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The role of angiotensin AT1 and AT2 receptors in the development of apoptosis and inflammation following experimental myocardial infarction

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List of Abbreviations

ACE	Angiotensin Converting Enzyme
AMI	Acute myocardial infarction
AIF	Apoptosis inducing factor
ATP	Adenosine triphosphate
AMCA	Amino methyl coumarin acetate
Ang II	Angiotensin II
Apaf	Apoptotic protease activating factor
AT1	AT1 receptor subtype 1
AT2	AT1 receptor subtype 2
Bax	Apoptosis regulator
Bcl-2	B-cell lymphoma/leukemia-2 gene
B2	Bradykinin receptor
ß-actine	Beta Actin Antibody
Caspase-3	Cystinoaspartic acid specific
cDNA	Complementary DNA
Cy3	Cyanine dyes
DNA	Deoxyribonucleic acid

DISC	Death-inducing signal complex
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
ED1	Anti-CD68 antibody [ED1]
ECG	Electrocardiogram
FADD	Fas-associated death domain protein
Fas-L	Fas ligand
FITC	Fluorescein Isothiocyanate
GS	Goat serum
H2O2	Hydrogen Peroxide
HCL	Goat serum Hydrochloric Acid
IL-1ß	Interleukin 1
IL-10	Interleukin 10
IGF	Insulin growth factors
JAK	Janus kinase
kDa	kilo Dalton
KCL	Potassium Chloride
IAP	Left Atrial Pressure

LDA	Left descending coronary artery
LV	Left Ventricular
LVFW	Left ventricular free wall
MI	Myocardial infarction
MAPK	Mitogen-activated protein kinases
MgCl2	Magnesium chloride
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic Acid
Na2HPO4	Sodium pyrophosphate
NaH2PO4	Natrium-beta-glycerophosphate
NF-kB	Nuclear factor-kappa B
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase chain reaction
PD123319	AT2 angiotensin II receptor antagonist
P53	Tumor suppressor gene
PKC	Protein kinase
PC12W	Rat pheochromocytoma cell line
PDGF	Platelet-derived growth factor

R3T3	Mouse fibroblast cell line
RAS	Renin angiotensin system
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription polymerase chain reaction
RV	Right Ventricular
RRX	Rhoda mine Red - X
STAT	Signal Transducer and Activator of Transcription
TAE	Trans arterial embolization
TGF	Transforming growth factor
T BID	Truncated Bid
TGF-ß1	Transforming growth factor-ß1
TNF	Tumor necrosis factor
TRITC	Tetra methyl Rhoda mine Isothiocyanate
VAL	Valsartan

1. Introduction

Myocardial infarction (MI) is one of the major public health problems and is associated with sudden death. After acute MI, the myocardium undergoes progressive dysfunction. The exact mechanisms responsible for progressive myocardial dysfunction are largely unknown. Recent studies have suggested that apoptosis and inflammation may play an important role in myocardial remodeling of MI-induced cardiomyopathy **(1)**. Indeed, the ischemic myocardium triggers an inflammatory reaction after MI. The inflammatory process includes macrophage infiltration, elaboration of inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, IL-1ß and IL-6 and fibroblast activation **(2, 3)**. Inflammatory reaction can severely damage the post-ischemic heart by inducing apoptosis **(4),** hypertrophy **(5)**, extracellular matrix alterations **(2, 6)** and contractile depression **(7)**. In the Animal models of MI induced heart failure, the cardiac renin-angiotensin system (RAS) was activated as confirmed by gene and protein upregulation of angiotensin converting enzyme (ACE), enhanced myocardial Ang II formation and angiotensin receptors upregulation **(8 - 12)**. The previous studies reported that cardiac non-cardiomyocytes involving infiltrated monocyte / macrophages as the main targets of cardiac Ang II. These cells are mainly responsible for the increased expression of cardiac AT1 and AT2 receptors **(13, 14, 15)**. However, the relevance of these increased angiotensin receptors in MI-induced apoptosis and inflammatory reaction is unclear.

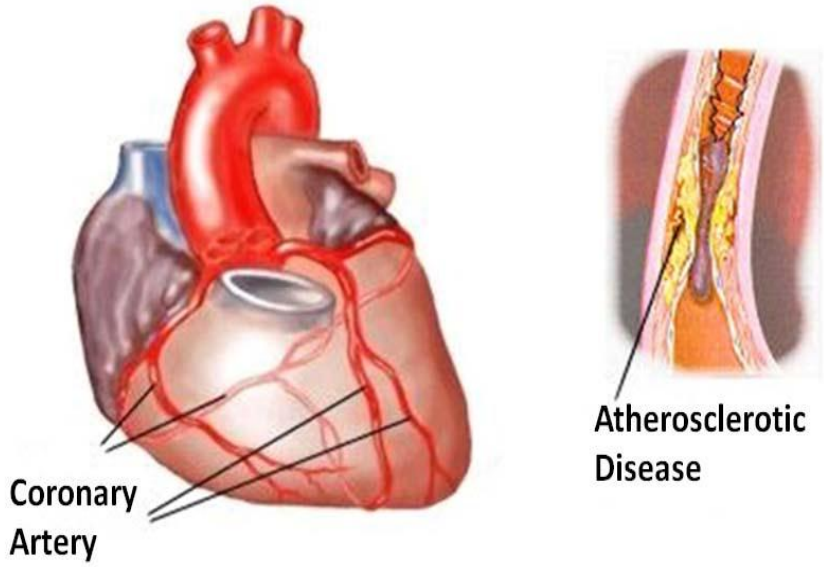
Our preliminary data demonstrated that upregulated AT1 receptors was co-localized with pro-apoptotic proteins in cardiomyocytes surrounding the infarct zone in rats with myocardial infarction. In contrast, AT2 receptors were detected in macrophages infiltrating cardiomyocytes **(16)**. The AT1 and AT2 receptors and their roles in cardiac remodeling after myocardial infarction have been examined extensively in Prof. Unger's group **(17-20)**.

We have previously provided evidence that both AT1 and AT2 receptors are expressed in adult rat cardiomyocytes constitutively as well as after MI-induced cardiac injury. However, it is currently unknown if angiotensin receptors mediate apoptosis and inflammation in cardiomyocytes in-vivo. We have assessed the regulation of AT1 and AT2 receptors in association with the expression of apoptosis markers such as P53, Bax and Caspase-3 and cytokines (i.e. IL-2, IL-4, IL-10 and TGF-ß1) by western blot and/or immunohistochemistry on days 1, 3, 7 and 14 after experimental MI in rats. Most of the known pathological effects of Ang II in the myocardium are attributed to the stimulation of its AT1 receptor while the function of the AT2 receptor is still a matter of discussion **(21)**. Pharmacological inhibition of RAS at ACE or AT1 receptor level has proven to be a useful tool to treat cardiovascular disease and to reduce cardiovascular mortality **(22, 23)**. Recent experimental data suggests that the AT2 receptor can be instrumental in preventing the consequences of ischemia after MI **(24, 25)**. A potential advantage of the AT1 receptor antagonists over ACE inhibitors is that they inhibit the RAS more selectively at the receptor site. The AT1 receptor antagonists do not antagonize the AT2 receptor but expose it to high levels of Ang II. This may contribute to the beneficial effects of these drugs following myocardial infarction. The most pronounced beneficial effects of AT1 receptor antagonists have been observed when therapy is initiated during the early remodeling phase post MI **(23)**. The remodeling is a relatively common post-acute MI event. The relevance of remodeling not only results in higher cardiac rupture prevalence, arrhythmia and aneurysm formation, but also modulates the onset and the progression of ventricular dysfunction, heart failure and the outcome of death following infarction. Additionally, this process is not homogenous in acute MI patients. Therefore, patients with diagnosed remodeling or under high risk of developing remodeling must be treated in an aggressive fashion to prevent, attenuate, or even revert the process.

