGF-β1 production and its actions are in the focus of antifibrotic therapy.

1.4. Renin-Angiotensin-System: Angiotensin II Receptor Type 2 in the Heart

The renin-angiotensin-aldosterone system (RAAS) plays a key role in the compensatory neurohumoral response to myocardial injury. Angiotensin II (Ang II), the major RAAS hormone, through its AT1-receptor (AT1R) induces myocardial hypertrophy, fibrosis and cardiomyocyte apoptosis. Consequently, both ACE inhibitors and AT1R antagonists are cardioprotective with regard to cardiac fibrosis and post-MI LV dysfunction [20,21]. Beneficial effects of AT1R blockade are known to be also attributed to the stimulation of unopposed angiotensin AT2 receptors (AT2R) [22]. In the heart, the AT2Rs are present in various cardiac cell types (fibroblasts, myocytes and endothelial cells) and promote anti-growth, anti-proliferation and anti-fibrosis [23].

The AT2Rs post MI are regulated in different ways, depending on the time after injury (for review see de Gasparo, 2000 [4] and Kaschina, 2015 [24]). However, in the intermediate post arrest phase, relative levels of the AT2Rs are known to decrease [25] and left ventricular dysfunction is also associated with AT2Rs down-regulation [26]. Therefore, it would be assumed that the activation of the AT2R post MI is cardioprotective.

The first available AT2R agonist CGP42112A was a peptide which rapidly degraded and was not orally active (reviewed by Jones ES 2008 [27]). Thus, its application *in vivo* has limitations in particular for long-term studies.

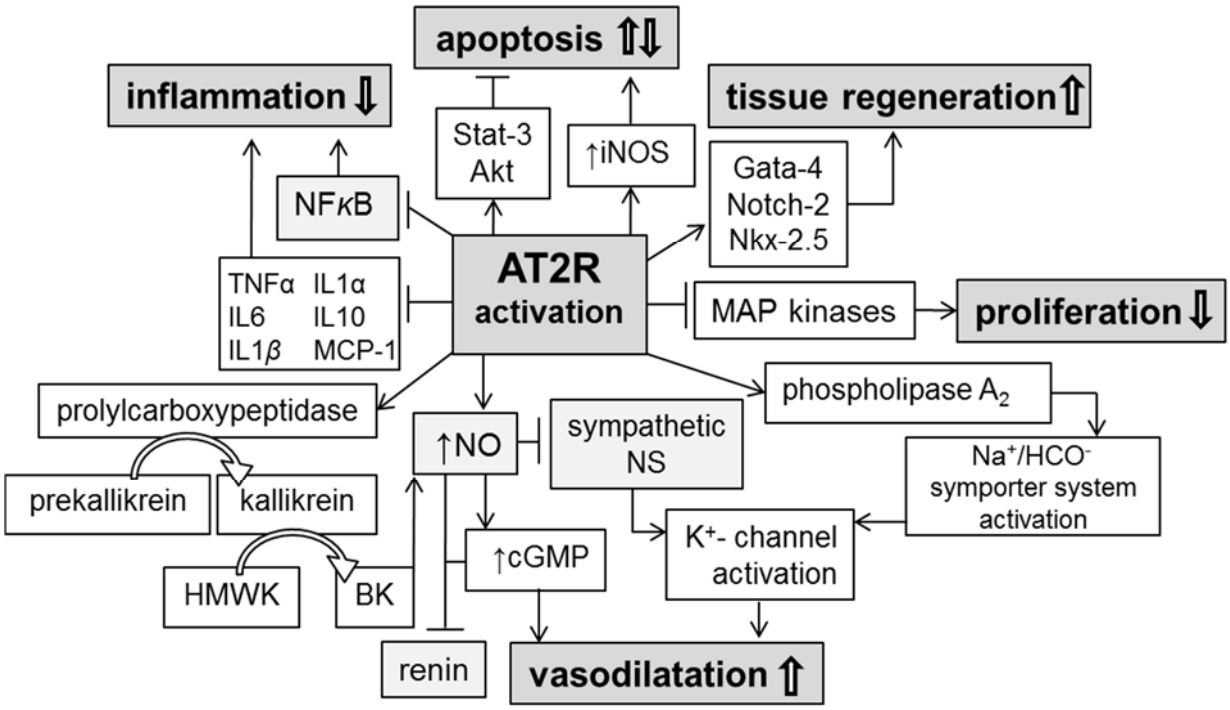

Figure 2: Schematic representation of the main AT2R-mediated signalling pathways in the heart

AT2R exerts beneficial effects in the heart via different signaling cascades: AT2R mediates vasodilatation through the activation of the bradykinin/NO/cGMP system [23] and downregulation of sympathetic nervous system [33]. Anti-inflammatory effects are released via the downregulation of NF-kB and interleukins [24,32]. Inhibition of MAPKs (ERK1/2) phosphorylation leads to anti-proliferative effects of AT2Rs [30]. Activation of the transcriptional factors Gata-4, Notch-2 and Nkx-2.5 evokes the tissue regeneration in the heart [35]. The activation of Stat-3 and Akt signaling as well as iNOS can differently regulate apoptosis [30].

Akt- protein kinase B (PKB); AT2R- angiotensin II receptor type 2; BK- bradykinin; cGMP- cyclic guanosine monophosphate; Gata-4 - transcription factor Gata-4; HMWK- high molecular weight kininogen; IL- interleukin; iNOS- nitric oxide synthase; MAPK - mitogen-activated protein kinase; MCP-1-monocyte chemotactic protein 1; TNFα- tumor necrosis factor α; NFκB- nuclear factor kappa-light-chain-enhancer of activated B cells; Nkx-2.5- homeobox protein; NO-nitric oxide; NS-nervous system; Notch-2- neurogenic locus notch homolog protein 2; Stat-3- signal transducer and activator of transcription 3.

The first non-peptide orally active specific and selective AT2R agonist, Compound 21 (C21) was developed by Hallberg et al. [28]. This agonist allows long-term, direct stimulation of the receptors *in vivo*. A bioavailability of 20−30% after oral administration and a half-life estimated to 4h in rat makes C21 a potential drug [28] which is expected to enter a phase I clinical testing in 2015 [29].

In acute phase of experimental MI, direct AT2R stimulation by C21 improves systolic and diastolic functions coinciding with a smaller infarct size [30]. These improvements were attributed to anti-apoptotic und anti-inflammatory mechanisms of the AT2 receptor activation [30-32]. The rescue of p38 and p44/42 MAPK (mitogen activated protein kinase) by C21 as well as an activation of Stat-3 and Akt signaling pathways were shown to be involved in its anti-apoptotic effect post MI [30]. The anti-inflammatory action of direct AT2R stimulation by C21 is attributed to inhibition of JAK/STAT signaling, inhibition of NF-kB and inhibition of COX2 synthesis (reviewed by Kaschina and Unger 2003 [33]; Namsolleck et al., 2014 [34]). Moreover, AT2Rs are implicated in cardiac tissue regeneration processes via up-regulation of transcription factors responsible for cardiogenic differentiation (Gata-4, Notch-2, Nkx-2.5) and genes required for self-renewal [35]. Although, many studies in genetically altered mice showed cardioprotective effects of the AT2R overactivation [36,37], ATR2-deficiency had no impact on post MI remodeling and fibrosis [38].

The results on AT2R expression in human hearts are conflicting. Nevertheless, the major cell types are the fibroblasts which are expressed in the interstitial regions [39,40]. Moreover, the relative increase in AT2Rs corresponds with areas of fibroblast proliferation and collagen deposition [41]. Altogether, the regulation of the AT2R in the heart depends on the remodeling phase.

Left ventricular remodeling post MI is also associated with hypertrophy in noninfarcted myocytes due to wall stress and activation of the local RAAS [42]. The AT2R activation counteracts growth effects of the AT1R in response to Ang II [4]. Although an antigrowth effect of the AT2R has been demonstrated in various cell types, e.g. endothelial cells, cardiomyocytes and fibroblasts (for review see Unger, 1999 [43]) the role of the AT2R in cardiac hypertrophy until now is inconsistent (for review see Steckelings 2010 [44]).
Summarising, current studies clearly demonstrate that the AT2R exerts beneficial effects on the heart function in the early phase after MI. On the other hand, the results concerning heart remodeling are conflicting or not investigated in the experiments by using direct AT2R stimulation with C21. Therefore, we studied the effects of chronic, long-term direct AT2R stimulation on cardiac function and structural myocardial alterations 6 weeks after MI.

1.5. Inhibition of Cannabinoid Receptor 1 in the Heart

The endocannabinoid system consists of the endocannabinoids (arachidonoyl-ethanolamide and 2-arachidonoylglycerol) and the cannabinoid receptors (CB1 and CB2), which are present in the heart and vessels [45]. CB1 receptor activation induces hypotension, bradycardia and negative inotropy, whereas CB2 receptor activation exerts positive inotropic effect on the heart [46,47]. In some experimental models, such as acute ischemia/reperfusion injury [48] and doxorubicin induced cardiotoxicity [49] the CB1 receptor blockade with rimonabant has been demonstrated to be cardioprotective. Moreover, the selective cannabinoid CB1 receptor antagonist rimonabant which was applied for obesity treatment, reduced cardiovascular risk in humans [50].

Thus, we hypothesized that CB1 receptor antagonism might produce beneficial effects independent from metabolic changes. We investigated its cardiovascular effects in the acute (7days) and chronic (6 weeks) models of myocardial ischemia. In addition, we were interested in the potential underlying mechanisms of rimonabant action on the heart in the context of remodeling processes and MMPs/TIMPs/TGF-β1 regulation of post-ischemic myocardium.

Aim of the study

Proteolytic degradation of cardiac ECM and fibrosis development seems to be the basic pathophysiologic mechanism of the heart remodeling development. Understanding of these processes suggests a potential area for therapy after MI.

This work summarizes our observations concerning the contribution of MMPs/TIMPs and TGF-β1 to post-MI cardiac remodeling and the potential utility of agents interacting with these proteins in affecting the outcome of cardiac fibrosis and left ventricular remodeling.