2. Review of Literature

2.1. Myocardial infarction (MI)

Myocardial infarction (MI) or acute myocardial infarction (AMI) is commonly known as a heart attack. MI results from the occlusion (blockage) of a coronary artery and consequently the rupture of a vulnerable atherosclerotic plaque, which is an unstable complex of lipids (cholesterol and fatty acids) and white blood cells (especially macrophages) in the wall of an artery causing heart cells to die. **(Fig.1)**

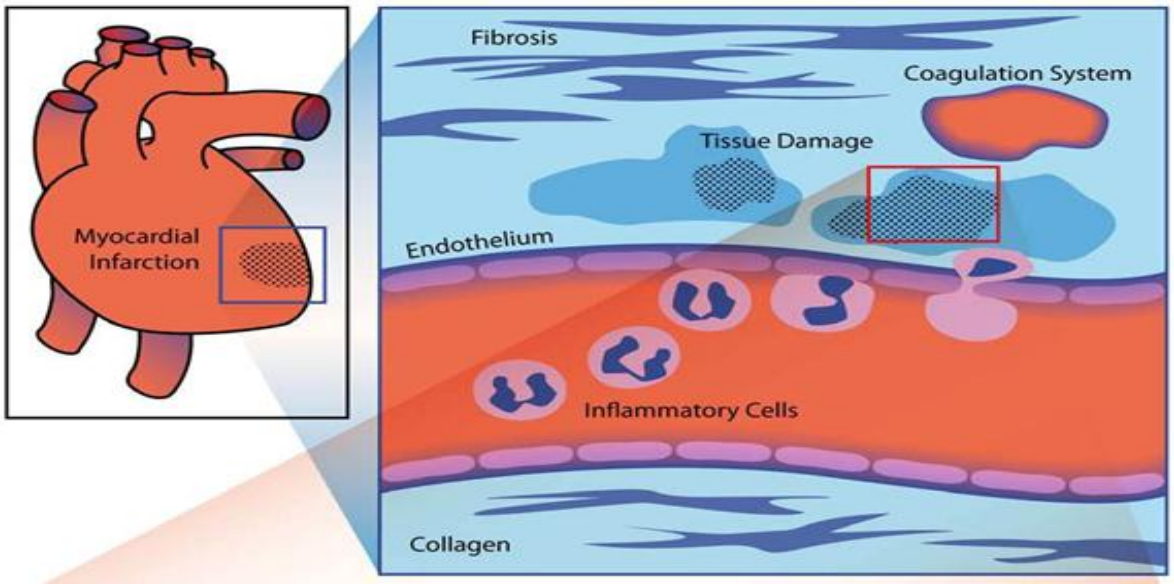


http://www.fmh.org/causesofaheartattack.

(Fig.1). The atherosclerotic disease effect on the coronary artery.

Following the MI, the segment of the heart which is normally fed by the coronary artery becomes ischemic and consequently cardiac cell death due to prolonged oxygen deprivation. If the resulting ischemia (restriction in blood supply) and prolonged oxygen deprivation is left untreated for a long period, invariably results in damage or death (infarction) of cardiac tissue.

Wound healing takes place in the infarct area after MI where the dead cardiac tissue is replaced by a scar. The wound healing process includes proliferation, differentiation and apoptosis of different cells. The previous study by Cleutjens et al., **(26)** reported that apoptosis also plays an important role in the process of tissue damage due to MI. The authors indicated that the various cellular activities during apoptosis are initiated and regulated by growth factors and finally lead to replacement of dead cells by scar tissue. Following apoptosis, macrophages, fibroblast like cells and endothelial cells invade the infarcted area. Then, macrophages remove dead cells and debris. However, fibroblasts and endothelial cells proliferate and lay new networks of collagen and small blood vessels. Finally, scar tissue matures into a collagen rich segment **(27, 28)**. Anversa et al **(29)** stated that the heart loses its contractile units and structure after MI resulting in reduction in the force of pumping and low performance.

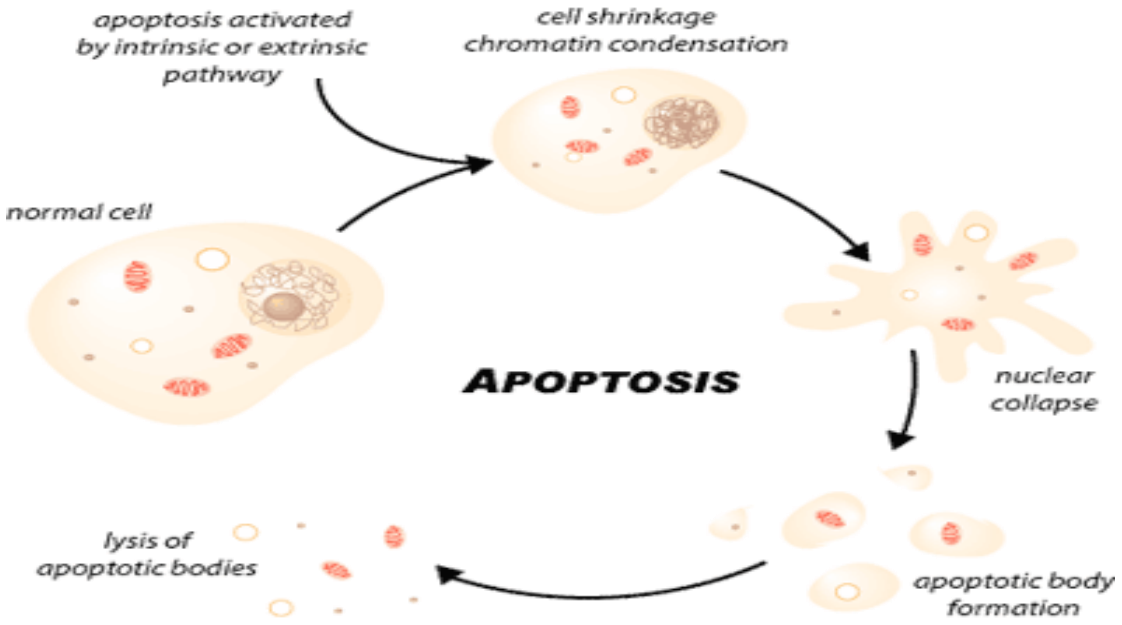


http://www.fmh.org/causesofaheartattack

(Fig.2). Physiological body responds to MI and the inflammatory respond to apoptosis mechanism.

2.2. Apoptosis in MI

Apoptosis, i.e. programmed cell death consists of several steps such as nuclear condensation, subsequent formation of membrane-bound apoptotic bodies, and DNA degradation. Apoptosis is a highly regulated process that allows a cell to self-degrade allowing the body to eliminate unwanted or dysfunctional cells. Macrophages phagocytosis apoptotic cell before its contents leak into the surrounding space and thereby **(30)** prevent unnecessary inflammatory response. Apoptosis can be triggered in a cell via the extrinsic or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of trans-membrane death receptors, such as the Fas. In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria.



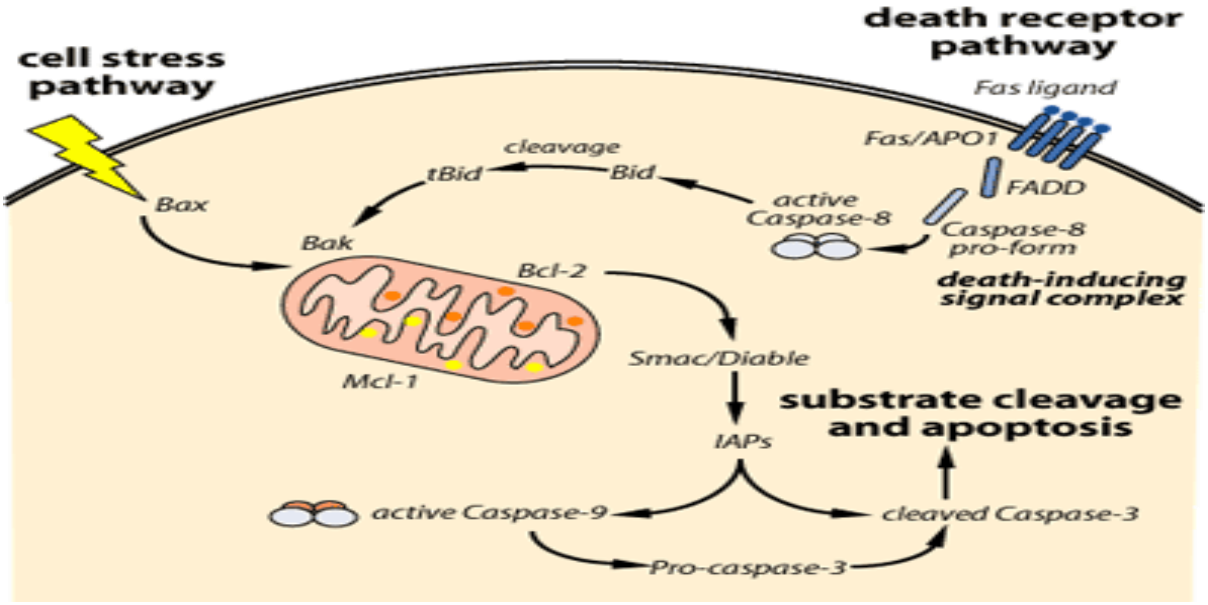
Apoptosis, by Philip Yau, (August 2004)

(Fig.3). Apoptosis - programmed death of cells.