One study was designed to assess the effect of chronic, long-term direct AT2R stimulation by C21 on cardiac function and structural myocardial alterations 6 weeks after MI. By using *in vitro* and *in vivo* approaches, we aimed further to investigate the potential underlying mechanisms of AT2R activation on the heart by exploring extracellular matrix turnover processes of the post-ischemic myocardium, especially in the context of the TIMP1/MMP9 axis and TGF-β1-mediated fibrosis.

We additionally investigated whether the cleaved form of high molecular weight kininogen, designated HKa, affects the expression of MMP9 and MMP2 and their tissue inhibitors (TIMPs).

We also studied the role of cannabinoid receptor 1 blockade with rimonabant in the rat model of acute and chronic MI with the emphasis on cardiac metabolic, fibrotic and remodeling processes, and the underlying regulatory mechanisms.

2 Methods

2.1. Experimental Induction of Myocardial Infarction (P1, P2)

MI was induced in anesthetized male normotensive Wistar rats (200-220g) by permanent ligation of the left anterior descending artery [51].

Under anaesthesia with ketamine/xylazine (80/10 mg/kg IP) and intubation, a left lateral thoracotomy was performed, and a suture was tightened around the proximal left anterior descending coronary artery. Sham - operated rats underwent the same surgical procedure with the exception of coronary ligature. Treatment of animals started 6 hours after induction of MI and continued 6 weeks until sacrifice. Animals with ejection fraction 35% < 85% were excluded from the experiment before assignment to groups (P1, P2).

Animals were randomly assigned to the following treatment groups: treatment with compound 21 (0.03 mg/kg/day IP) (P1), rimonabant (10 mg/kg/day IP) (P2), vehicle treatment (0.9% 0.5 mL NaCl, IP) and sham - operated controls (P1, P2).

2.2. Transthoracic Doppler Echocardiography (P1, P2)

Transthoracic Doppler echocardiography (M-mode and Doppler measurements) was performed 1 and 6 weeks after MI under 2 % isoflurane anaesthesia. Images were obtained by using a high- resolution imaging system Vevo 770 (VisualSonics Inc., Toronto, Canada). M-mode tracings were recorded from short- axis view of the left ventricle (LV) at the level of the papillary muscles with two-dimensional image guidance through the anterior and posterior walls (P1: Figure S 1A; P2: Figure 2G). Left ventricular internal dimensions were measured through the largest diameter of the left ventricle, both at the end of diastole (LVIDd) and systole (LVIDs). Ejection fraction (EF) and fractional shortening (FS) were calculated from the linear measurements of LVIDd and LVIDs.

Pulsed wave Doppler spectra of mitral inflow was recorded from the apical four- chamber view to evaluate diastolic parameters (P1: Figure S-B, P2): E-wave-early passive ventricular filling velocity, A-wave-late atrial ventricular filling velocity, deceleration time (EDT) and E/A-ratio.

2.3. Hemodynamic Parameters and Pulse Wave Analysis (PWA) (P1, P2)

Hemodynamic assessment was performed at the end of the study in anesthetized rats, using fiber-optic pressure transducer- Samba catheter (Samba Sensors, Västra Frölunda, Sweden) and Chart 5 software for analysis. LV pressure and pressure time indices (dP/dt_{min}, dP/dt_{max}) were determined and averaged on the LV pressure waveforms.

Pulse pressure (PP), augmentations pressure (AP), mean arterial pressure (MAP) and augmentations index (AIx) were calculated and recorded on the central aortic pressure waveforms from at least 20 cardiac cycles. AIx was calculated as a ratio of AP to PP (P2: Figure 3C).

2.4. Cell-Culture Experiments (P1, P2, P3)

Primary cardiac fibroblasts (CFs) (P1, P2) and vascular smooth muscle cells (VSMCs) (P3) were isolated by enzymatic digestion from the healthy adult euthanized rats. The origin of isolated cells was confirmed with vimentin s-20 immunostaining for CFs (P1, P2) and with smooth muscle actin immunostaining for VSMCs (P3). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate, D-glucose and 10% foetal bovine serum and supplemented with interleukins: (IL-1α 20 ng/mL) (P1, P2, P3) for 48 hours to induce secretion of MMPs. Incubation was performed with or without co-incubation with C21 (1.0μM, 0.5μM, 0.25μM) (P1), PD123319 (10μM) (P1), rimonabant (1.0μM, 0.75μM, 0.5μM, 0.25μM) (P2) or cleaved high molecular weight kininogen (HKa, 1.0μM, 0.1μM and 0.01μM) (P3).

2.5. Molecular Biology Methods

2.5.1. MMPs Enzymatic Activity Analysis by Gelatin Zymography (P1, P2, P3)

Culture media harvested from cardiac fibroblasts and vascular smooth muscle cells (P1, P2, P3) or extracts from the left ventricles (P1) were analysed for proteins with gelatinolytic activity (MMP2, MMP9) by gelatin zymography. Aliquots of conditioned media were resuspended in non-reducing sample buffer and applied to SDS–PAGE copolymerized with gelatin. After electrophoretic separation of proteins, gels were washed with Triton X-100 and subsequently incubated in enzyme buffer. The gels were then stained with Coomassie Brilliant Blue G-250 with a following destaining in methanol and acetic acid. Proteins having gelatinolytic activity were visualised as clear bands against a blue-stained background.

Gels were scanned and densitometric analysis was performed using the image analysis programme (Image J). Molecular sizes of bands were characterized by comparison with prestained molecular weight markers.

2.5.2. Hydroxyproline Content in the Heart (P1, P2)

The amount of collagen in cardiac tissue (board zone of MI) was analyzed by quantification of the tissue hydroxyproline content using the modified method based on alkaline hydrolysis as previously described [52].

2.5.3. Western Blotting (P1, P2, P3)

Protein lysates were prepared according to standard protocols. Proteins were diluted with reduced sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibody diluted in milk powder solution overnight. The following primary antibodies were used: TGF-β1, MMP2, MMP9, TIMP1, TIMP2, IL-1α (P1, P2, P3). Protein expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Immunoreactive bands were detected by enhanced chemiluminescence (GE Health Care, Germany) and quantified with Image J software.

2.6. Histology

2.6.1. Analysis of Interstitial Fibrosis by Sirius Red Staining and Measurement of Cardiac Myocytes Diameter (P1, P2)

Cardiac fibrosis was evaluated in paraffin sections stained with Sirius red (P1, P2). Collagen fibres were stained red. The quantification of fibrosis was carried out using image analysis software. Results were calculated as percentage of Sirius-Red-positive areas in randomly chosen pictures from cardiac sections.

The diameter of cardiac myocytes in noninfarcted regions was quantified by means of computer-aided histomorphometry by using of a 40x objective (P1).

2.6.2. TGF-β1 Immunostaining (P1, P2)

Immunohistochemistry of TGF-β1 was performed in paraffin-embedded tissue sections (5μm) using the avidin-biotin complex method according to the manufacturer's instructions (Vectastain ABC; Vector Laboratories, USA).

Peroxidase activity was visualized by 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Primary antibodies against TGF-β1 and goat anti-rabbit second antibodies were used (P1, P2).

2.7. TGF-β1 - Linked Immunosorbent Assay (P1)

TGF-β1 concentration was determined in rat plasma collected 6 weeks after MI using a commercially available ELISA- kit according to the manufacturer's protocol.

2.8. mRNA Analysis: Quantitative Real Time - Reverse Transcription Polymerase Chain Reaction and Relative Quantification of Gene Expression (P1, P2, P3)

Total RNA from peri-infarcted left ventricular tissue (P1, P2), primary CFs (P1, P2) and primary VSMCs (P3) was isolated with Trizol® reagent and subsequently treated with DNase-I. qRT-PCR was performed with MX3005p QPCR System (Stratagene) using SYBR green reaction mix and primers for MMP2, MMP9, TIMP1, TIMP2 and TGF-β1. All samples were measured in triplicate, and expression values were normalized to 18s rRNA. Data analysis was done using the MxPro™ ET QPCR software (Stratagene) and ΔΔCt-method.

2.9. Fluorescence Microscopy (P1)

MMP9 and TIMP1 release was analysed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Japan) in single CFs after stimulation with IL-1α (20ng/mL) in presence or absence of C21 (1μM) for 1, 4, 24, and 48 hours. The cells were fixed with paraformaldehyde, labelled with rabbit MMP9 antibodies and co-labelled with Cy3 conjugated anti-rabbit antibodies. Then the cells were labelled with mouse TIMP1 antibodies, co-labelled with FITC conjugated anti-mouse antibodies and counterstained with Hoechst stain.

2.10. Statistical Analysis (P1, P2, P3)

Results were expressed as mean ± S.E.M. Multiple comparisons were analyzed with one-way ANOVA followed by the Bonferroni post-hoc test. Two-group comparisons were analyzed by the 2- tailed Student unpaired T-test for independent samples. Differences were considered statistically significant at the value of $p<0.05$.

3 Results

3.1. Hemodynamic parameters

3.1.1. Functional alterations after myocardial infarction

Post infarct mortality was 31%. Animal losses all occurred within the first 6 hours after MI. Hemodynamic parameters, measured 6 weeks after MI via transthoracic echocardiogram (TTE) and intracardiac Samba catheter, demonstrated a pronounced cardiac dysfunction (P1, P2). Left ventricle dilation was evidenced by increased systolic and diastolic left ventricular inner diameter (P1: Figure S1A; P2: Figure 2, Table 3.2). Decreased heart contractility was indicated by reduction of ejection fraction (EF), fractional shortening (FS) and stroke volume post MI (P1: Table, Figure S1A; P2: Table 1). Decreased contractility index and maximal peak rate of left ventricular pressure increase (dP/dt_{max}) which were measured by intracardiac Samba catheter also confirmed systolic function deterioration (P1: Figure 1A, 1B; P2: Table 3).