The Extrinsic Pathway: Extrinsic pathway is activated upon binding of ligands, released by other cells, to trans-membrane death receptors on the targeted marked for cell apoptosis. For example, the immune system's natural killer cells possess the Fas ligand (Fas L) on their surface **(31)**. The binding of the Fas L to Fas receptors (a death receptor) trigger multiple receptors to aggregate together on the surface of the target cell. Subsequent aggregation of these receptors recruits cytoplasmic adaptor protein known as Fas-associated death domain protein (FADD). FADD, in turn, recruits Caspase-8, an initiator protein, to form the death-inducing signal complex (DISC). The recruitment of Caspase-8 to DISC, activate Caspase-3, an effector protein, to initiate degradation of the cell. Active Caspase-8 can also cleave BID protein to t BID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway **(32)**.

The Intrinsic Pathway: Intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock **(32)**. Upon receiving the stress signal, the pro apoptotic proteins in the cytoplasm, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of the internal content. However, the signal of BAX and BID is insufficient to trigger a full release. To achieve 'full activation' BAK, another pro apoptotic protein that resides within the mitochondria, is required to fully promote the release of cytochrome c and the intra-membrane content from the mitochondria **(33)**. Following the release, cytochrome c forms a complex in the cytoplasm with adenosine triphosphate (ATP), and Apaf-1, an enzyme. Following its formation, the complex will activate Caspase-9, an initiator protein. In return, the activated Caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates Caspase-3, the effector protein that initiates degradation. Besides the release of cytochrome c from the intra-membrane space, the intra-membrane content released also

contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) **(33)**.



http://www.scq.ubc.ca/apoptosis/

Apoptosis, by Philip Yau, (August 2004).

(Fig.4). The intrinsic pathways leading to apoptosis.

More generally, apoptosis also involves inflammation, which is part of the complex biological response of vascular tissues to harmful stimuli, such as (pathogens, damaged cells, or irritants). Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process **(34)**. It is a stereotyped response, and therefore considered as a mechanism of innate immunity, without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases

(atherosclerosis, rheumatoid arthritis), and even cancer. Therefore, because of these associated risks that inflammation is closely regulated by the body. Inflammation can be classified as either acute or chronic inflammation **(35).**

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue.

Chronic inflammation also known as prolonged inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Assessment of apoptosis is usually performed using a combination of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) by light microscopy and genomic deoxyribonucleic acid (DNA) ladder detection **(36)**.

In the phase preceding the execution, or degradation phase of apoptosis, a particular class of aspartate-specific cysteine proteases called Caspases is activated in a self-amplifying cascade **(37)**. Currently, two major pathways leading to Caspase activation are characterized. One is a mitochondrial pathway, which involves mitochondrial release of cytochrome C, and probably other factors into the cytosol **(38)**. The second pathway leading to activation is initiated by the ligation of death receptors such as Fas ligand and tumor necrosis factor (TNF)-α **(39)**.

Caspase 8 is the most upstream in these pathways and is activated by a signaling complex. The best known factors controlling apoptosis are multi gene family of Bcl-2-like proteins with homologous structure, some of which, e.g. Bcl-2 itself inhibit apoptosis and while others such as Bax promote it. A determining factor for a cell continuing to exist without going into mitosis or apoptosis may be the ratio of different components of the extended Bcl-2 family that are being produced **(40)**. MI is the leading cause of death from cardiovascular diseases **(41)**. Since the first report documented **(42)**, reperfusion-induced apoptosis in rabbit cardiomyocytes, many reports focused on confirming the appearance of apoptosis in ischemic cardiomyocytes and on its distribution in- vivo and humans **(43,44,45)**. In humans MI **(46,47,48)** induced apoptosis has been observed in three different regions including the core of the ischemic myocardial area, the border zone of the infarction and the viable myocardium, remote from the ischemic area **(49)**. The highest number of TUNEL-positive cells is consistently present in the border zone of the infarct but few positive cells can be observed in the central infarction region **(50)**. The previous studies showed that after AMI in humans, 12% of cardiomyocytes were TUNEL-positive in the border zone of the infarction, and less than 1% in areas remote from the infarction zone. Recently, it was shown in an animal model that the increased apoptosis in remote areas after MI is associated with an elevated expression of pro-apoptotic proteins P53 and Bax and of caspase-3. TUNEL-positive cells increase during day 1-14 after permanent coronary occlusion and then decrease over 4 weeks **(43)**. TUNEL-positive cells in MI are not limited to ischemic cardiomyocytes. Abundant numbers of non-cardiomyocytes such as coronary endothelial cells, interstitial macrophages, myoblasts and infiltrated neutrophils also undergo apoptosis **(51).**

2.3. Angiotensin II, Angiotensin receptor subtypes and apoptosis

Ang II mediates its physiological function by binding to highly specific receptors located on the cell membrane. In humans, two main subtypes of Ang II receptors have been identified, AT1 and AT2 receptors **(52)**. It is generally accepted that Ang II exerts the actions of promoting cellular proliferation and apoptosis, however, the precise details remain controversial. It was initially considered that Ang II induces apoptosis via the AT2 receptor. However, more recent ex-vivo and in-vivo studies have suggested that AT1 and AT2 receptors could be involved in apoptosis **(53,54)**. The cellular and molecular mechanism underlying Ang II induced apoptosis have recently been further investigated. A number of studies conducted in different types of cell lines have demonstrated that the AT2 receptor mediates apoptosis by inhibiting the mitogen-actived protein kinase activity **(55)**. This action is mediated ex-vivo assays by activation of phosphatase like mitogen-activated protein kinase phosphatase 1 in PC12W and R3T3 cells **(56,57)**. We have demonstrated that AT2 receptor activation induces apoptosis by dephosphorylation and thereby inactivate the anti-apoptotic protein Bcl-2. Furthermore, AT2 receptor stimulation also upregulated expression of both P53 and Bax genes which are potent inducers of apoptosis **(55,58)**. Moreover, the potential role of AT2 receptor through Nitric oxide (NO) production in the induction of apoptosis has also been suggested **(21).** Recently, AT2 receptor induced Caspase 3 activation and DNA fragmentation have been also shown to be mediated by Ceramide accumulation in PC12W cells, suggesting an additional pathway by which angiotensin II may regulate apoptosis **(20, 59).** Furthermore, Ang II induced AT1 receptor stimulation has been reported in vitro to activate P53 DNA binding activity, leading to subsequent upregulation of the pro-apoptotic Bax gene. Consistently, both AT1 receptor antagonism and Ang II antibody administration prevented P53-induced apoptosis **(60)**. A recent study has suggested that the AT1 receptor-mediated myocyte apoptosis may be affected via a protein kinase C dependent pathway **(61)**.

2.4. The immune response following MI

MI is associated with an immune response. Physiological inflammation response causes self-repair and protection, while pathological autoimmune response leads to ventricular remodeling and cardiac injury **(62)**. In the early phase following MI, cytokine production participates in the recruitment of inflammatory cells such as neutrophils and macrophages at the border of the infarcted myocardium thus promoting the physiological process of myocardial healing. Acute inflammatory response post MI is the hallmark of wound healing **(63)**. During the inflammatory phase, the infiltration of inflammatory cells is followed by removal of necrotic tissue and degradation of extra-cellular matrix components. The marked neutrophil infiltration at the infarct border occurs at first, second and fourth day after MI, while massive macrophage infiltration takes place after four days, which comprised the acute inflammatory response.

Macrophages, which have a strong phagocytic function, clear away necrotic myocardium and produce cytokines (TGF-β, PDGF, β-FGF) which stimulate neovascularization and fibroblast proliferation. Therefore, they may play a pivotal role in the transition between inflammation and myocardial repair. Lymphocytes, which are chronic inflammatory cells, appear by day two, and reach a maximum level at 7-14 days suggesting that they participate in the transformation from acute to chronic inflammation and the transition between inflammation and wound repair **(64)**. In acute MI, an inverted CD4/CD8 ratio, i.e. Low CD4 cell count, is detected and it strongly correlates with low ejection fraction and high myocardial mass destruction **(65)**. It has also been shown that CD8 T-lymphocyte numbers increase and mediate an essential part of the immune response observed following MI. However, an inappropriate inflammatory response may target at healthy cardiac cells, resulting in myocardial injury. Indeed, in-vivo evidence shows that donor lymphocytes derived from MI rats induce myocarditis in the recipient rats **(66)**.

2.5. Angiotensin receptors in the MI

AT2 as well as AT1 receptor is a seven transmembrane domain receptor, but there is only 34% identity between them. AT2 receptor expression declines after birth, suggesting that it may play an important role in fetal development, and can be induced later in adult life under pathological conditions. The previous studies **(67)** reported that AT1 but not AT2 receptor stimulation induces programmed cell death in cardiomyocytes by activating P53. Indeed, Ang II is capable of promoting both apoptosis and neuronal regeneration via its AT2 receptor **(67)**. It has been shown that AT2 receptor expression is significantly increased after myocardial infarction and is involved in mediating vasodilatation, anti-inflammation, anti-growth, differentiation and neuronal survival **(14)**. However, AT2 receptors have been associated with cell protection but there is also strong evidence that Ang II acting through AT2 receptor induces apoptosis **(68)**. The AT1 is the predominant receptor in the adult heart and considered to be the major mediator of the Ang II induced effects in the cardiovascular system **(52)**. In contrast, AT2 activation has been reported to inhibit cell proliferation **(17)**, to induce differentiation **(18)**, apoptosis and regeneration **(56, 69)**. Ang II has been implicated in ventricular remodeling after MI. After the induction of MI by coronary ligation in rats, a time-dependent increase of AT1 and AT2 receptor mRNA levels in the heart are observed during the acute phase of MI **(11)**. AT1 and AT2 receptor gene expression has been shown to be significantly increased at 30 min and peaking at 24h post MI. The data demonstrated that cardiac left ventricular AT1 and AT2 receptor gene expression is transiently elevated after MI. This in combination with increased Ang II receptor density may serve as an enhancer of the acute cardiac effects of angiotensin peptides post MI and thereby initiate the remodeling phase. Recent investigation, using a single-cell reverse transcriptase-polymerase chain reaction, demonstrated that approximately 40% of adult rat cardiomyocytes expresses AT1 receptors and approximately 10% expressed AT2 receptors **(14)**.

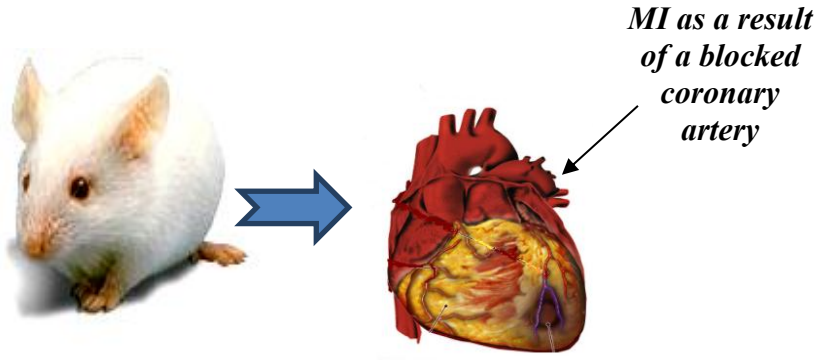
2.6. Cardiac effects mediated by the AT1 and AT2 receptors

Based on the idea that most of the effects of Ang II are mediated by its type 1 receptor, several experimental and clinical studies have demonstrated that AT1 receptor antagonist are more specific blockers of the RAS other than ACE inhibitors and attenuate most of the deleterious effects of Ang II in heart**(70-75)**. A number of studies have demonstrated that Ang II-induced apoptosis of ventricular myocytes was inhibited by angiotensin AT1 antagonist losartan **(68)**. Treatment of AT1 receptor antagonist has been shown to reduce infarct size, left ventricular loading, and to improve hemodynamics, coronary angiogenesis and anti-apoptosis. However, there is concern regarding application of the AT1 receptor antagonists as therapeutic agents in cardiac diseases as accumulated Ang II may excessively stimulate the AT2 receptors leading to unexpected effects **(76, 19)**. Intensive efforts have been made to clarify the role of AT2 receptor during cardiac remodeling since the middle of 1990s, which resulted in many controversial reports. Evidence has now emerged that many of the actions of Ang II exerted via the AT2 receptor are directly opposed to those mediated by the AT1 receptor. The cardio-protective role of AT2 receptors was demonstrated in rats two months after MI treated with an AT1 receptor antagonist L-158809. Left ventricular remodeling and cardiac function were significantly improved and this effect was attenuated either by the AT2 receptor antagonist PD123319 or the bradykinin B2 receptor antagonist. The data suggest that accumulated Ang II induced by AT1 receptor blocker may exert cardio protective effects by stimulating the AT2 receptor via kinins and other autacoids **(77)**. Accordingly, in transgenic mice overexpressing AT2 receptor in heart, the end-systolic volume indices were significantly lower and ejection fraction, blood pressure, and LV dP/dt significantly higher in 28 days post MI when compared with wild-type mice, suggesting a cardio protective role of the AT2 receptor in improving LV function during post-MI remodeling **(25)**.

In contrast, several studies have shown that AT2 receptor mediates actions in coordination with AT1, therefore, the cardio protective effects of AT2 antagonist. In isolated working rat hearts, Ford et al (1996) showed that PD123319 an AT2 receptor antagonist given before ischemia significantly improved the recovery of LV work and efficiency **(78)**. Similarly, PD123319, improved functional recovery in isolated working hearts with **(79)** or without ischemia-reperfusion **(80,81)**. In respect of the growth promoting actions, AT2 receptor blockade, but not AT1 receptor antagonist inhibits interstitial DNA synthesis early after myocardial infarction in rat suggesting a growth-triggering role of the AT2 receptor **(82)**. Furthermore, AT2 receptors also mediate the release of growth factors, e.g. the AT2 receptor antagonist PD123177 decreased secretion and immunostaining of transforming growth factor-beta1 (TGF-beta1) in valvular interstitial cells of the heart, although to a lesser extent than losartan **(83)**. However, in several experimental paradigms, a role of AT2 receptors in mediating Ang II effects in heart could not be validated. In a long-term treatment report, SHR were treated for 12 weeks with the AT1 receptor antagonist candesartan or in combination with an AT2 receptor antagonist PD 123319. AT2 antagonism was not able to blunt or enhance the coronary hemodynamic improvement such as a reduction of minimal coronary vascular resistance of heart or left ventricular mass induced by AT1 receptor antagonism despite a partial prevention of the candesartan-induced hypotensive effect by Coad ministration of PD 123319 **(84)**. Accordingly, the AT2 receptor blocker PD 123319 was unable to abolish the cardio protective effects of Ang II pretreatment in limiting infarct size in isolated rabbit hearts that could be antagonized by losartan **(85)**.

3. Material and Methods

3.1. Subjects and materials



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC295531/

(Fig.5). Performing MI in Wistar rats.

Experiments were performed on male Wistar rats weight between 250 to 300g. The rats were housed under climate-controlled conditions with a 12-hour light/dark cycle and were provided with standard rat chow and tap water. The animals were housed individually at controlled temperature and humidity under a 12-hour light/dark cycle and had free access to a standard diet and drinking water. The study was performed in accordance with German law on animal protection as released in its new version in 1993.

3.2. Operation procedure and induction of MI

MI induced by permanent ligation of the left descending coronary artery as described previously by Kaschina et al., **(86)** (see fig. 6). Briefly, after induction of anesthesia with an intra-peritoneal injection of ketamine/xylazine (Sigma Aldrich Chemie, Steinheim, Germany*)* (80 mg/10 mg/kg), rats are artificially incubated, ventilated, and connected to an electrocardiogram (ECG) recorder for continuous monitoring during surgery. A left thoracotomy was carried out by cutting the third and fourth rib and a rib-spreading chest retractor was inserted. Then, the left descending coronary artery was ligated. Successful ligation of coronary artery was verified by the occurrence of arrhythmia in the ECG and, visually, by the color change of the ischemic area. Rectal temperature was maintained at 37 °C by means of a heating blanket during surgery. Control rats were sham operated by omitting only the occlusion of the coronary artery. At various time points up to 2 weeks after the operation, the animals were decapitated and the hearts quickly dissected into five distinct regions, namely the left ventricle (LV, surrounding area of necrosis), septum and right ventricle (RV). Tissues were frozen immediately in liquid nitrogen and stored at -70°C.

3.3. Treatment with AT1 and AT2 receptor antagonists

Animals were randomly divided into five groups as following: a)- sham operation without treatment b)- MI subjected to placebo treatment c)- MI subjected to AT1 receptor antagonist treatment d)- MI subjected to AT2 receptor antagonist treatment e)- MI subjected to both antagonists.