Diastolic function post MI was also impaired. Pulsed wave Doppler spectra of mitral inflow measurements demonstrated an increase of early passive ventricular filling velocity (E-wave) followed by enhanced E/A-ratio in vehicle treated animals compared to sham operated group (P1: Table 1; P2: Figure 2). Impairment of the diastolic function was confirmed by Samba catheter measurements: left ventricular end diastolic pressure (LVEDP), minimal peak rate of left ventricular pressure increase (dP/dt_{min}) were elevated and diastolic duration time (EDT) was shortened (P1: Figure 1D, 1E, 1F) in vehicle treated animals compared with sham-operated rats. Arterial stiffness was also impaired post MI as demonstrated by increased augmentations index (AIx) and wave reflection (P1, P2).

3.1.2. Improvement of systolic and diastolic function after myocardial infarction by direct AT2 receptor agonism

Chronic daily administration of the direct AT2 receptor agonist Compound 21 (C21) improved systolic and diastolic functions 6 weeks after MI (P1). C21 prevented left ventricular dilation by reduction of systolic and diastolic left ventricular inner diameters, improved anterior wall motion and contractility of the left ventricle (P1: Figure S1A). Beneficial effect of AT2R activation on the systolic function was shown by enhanced EF by 17.8% ($p<0.001$) and FS by 9.9% ($p<0.05$) (P1; Table 1). Contractility index and dP/dt_{max} (P1: Figure 1A, 1B) measured by intracardiac catheter were also significantly improved after treatment and confirmed the echocardiographic data.

C21 also attenuated post MI diastolic dysfunction by decreasing E/A-ratio and by increasing diastolic duration time (P1: Table 1). C21 also reduced E/A-ratio, LVEDP and dP/dt_{min} and increased EDT (P1: Figure 1D, 1E, 1F) indicating better relaxation of the left ventricle after systole. Arterial stiffness was also normalized after C21 treatment as evidenced by decreased AIx (P1).

3.2. Cardiac fibrosis after myocardial infarction

Collagen content was studied by measuring tissue hydroxyproline (HP) concentration in the left ventricle. HP concentration was increased in peri-infarct zone 6 weeks post MI (P1: Figure S4B; P2: Figure 4). Increased interstitial fibrosis post MI was also demonstrated by picro-sirius red collagen staining (P1: Figure S3, S4A; P2: Figure 4). C21 prevented fibrotic remodeling (P1: Figure S3, S4A) and significantly attenuated HP increase (P1: Figure S4B).

Interestingly, MI also induced cardiac myocytes enlargement in the peri-infarct zone and this hypertrophy was further aggravated by C21 treatment (P1: Figure S2).

The regulation of transforming growth factor-β1 (TGF-β1), a pro-fibrotic cytokine, was investigated in the heart and in the serum. TGF-β1 expression was up-regulated in the peri-infarct zone both on the mRNA and particularly on protein levels in vehicle treated animals compared to sham group (P1: Figure 2B, 2C; P2: Figure 4). Furthermore, serum concentration of active form of TGF-β1 was elevated 6 weeks post MI, while the latent form of TGF-β1 was decreased (P1: Figure 2B, 2C).

TGF-β1 regulation was also investigated *in vitro* (P1, P2). In primary cardiac fibroblasts, IL-1α stimulation induced TGF-β1 mRNA expression (P1: Figure S7; P2: Figure 4). Treatment with the AT2 receptor agonist C21 abolished the over-expression of TGF-β1 in the cells (P1; Figure S7). Moreover, cardiac TGF-β1 expression in the heart tissues 6 weeks post MI was completely abolished by C21 on both transcriptional and translational levels (P1: Figure 2A, 2B, 2C). C21 also normalized serum concentration of both active and latent TGF-β1 forms up to the control levels (P1: Figure 2D, 2E).

3.3. Regulation of MMP/TIMP axis in cardiac remodeling after myocardial infarction

The activity of matrix metalloproteases MMP9 and MMP2 was first investigated by zymography in vascular smooth muscle cells (P3). Pro-inflammatory cytokine IL-1α stimulated gelatinolytic activity of MMP9 and MMP2 (P3: Figure 1A, 1B).

In cardiac fibroblasts, IL-1α similarly stimulated MMP9 and MMP2 activity (P1: Figure 4; P2: Figure 5).

The release of MMP9 was extremely increased in the heart 6 weeks post MI (P1: Figure 2A, 2B; P2: Figure 5). MI also induced protein expression of MMP2 but not MMP2 mRNA (P1: Figure S5A, S5B). Zymographic analysis of the left ventricle extracts from the peri-infarct zone also demonstrated proteolytic activation of MMP9 after MI, whereas MMP2 activity was tended to increase (P1: Figure 3).

C21 administration abolished increased MMP9 activity, mRNA and protein expression 6 weeks post MI (P1: Figure 3C, 3D, 3E). C21 also normalised MMP2 mRNA release almost to the levels of sham operated rats (P1: Figure S5B). MMP2 protein expression was down-regulated in C21 treated animals, although MMP2 activity was regulated only by tendency (P1: Figure 3E, Figure S5A).