3.3.1. Treatment procedure

Infarcted and sham-operated animals were subjected to the following treatment as detailed in **Table 1.**

Drugs or Therapy	Dosage	Number of Rats
Sham	_	10
MI+ vehicle	10mg/kg/day	32
MI+ valsartan	10mg/kg/day	24
PD123319	30mg/kg/day	24

For accuracy, the whole procedure was single blinded. After one week of treatment, the rats underwent a permanent ligation of the left descending coronary artery (LDA), which is a widely used surgical procedure to induce MI in animals. Treatment was continued for another 2 weeks after the surgery. For subcutaneous AT1 receptor (valsartan) or AT2 receptor (PD123319) antagonist delivery Alzet osmotic mini pumps (AP-2001, 0.5 µl/hr, 7 days, Alzet Corporation, Cupertino, CA) were filled with sterile 0.9% saline with or without AT1 receptor (valsartan) or AT2 receptor (PD123319) antagonist to administer antagonist or vehicle continuously at 1 µl/h. The mini pumps were implanted subcutaneously at the back directly after MI induction under anesthesia. To minimize the tissue damage at the site of pump implantation, the pump was gently moved around within the subcutaneous pocket every 2-3 days. All tests were performed on the 8^{th} day after pump implantation. At the end of the treatment period, infarcted and sham-operated animals were decapitated and the hearts were rapidly excised and placed on a preparation chamber at 4c. The left ventricle (LV) was separated from atrial and the right ventricle (RV) and then divided into the inter ventricular

septum (IS) and the left ventricular free wall (LVFW) including scar tissue and area at risk. The tissue samples were divided into two parts of equal size, rapidly frozen in liquid nitrogen and separately stored at-80. Another group of hearts was collected for morphological examinations.

3.4. Analysis of infarct size

Animals after MI at different time points will have the hearts removed for preparation of coronal sections (2-mm thickness). The fresh heart slices will be immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline at 37 °C for 30 minutes and then fixed in 10% phosphate-buffered formalin at 4 °C. The TTC-stained heart slices will be photographed using colour film, and the volume of an infarct will be calculated using NIH Image (version 1.62) system.

3.5. Measurement of infarct size

Measurement of infarct size was performed as described previously by Takagawa et al., **(87).** Briefly, all histological sections were examined with a Leica microscope using a 1x objective. Images were captured with a Retiga CCD camera with the use of Open lab software (Improvision, Lexington, MA). Image J 1.62 software (National Institute of Health. Image J: Image Processing and Analysis in Java. Available at: http://rsb.info.nih.gov/ij/) was used to measure areas of infarct and LV by an investigator who was blinded to the identity of the sections. The infarct area and the total area of LV myocardium were traced manually in the digital images and measured automatically by the computer. Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections and multiplying by 100.

3.6. Immunostaining

3.6.1. Single Immunohistochemical staining

Immunohistochemical staining of heart sections were performed with a vectastain avidin-biotin peroxidase complex (ABC) Kit, Vector Laboratories, Burlingame, CA), as described. Unless otherwise stated all incubations steps were at room temperature and PBS was used for washing (three times for 10 min) after each step. The sections were incubated with PBS, 0.3 % H_2O_2 and 10 % methanol for 45 min to block endogenous peroxidase. To prevent nonspecific binding, the sections were incubated for 60 min in PBS containing 0.3 % Triton X-100, 1 % BSA, 4 % goat serum (GS) (block solution). The sections were then incubated overnight at 4^{o}C with the following antibodies: Anti-rat Bcl-2 mouse monoclonal antibody (Cat:MS-598-P, Neo Markers, Washington), anti-rat Bax polyclonal antibody (Cat: 554106, BD Bioscience, USA) and Fas anti-rat monoclonal antibody Cat:F22120, BD Bioscience, USA) were used at 1:100 dilution The AT1 receptor polyclonal antibody from rabbit (Santa Cruz, sc-579) and AT2 receptor polyclonal goat antibody (Santa Cruz, sc-7420) diluted in 1:20 in the PBS for 1 hour at room temperature. Thereafter, the sections were incubated for 1hr with goat anti-rabbit, horse anti-mouse or anti-goat biotinylated secondary antibody (Vector Laboratories). Sections were then incubated with avidin-biotin-conjugated peroxidase (PK-400, vector Laboratories) for 45 min. Finally, the sections were washed and stained with 3`, 3`- diaminobenzidine tetra hydrochloride (DAB) (Sigma) containing 0.01 % H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6) for 3-5 min. After the enzyme reaction, the sections were washed in tap water, counterstained with hemaxylin, then dehydrated in alcohol, cleared in xylene and mounted in DPX. Score analysis of results was dependent on immunostaining density in each type of cell as in **(table 3-11)**. The specificity of immunoreaction was evaluated in comparison with a negative control specimen without primary antibodies.

Table 2. Antibodies for immunofluorescence staining

1st Antibody	Source / Clone / Concentration	Dilution
Polyclonal goat anti-rat AT2 receptor	Santa Cruz Biotechnology, Inc, California, USA / C-18 / 0.2 mg/ml	1:150
Polyclonal rabbit anti-rat AT1 receptor	Santa Cruz Biotechnology, Inc, California, USA / 0.2 mg/ml	1 : 200
Polyclonal rabbit anti-rat Bax	Santa Cruz Biotechnology, Inc, California, USA / P-19/0.2 mg/ml	1 : 200
Polyclonal goat anti-rat P53 (C-19)	Santa Cruz Biotechnology, Inc, California, USA / C-19/0.2 mg/ml	1 : 200
Polyclonal goat anti-rat Caspase-3	Santa Cruz Biotechnology, Inc, California, USA / K-19/0.2 mg/ml	1: 200
Monoclonal mouse anti-rat CD11b	BD Biosciences pharmingen, USA / 0,5 mg/ml	1:400

Monoclonal mouse anti-rat CD68 (ED1)	AbD Serotec / UK / 0.25 mg/ml	1:200
Monoclonal mouse anti-rat OX-62 (CD8)	AbD Serotec / UK / 2.0 mg/ml	1:200

2nd Antibody	**Source**	**Dilution**
Cy3 Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch, Hamburg, Germany	1:300
Cy3 Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch, Hamburg, Germany	1:300
FITC Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch, Hamburg, Germany	1:150
AMCA Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch, Hamburg, Germany	1:200
RRX	Jackson ImmunoResearch, Hamburg, Germany	1:200

Table 3. Microscope and software

Instrument	Company
Leica DMIRE2 fluorescence microscope LSM confocal microscope (LEICA SP2 with AOBS)	Leica, Wetzlar, Germany
OpenLab Imaging Software	Improvision, Tubingen, Germany

Table 4. Buffers and solutions:

Description	Chemical composition
PBS* (Calcium and magnesium-free phosphate-buffered saline)	130 mM NaCl, 10 mM Sodium-phosphate buffer (100 mM Na2HPO4. 20 mM NaH2PO4), pH 7.4
MACS-Buffer	PBS-Buffer with 0.5% (w/v) BSA. pH 8.0
FACS-Buffer	PBS-Buffer with 0.5% (w/v) Bovine serum albumin (BSA) and 0.1% (w/v) NaN3
Chloroform	J.T.Baker, Griesheim, Germany
* **Company**: PAN Biotech GmbH, Aidenbach, Germany	

Table 5. Reagents for RNA isolation:

Reagent	**Company**
Trizol® Reagent kit	Invitrogen GmbH, Karlsruhe, Germany
Ethanol	J.T.Baker, Griesheim, Germany
Isoproponanol	J.T.Baker, Griesheim, Germany

Table 6. Reagents for Reverse Transcription:

Reagent	Company	Chemical composition
Random primer	Promega GmbH, Mannheim, Germany	0.5 μg/μl
dNTPs mix	Promega GmbH, Mannheim, Germany	10 mM of each 4 dNTP. pH 7.0
5x M-MLV Reaction Buffer	Promega GmbH, Mannheim, Germany	100mM Tris-HCl pH 8.8; 500 mM KCl 1% Triton® X-100
Recombinant RNasin® Ribonuclease inhibitor	Promega GmbH, Mannheim, Germany	25 mM
M-MLV-RT (reverse transcriptase)	Promega GmbH, Mannheim, Germany	0.1 M Potassium phosphat pH 7.2. 0.2% TritonX®-100. 2 mM DTT and 50% glycerol

Table 7. Reverse transcription reaction:

Reagent	Final concentration	Final volume
5x M-MLV Reaction buffer	1x	5 µl
dNTPs mixture	25 mM	2 µl
Recombinant RNasin® Ribonuclease Inhibitor	25 Units	0.5 µl
M-MLV-RT (Reverse Transcriptase)	200 Units	0.5 µl

Table 8. Primers synthesis:

Company
TIB MOLBIOL Berlin, Germany

Table 9. Miscellaneous:

Instrument	**Company**
HeraeusTM Multifuge 3L-R	Thermo Scientific, Germany
Incubator (Binder CB210)	Binder, Germany
Tj-6 centrifuge with inserts for 96-well Plate	Beckman, Germany
Cryostat Leica CM3050S	Leica Microsystems, Germany
15ml / 50ml conical polypropylene tubes	Greiner bio-one, Germany
0.45 $m seringe-filters	Greiner bio-one, Germany
Neubaurer improved counting chamber	Carl Roth, Germany
Paraformaldehyde	J.T.Baker, Griesheim, Germany
Acetone	J.T.Baker, Griesheim, Germany

3.6.2. Single Immuonofluorescent staining

The heart tissue sections were incubated for 20 min with blocking solution in humidified chamber and subsequently incubated with following antibodies: Rabbit polyclonal anti-AT1 receptor, mouse monoclonal anti-ED1 (Serotec, Oxford, UK) or mouse monoclonal anti-IL-10 (Santa Cruz, sc-32815), diluted in PBS containing 5% blocking serum for ~45 min in a dark humidified chamber. After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies; a) FITC conjugated goat anti-rabbit antibody. b) AMCA or CY3 conjugated donkey anti-mouse antibody. Thereafter, sections were washed with PBS, mounted in vectashield (Vector Laboratories) and viewed under a fluorescence microscope with appropriate filters (#9 or #15 or #00 or #20) in a dark room, and the slides stored in the dark at RT or at 4°C.

3.6.3. Double Immuonofluorescent staining

The heart tissue sections were incubated for 20 min with blocking solution in humidified chamber then incubated with the following antibodies: a) Rabbit polyclonal anti-AT1 receptor in combination with mouse anti-P53 (Santa Cruz) or polyclonal goat anti-AT2 receptor; b) rabbit polyclonal anti-AT2 receptor in combination with mouse monoclonal anti-ED1 or goat polyclonal anti-IL-10 diluted in PBS containing 5% blocking serum for ~45 min in a dark humidified chamber. Subsequently, the tissue sections were washed with PBS and incubated with the appropriate secondary antibodies; FITC conjugated donkey anti-mouse or anti-goat antibody in combination with Texas red conjugated donkey anti-rabbit antibody. Thereafter, sections were washed with PBS, mounted in vectashield (Vector Laboratories) and viewed under a fluorescence microscope with appropriate filters (#9 or #15 or #00 or #20). The slides were stored in the dark at room temperature or at 4°C.

3.6.4. Triple immunofluorescent staining

The heart tissue sections were incubated for 20 min with blocking solution in a humidified chamber then incubated with the following antibodies: rabbit polyclonal anti-AT2 receptor in combination with mouse monoclonal anti-ED1 and goat polyclonal anti-IL-10 diluted in PBS containing 5% blocking serum for ~45 min in a dark humidified chamber. After incubation with primary antibodies, the tissue sections were washed with PBS and incubated with the appropriate secondary antibodies; FITC conjugated donkey anti-goat antibody in combination with Texas red conjugated donkey anti-rabbit antibody and AMCA conjugated donkey anti-mouse. Thereafter, sections were washed with PBS, mounted in vectashield (Vector Laboratories) and viewed under a fluorescence microscope with appropriate filters (#9 or #15 or #00 or #20). As above. The slides were stored at RT or at 4°C in the dark.

3.7. The Process of RT-PCR

3.7.1. Sample preparation

Each of the hearts was separated into 3 parts known as left, right ventricles and inter ventricular septum immediately after the hearts were removed from sham, MI, MI+ Vehicle, MI+ Valsartan, MI+ PD123319 rats. These myocardial tissues were frozen in liquid nitrogen. Tissues from the same section of the 6 hearts from each group were stored as together as one sample at -80^{o}C for further analysis.

3.7.2. Ribonucleic Acid (RNA) isolation

The 12 samples were homogenized in a 10-fold volume (wt/vol.) of ice-cold Trizol reagent (Gibco, BRL) using a Polytron homogenizer (Janke & Kunkel, Germany) and total RNA extracted according to manufacturer's instructions. Briefly, following centrifugation (12,000rpm, 4^{o}C, for 20min) of Trizol containing tissue homogenate, the aqueous phase was transferred to a new tube. Total RNA was precipitated through two consecutive ethanol precipitations separated by an additional phenol/chloroform extraction step. Finally, total RNA yield was quantified by UV spectrophotometer measured at 260 nm. The concentration and ratios of RNA/DNA of all the total RNA samples were measured by UV spectrophotometer through a special programmed computer card and recorded. Concentrations and ratios of total RNA were about 0.7 μg/μL and 1.6 - 1.8 respectively. This showed that the RNA extraction was successful and the quality of total RNA from the samples was good **(11)**. The RNA samples were stored in DEPC water at -80^{o}C for reverse transcriptase - polymerase chain reaction (RT-PCR).

3.7.3. RT-PCR

5µg total RNA of each of the 12 samples was reverse-transcribed into first-strand complementary DNA (fs cDNA) using oligo-dT primers (Gibco, BRL). The fs cDNA was amplified by polymerase chain reaction (PCR). The PCR was carried out in a total volume of 100µL containing Tris-HCL 20mmol, KCL 50mmol, MgCl2 1.5mmol, dNTP 0.2 mmol, 0.6 mmol of corresponding sense and antisense primers including ß-actin, ACE, AT_1, AT_2 and VEGF respectively, and 2.5 units of Tag DNA polymerase. ß-actin mRNA was included as an endogenous control. The PCR was performed using Perkin Elmer 9600 thermocycler. The PCR conditions included 30 cycles with denaturation step at 94^{o}C for 1 min and target gene specific annealing temperature for 1 min; at 60^{o}C (ß-actin); 64^{o}C (P53); 57^{o}C (AT_1 receptor); and 53^{o}C (AT_2 receptor) for 1min, respectively.

3.7.4. Analysis of PCR Products

Gel electrophoresis was used to analyse PCR products were analysed by gel electrophoresis. Briefly, 1.5% agarose gel was prepared in 10% TAE with EB (2µL EB in 50ml 1.5% gel). A 25µL aliquot of PCR product with 2µL DNA dye were loaded and electrophoresed through the prepared gel (Bio-Rad Sub cell* GT) at 90 volts for 45min. The results of electrophoresis were analysed with a UV lamp and a photo taken by a UV camera (UVL BER LOURMAT FRANCE TFX-35m). The scanned images were subjected to quantification using a software designed to measure the target gene expression relative to the endogenous control gene, ß-actin, with the specified RT-PCR conditions; software program through RT-PCR fragments which were scanned in photos **(88)**. These RT-PCR conditions were as outlined above. The PCR will be carried out in a total volume of 100 µl containing 20 mM Tris-HCL, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 mmol of each primer, and 2.5 units of Taq DNA

polymerase (Gibcol, BRL). The expression of the house-keeping gene, GAPDH mRNA, will be considered as an internal standard. The primer sequences were:

sense 5'- ACG TGG TGA GAC GCT GCC CT-3', antisense 5'- TGA TCG CTG GTT CCC CTC AAC-3') for P53, sense 5'- CAAGACGCAGGCTTTTTGGCC-3', antisense 5' ATACCGCTATGGAGTA CCGCTGGC-3' for AT1 receptor and sense 5'- TTGCTGCCACCAGCAGAAAC-3', antisense 5'- GTGTGGGCCTCCAAACCATTGCTA-3' for AT2 receptor cDNA. And sense 5'- ATCTGGCACCACACCTTCTA CAAT GAGCTGCG-3', antisence 5' - CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' for β - actin. For ACE, the sense primer will be 5'- GACTGGTCCAACATCTATG-3', and antisense will be 5'- ATGAAGCTGACGAAGTACCT-3'. PCR will include 25 cycles for β - actin, ACE, AT1 and AT2 receptors cDNA in a Perkin Elmer 9600 thermocycler. Three Steps PCR of denaturing, annealing, and extension reactions will be proceeded at 94 °C for 1 min, at 60 °C (β-actin) or at 64 °C (ACE) or at 57 °C (AT1-) or at 53 °C (AT2-) receptor for 1min, and 72 °C for 1 min, respectively.