Additionally, the effect of C21 on MMPs activity was studied in cardiac fibroblasts *in vitro* (P1). C21 prevented the cytokine-induced MMP9 secretion in a concentration dependent manner. MMP2 activity was regulated similarly by C21 (P1: Figure 4). Importantly, treatment with the AT2R antagonist PD123319 in combination with agonist C21 completely abolished these effects, confirming the AT2R specificity of C21 (P1: Figure S6).

Since overall proteolytic effect is known to be dependent from the ratio between MMPs and its native inhibitors, the release of TIMP1 was investigated in various experimental settings (P1, P3). TIMP1 mRNA release after IL-1α stimulation was first demonstrated in VSMCs (P3: Figure 3 A). In the heart peri-infarct tissues, TIMP1 mRNA expression was also enhanced in comparison with sham group (1.7-fold, $p<0.001$), whereas TIMP1 protein expression was strongly reduced by 90.5% (P1: Figure 3F). Increased MMP9/TIMP1 ratio confirmed the proteolytic imbalance in the heart post MI (P1: Figure 3G). The AT2R activation with C21 abolished TIMP1 protein decrease (17.7-fold, P1: Figure 3F) and attenuated cardiac proteolysis caused by MMP9 activation (P1: Figure 3G).

To investigate the primary mechanism of anti-proteolytic effect of C21 treatment, single cardiac fibroblasts were immunostained against TIMP1 (green stain) and MMP9 (red stain) at different time points after IL-1α stimulation (P1: Figure 5). Control cardiac fibroblasts exhibited low levels of MMP9 and TIMP1, their expression gradually increased in 48 hours (P1: Figure 5).

After IL-1α stimulation, MMP9 protein was expressed in the cytoplasm in 5 hours, and reached a maximum of expression at 48 hours. TIMP1 protein appeared later, 24 hours after stimulation, and was colocalized (yellow stain) with MMP9. TIMP1 expression was strongly decreased compared to control (P1: Figure 5).

In the cells treated with C21, TIMP1 was strongly expressed already 5 hours after IL-1α stimulation and partly colocalized with MMP9. TIMP1 expression reached maximum after 24 hours and was distinguishable until 48 hours in a colocalization with MMP9 as well. Importantly, in C21 treated cells, MMP9 was not expressed at all, even 48 hours after cytokine stimulation.

4 Discussion

We studied pharmacological effects in an animal experimental model by using permanent ligation of the left anterior descending artery (LAD) in the rat [51]. LAD ligation closely reproduces the pathophysiological conditions occurring in infarction-related myocardial ischemia. In two independent studies (P1, P2), we observed systolic and diastolic dysfunctions in parallel with an expansion of cardiac fibrosis 6 weeks post MI. Hemodynamic parameters which were measured via two methods e.g. transthoracic echocardiogram and intracardiac Samba catheter, clearly indicated the presence of left ventricular remodeling followed by cardiac dysfunction. LV dilation and decreased heart contractility delineated systolic dysfunction. Impaired diastolic function post MI was characterized by enhanced diastolic parameters (E/A-ratio, LVEDP, dP/dt_{min}) and shortening of diastolic duration time (EDT). Interestingly, arterial stiffness was also impaired post MI in both studies (P1, P2).

The data presented in the first study (P1) demonstrate for the first time cardioprotective effects of direct AT2R stimulation in a long-term period after experimental MI (P1). Previously, the role of the AT2R in the post-ischemic remodeling has been explored in mice either overexpressing or lacking the AT2Rs [37,38]. Although most investigations proposed cardioprotective effects of the AT2R stimulation, some studies could not confirm an impact of this receptor to cardiac improvement [38,53]. Due to synthesis of a non-peptide direct AT2R agonist Compound 21 (C21) [28] recent studies could demonstrate a protective role of the AT2R stimulation in the heart and other organs pointing to its anti-inflammatory, anti-apoptotic, and anti-oxidative actions [30,36,38,54-57].

In our study, C21 prevented LV dilatation and systolic dysfunction as evidenced by an improvement in cardiac parameters (LVIDd, LVIDs, EF, FS, dP/dt_{max}, dP/dt_{min}) and contractility index. Moreover, a reduction in the E/A-ratio and LV EDP demonstrated an improved diastolic function of the heart. These findings confirm previous data obtained 7days post MI [30] and are in accordance with the other investigations describing the role of the AT2R overexpression in the peri-infarct zone [37]. Since there is no specific therapy to improve LV diastolic function [58], C21 may be suggested as potential compound for the treatment of diastolic heart failure.