5 µl MgCl2

2 µl dNTP

1 µl Antisense primer

1 µl sense primer

1 µl cDNA

and 0.5 µl Tag DNA polymerase. The final volume was completed to 100 µl with dist. water, mixed, placed in PCR machine at 95 degree for 5 min and 95 degree 1 min,

Annealed (each primer may different, e.g. for beta actin is 60 degree), Then it is extended at 72 degree and these step was repeated for 25-35 cycles at72 degree for 5min hold at 4 degree.

3.7.5. Quantitation of PCR products.

15 µl of PCR products will be diluted with 35 µl Tris/EDTA and submitted to ionic exchange chromatography on a Mini Q-column (Pharmacia) using the SMART micro chromatography apparatus (Pharmacia). PCR products were quantified by measuring the absorption at 260nm (peak integration against a calibration standard). Ratios of the corresponding peak areas (P53/β - actin, or AT1 receptor /β - actin or AT2 receptor /β - actin) then calculated for each sample.

3.8. Protein extraction and Western blot analysis.

In the second set of the experiments, protein levels of cardiac A and B were determined in additional sham operated and placebo-, PD123319-treated infarcted groups. At day 1 and day 7 after MI, the three tissue samples (RV, IS, LV free wall) of each animal were homogenized in 5 ml of ice cold lysis buffer containing 50mM Tris-HCL (pH 7.4), 0.5 M EDTA, 150 mM NaCL, 0.1% Triton-100, freshly added proteinase inhibitor (100 µg/ml PMSF, 1mg/ml Trasylol) followed by centrifugation at 14,000 rpm at 4 C° for 1 min. The supernatant was collected, and aliquots mixed with loading buffer (1M Tris-HCL [pH 6.8], 1% SDS, 30% glycerol, 0.8M DTT, 2% Bromophenol blue) and solubilized for 10 min at 95 C° and then centrifuged 30 min at 14,000 rpm at 4 C°. Protein concentration was determined as previously described by Bradford, using bicinchonic acid system (Pierce, Rockford, IL, USA) and bovine serum albumin as a standard. All preparations were carried out at 4 C°.

For Western blotting, the samples were subjected to SDS-PAGE under reducing conditions. Briefly, 80 µg protein from the supernatant were precipitated by TCA and solubilized in modified SDS-PAGE sample buffer consisting of 5 M urea, 0.17 M SDS, 50 mM Tris, and 5% mercapthoethanol and loaded onto a 15% polyacrylamide Gel. Proteins were electrophoresed as previously reported by Laemmli (1970) and transferred to nitrocellulose

membranes (Bio Rad, München, Germany) using a Bio-Rad Trans-Blot apparatus (120 Volt, 80 min). The membrane was washed three times for 20 min in TTBS (0.1% Tween 20, 100mM Tris-HCL, 150 mMHCL NaCL, pH 7.5), blocked for 1h in 5% nonfat milk/TTBS. After three washes in TTBS, the membrane was incubated with the following primary antibodies: anti-P53, anti-AT2 receptor, anti-Bax or mouse anti-Caspase 3 (Santa Cruz, sc-56055) 1:1000 dilutions of the anti-rabbit. Second antibody for 30 min at the room temperature. After extensive washes in TTBS, detection of immunoreactive bands was carried out using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Freiburg, Germany), and the blot was immediately exposed to autoradiograph film for 30 sec to 3 min. Each experiment was repeated three times.

The Western blot bands of P53, AT2 receptor, Bax or caspase 3 were quantified by Java Image processing and analysis software. The area and density of pixels within the threshold values representing immunoreactivity were measured, and the integrated density (the product of the area and mean of gray value) was calculated. Integrated immunodensities of controls and treated groups were compared and statistically analyzed.

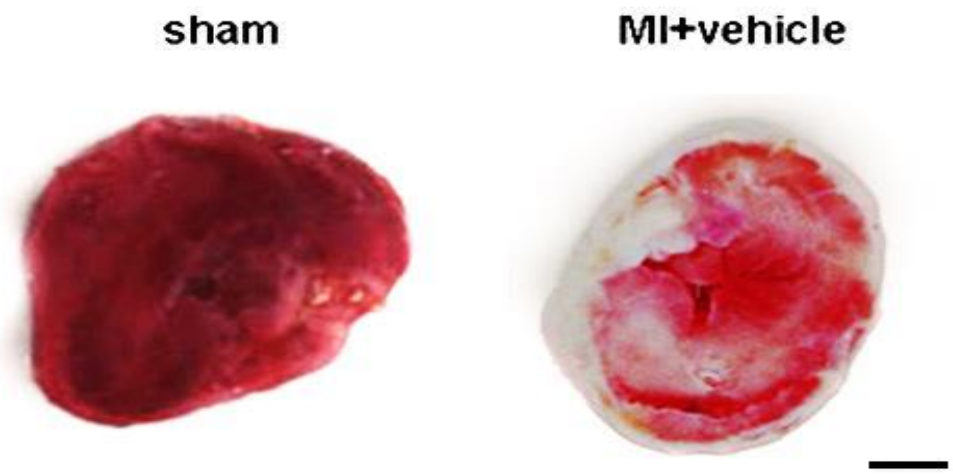
4. Statistical analysis

Results were reported as means ± SE multiple, while the statistical analysis was performed using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-kramer test. Two-group comparisons were analyzed by the two-tailed Student's unpaired t test for independent samples. For all procedures, P values <0.05 were considered statistically significant.

5. Results

5.1. Myocardial infarction (MI)

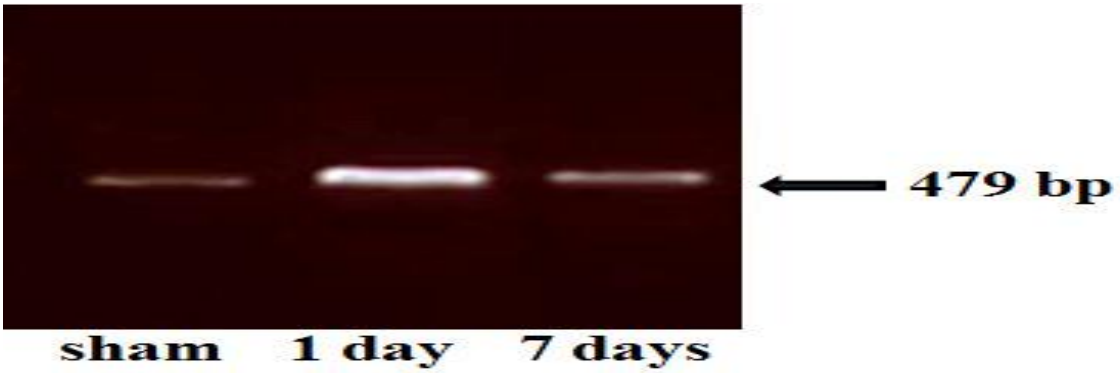
MI, commonly known as a heart attack occurs when the blood supply to part of the heart is interrupted. This is most commonly due to occlusion (blockage) of a coronary artery. The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period, can cause damage and/or death (infarction) of heart muscle tissue (myocardium). We established animal model of MI in rats and we performed our experiments 7 days after MI.



(Fig. 6). Sagittal sections through adult rat heart showing the infarct area in left ventricle of MI compared to sham-operated animals. Bar = 100 μM.