Diastolic function is known to correlate with cardiac fibrosis [59]. Post-ischemic fibrotic processes and collagen deposition in the remote myocardium are followed by the left ventricular dilation at the cost of contractility function [60]. Direct AT2R activation significantly decreased collagen accumulation in the remote myocardium and interstitial fibrosis after ischemia (P1). Our findings are in accordance with the other investigations demonstrating an anti-fibrotic effect of C21 in the aorta of L-NAME-treated hypertensive rats [61] as well as in the vessels and the heart of hypertensive SHR rats [62]. Thus, the AT2R agonist C21 may be a promising target for the prevention of cardiac fibrosis.
Post-ischemic fibrosis in the heart is closely correlated with TGF-β1, which acts as a signal-transducer-effector to collagen synthesis and is highly up-regulated in the heart after RAAS activation [13,18,19]. In the first study we have demonstrated that the AT2R activation prevents TGF-β1 up-regulation in left ventricle after ischemia, both at the protein and transcriptional levels. Further *in vitro* experiments with primary CFs demonstrated that C21 attenuated cytokine induced TGF-β1 transcription suggesting its regulation through the AT2R (P1). Our results correlate with the findings obtained in the other experimental models such as hypertrophic cardiomyopathy mice model [63] and stroke-prone SHR model [61].

The degradation of extracellular matrix by MMPs is another mechanism that contributes to LV wall-thinning in the remote myocardium post MI [11,60]. MMP2 and MMP9 are known to be the most important gelatinases in the heart [11]. Their release is tightly regulated by native inhibitors - tissue inhibitors of MMPs (TIMPs) [11]. In our first study, the increased MMP9/TIMP1 protein expression ratio in cardiac tissue post MI evidenced the proteolytic dysbalance which was completely ameliorated by treatment with C21. Furthermore, experiments performed in single cardiac fibroblasts showed that C21 primarily activates TIMP1 which, in turn, inhibits MMP9. Since TIMP1 deficiency is known to promote LV dilatation and increase LVEDV [11], TIMP activation may be cardioprotective. Therefore, it could be suggested that left ventricular remodeling can be treated by modulating MMPs/TIMPs axis via the AT2R activation.

We have also investigated the role of the kallikrein-kininogen-kinin system in the regulation of MMP2, MMP9 and TIMPs (P3) while this system is closely connected with RAAS via bradykinin (Figure 2), [23].

Bradykinin B1 and B2 receptors are involved in the AT2R-mediated anti-fibrosis in the rat HF model [64]. There is also a relationship between AT2R, B1 and B2 receptors, and cardiac protection via increased NO production [54].
Using an *in vitro* model we could show that cleaved form of high molecular weight kininogen (HKa) regulates the expression of MMP9, MMP2 and TIMPs in vascular smooth muscle cells. HKa reduced IL-1α - stimulated release of MMP9 and MMP2. Decrease of corresponding MMP9 mRNA levels did not result from MMP9 mRNA degradation. Moreover, HKa increased TIMP1 levels. These results demonstrate for the first time that HKa affects the regulation of MMPs/TIMPs axis and, thus, HKa may be also a potential drug target for treatment of cardiovascular diseases. These findings were confirmed in endothelial cells [65] and require further investigations in *in vivo* models.

In the second study we focused on the cannabinoid receptor inhibition after MI. The effects of the selective cannabinoid-1 receptor (CB1) antagonist rimonabant on cardiac function were investigated after experimental MI and in experimental metabolic syndrome.

Blocking of CB1 receptor with rimonabant continually improved systolic and diastolic cardiac functions in the early and late stage after myocardial infarction, decreased pulse wave reflections and reduced cardiac remodeling. Arterial function parameters, such as pulse pressure, augmentation pressure- and index, were positively influenced by rimonabant in the late phase post MI. Moreover, CB1R blockade was also cardioprotective in rats characterized by metabolic syndrome.
We further show that inhibition of proteolysis and reduced expression of pro-fibrotic cytokine TGF-β1 as well as decrease of collagen accumulation in the myocardium are likely mechanisms involved in the protective action of CB1 receptor blockade in the heart.

Unfortunately, rimonabant demonstrated neurological and psychiatric side effects in patients resulting from the actions at CB1 receptors in the brain [66]. Despite of this fact, cardiovascular protective effects of rimonabant merits attention. Affecting endocannabinoid system could be of clinical relevance in cardiology and further development of CB1 receptor inhibitors, which do not penetrate the blood-brain barrier would be important.

Summarising,

In the late phase of myocardial infarction, prevention of extracellular matrix degradation and fibrosis attenuates adverse myocardial remodeling and contributes to functional cardiac improvement.

Left ventricular remodeling can be prevented by modulating extracellular matrix turnover, especially via the regulation of MMP/TIMP axis.

The AT2 receptor stimulation represents a novel pharmacological target in post-ischemic left ventricular remodeling and heart failure treatment. Further investigations should be focused on the effects of the ATR2 stimulation in heart failure and combined treatment of the AT2R agonist with the AT1R antagonists.

High molecular kininogen affects the regulation of MMPs/TIMPs and requires further investigations in the field of cardiac and vascular remodeling.

Cannabinoid receptor 1 antagonism could also be a novel therapeutic concept in post-ischemic and vascular remodeling. Further development of CB1 receptor inhibitors, which do not penetrate the blood-brain barrier would be important.

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Affidavit

I, Dilyara Lauer certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "*Prevention of cardiac remodeling after experimental myocardial infarction. Role of the angiotensin II type 2 receptor stimulation and modulation of MMP/TIMP axis*". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date

Signature

Declaration of any eventual publications

Dilyara Lauer had the following share in the following publications:

Publication I: Dilyara Lauer, Svetlana Slavic, Manuela Sommerfeld, Christa Thöne-Reineke, Yuliya Sharkovska, Anders Hallberg, Bjorn Dahlцf, Ulrich Kintscher, Thomas Unger, Ulrike Muscha Steckelings, Elena Kaschina. Angiotensin Type 2 Receptor Stimulation meliorates Left Ventricular Fibrosis and Dysfunction via Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1/Matrix Metalloproteinase 9 Axis and Transforming Growth Factor β1 in the Rat Heart. Hypertension 2014;63(3):e60-7.
Impact Factor: 7.632

Contribution in detail: Share in the development of the concept, and design of the study; selection of the test method, participation in animal and cell culture experiments, histological evaluation of the samples, biomolecular analysis of the samples, statistical analysis of results, creation of the image material, writing of manuscript.

Publication II: Svetlana Slavic, **Dilyara Lauer**, Manuela Sommerfeld, Ulrich Rudolf Kemnitz, Aleksandra Grzesiak, Manuela Trappiel, Christa Thöne-Reineke, Johannes Baulmann, Ludovit Paulis, Kai Kappert, Ulrich Kintscher, Thomas Unger, Elena Kaschina. Cannabinoid receptor 1 inhibition improves cardiac function and remodelling after myocardial infarction and in experimental metabolic syndrome. Journal of molecular medicine. 2013;91(7):811-23.
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Contribution in detail: participation in animal and cell culture experiments, histological evaluation of the samples, biomolecular analysis of the samples, statistical analysis of results, creation of the image material.

Publication III: Uwe Vosgerau, **Dilyara Lauer**, Thomas Unger, Elena Kaschina. Cleaved high molecular weight kininogen, a novel factor in the regulation of matrix metalloproteinases in vascular smooth muscle cells. Biochemical Pharmacology, 2010;79(2):172-9.
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Contribution in detail: participation in cell culture experiments, biomolecular analysis of the samples, creation of the image material.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Publication I:

Dilyara Lauer, Svetlana Slavic, Manuela Sommerfeld, Christa Thöne-Reineke, Yuliya Sharkovska, Anders Hallberg, Bjorn Dahlцf, Ulrich Kintscher, Thomas Unger, Ulrike Muscha Steckelings, Elena Kaschina. Angiotensin Type 2 Receptor Stimulation meliorates Left Ventricular Fibrosis and Dysfunction via Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1/Matrix Metalloproteinase 9 Axis and Transforming Growth Factor β1 in the Rat Heart. Hypertension 2014;63(3):e60-7.

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DOI: http://dx.doi.org/10.1016/j.bcp.2009.08.004

Curriculum Vitae

In the electronic version my *Curriculum Vitae* is not published due to data protection reasons.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete List of Publications

1. Vosgerau U, **Lauer D**, Unger T, Kaschina E. *Cleaved high molecular weight kininogen, a novel factor in the regulation of matrix metalloproteinases in vascular smooth muscle cells*. Biochem Pharmacol 2010;79(2):172-9.

2. Slavic S, **Lauer D**, Sommerfeld M, Kemnitz UR, Grzesiak A, Trappiel M, Thöne-Reineke C, Baulmann J, Paulis L, Kappert K, Kintscher U, Unger T, Kaschina E. *Cannabinoid receptor 1 inhibition improves cardiac function and remodelling after myocardial infarction and in experimental metabolic syndrome*. Journal Mol Med 2013;91(7):811-2310.

3. **Lauer D**, Slavic S, Sommerfeld M, Thöne-Reineke Ch, Sharkovska Y, Hallberg A, Dahlöf B, Kintscher U, Unger Th. *Angiotensin Type 2 Receptor Stimulation Ameliorates Left Ventricular Fibrosis and Dysfunction via Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1 / MatrixMetalloproteinase 9 Axis and Transforming Growth Factor β1 in the Rat Heart*. Hypertension 2014;63(3):60-7.

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Oral Presentations

1. **Lauer D**, Slavic S, Sommerfeld M, Steckelings UM, Unger T, Kaschina E. *AT2 receptor stimulation improves cardiac function 6 weeks after myocardial infarction in the rat.* 22nd European Meeting on Hypertension and cardiovascular protection. Abstractband. London, 2012.

2. **Lauer D**, Slavic S, Sommerfeld M, Steckelings UM, Unger T, Kaschina E. *AT2 receptor stimulation improves cardiac function 6 weeks after myocardial infarction in the rat.* 24th Scientific Meeting of the International Society of Hypertension (ISH 2012). Abstractband. Sydney, 2012.

Poster Presentations

1. **Lauer D**, Slavic S, Sommerfeld M, Steckelings UM, Unger T, Kaschina E. *Two-chain high molecular weight kininogen prevents secretion of MMP-9 in primary cardiomyocytes.* 34 Wissenschaftliche Kongress der Deutschen Hochdruckliga e.V. DHL® – Deutschen Hypertonie. Abstractband, s.150. Berlin, Germany, 2010.

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