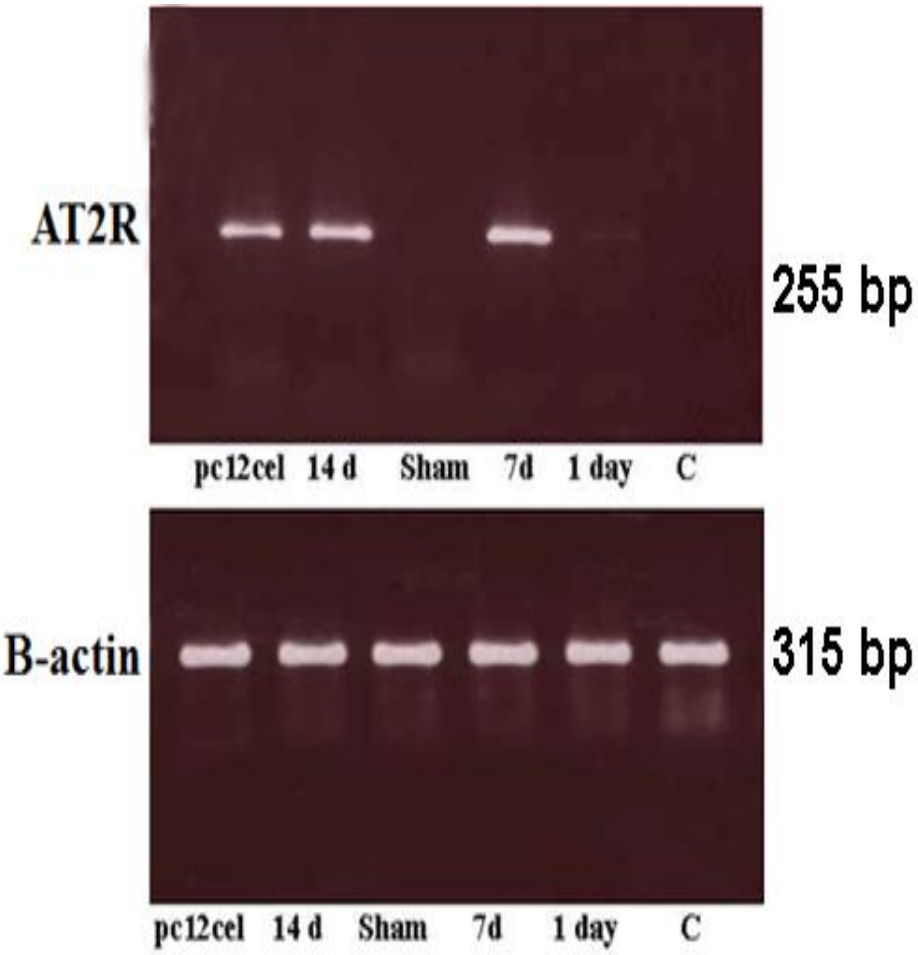
5.2. The changes in AT1 and AT2 receptors mRNA during experimental MI-induced cardiac injury

In the present study, the changes in the expression of AT1 receptor at 1 day, 3 days, 7 days and 14 days after experimental MI-induced cardiac injury was measured by reverse transcription–polymerase chain reaction (RT-PCR). Interestingly, our results demonstrated that ventricle wall AT1 receptor mRNA levels were increased at 1 day but return back to normal level at 7 days after experimental MI-induced cardiac injury **(Fig. 7)**. Also, the results showed that cardiac AT2 receptor mRNA levels were increased from 1 day, 3 days, and 7 days till 14 days after experimental MI **(Fig. 8)**. The data suggest MI leads to an upregulation of AT1 and AT2 receptors which peaked at day 7 after MI. Therefore, we performed the further AT2 receptor-related experiments 7 days post MI.



(Fig.7). Effect of MI-induced cardiac injury on ventricle AT1 receptor mRNA level.

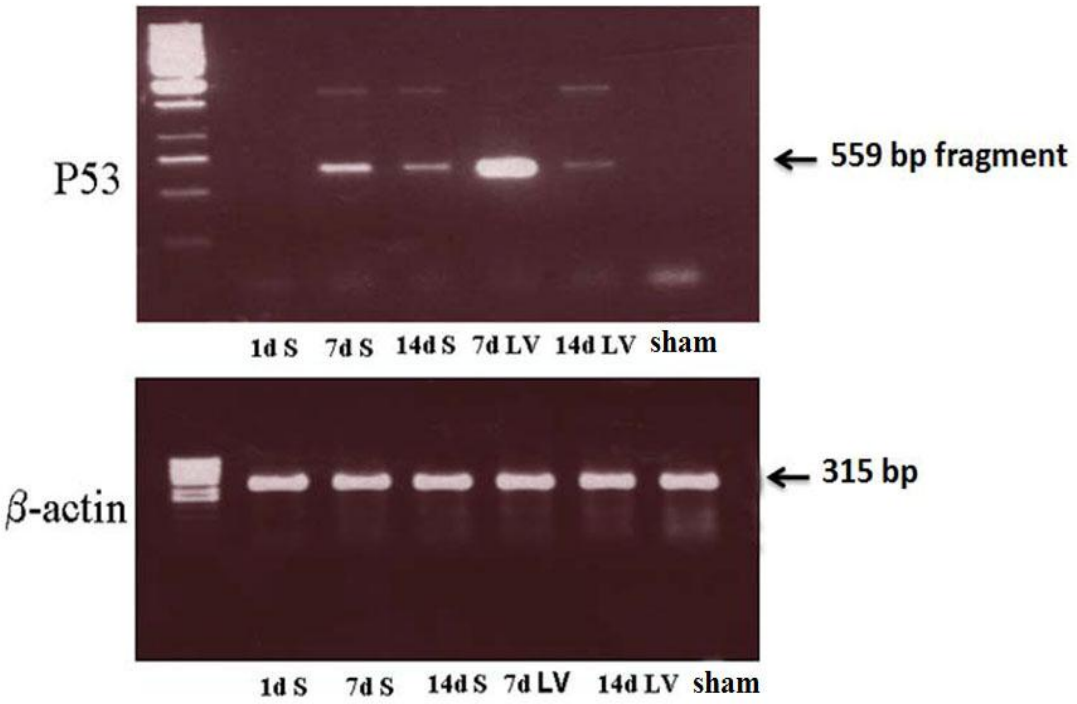
AT1 receptor mRNA levels were determined by RT-PCR with an internal cRNA standard. MI induced an upregulation of AT1 receptor mRNA formed at the 1st day but return back to nearly normal after 7 days from MI versus sham-operated animals.



(Fig.8). Effect of MI on cardiac AT2 receptor mRNA level. AT2 receptor mRNA levels were determined by RT-PCR. MI induced a gradual upregulation of AT2 receptor mRNA in rats 1 day, 7 days or 14 days after MI versus sham-operated animals. The pc12 cells served as positive control for the detection of AT2 receptor mRNA.

5.3. Upregulated mRNA levels of apoptotic marker P53 at different time points after MI

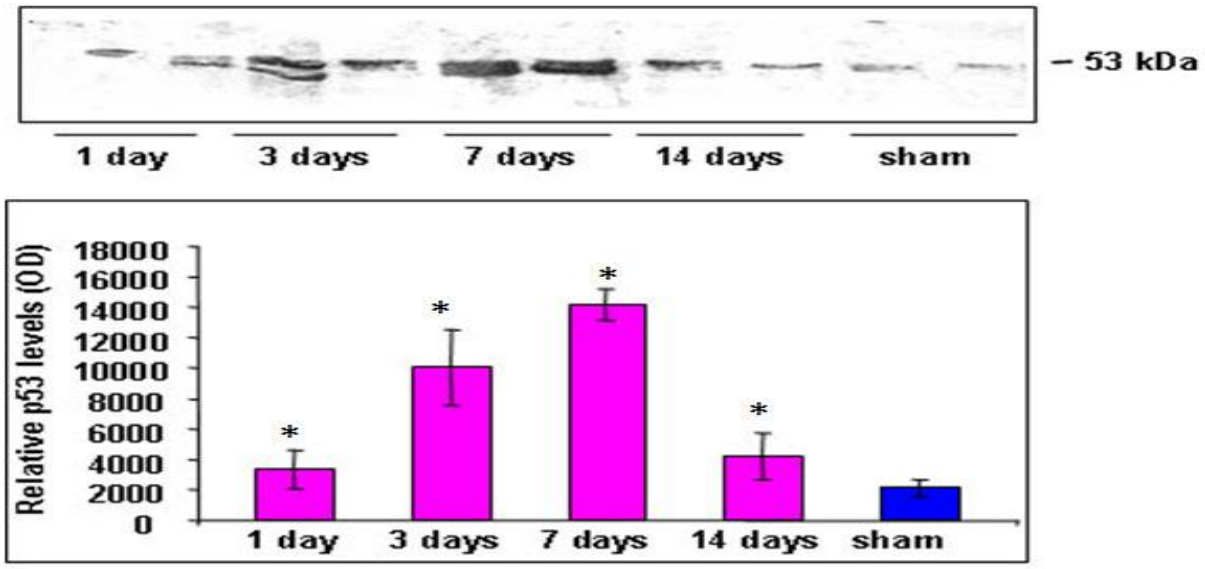
To further evaluate cardiac injury, we assessed the changes of apoptosis-related markers such as P53 in peri-infarct myocardium 1 day, 7 days and 14 days after experimental MI. Our RT-PCR analysis revealed that cardiac P53 mRNA levels were increased 1 day, 7 days and 14 days in response to MI-induced cardiac injury. The upregulation of P53 peaked at 7 day post infarct **(Fig. 9)**. To study the adaptive response of AT1 and AT2 receptor to cardiac injury, we performed all our experiments at day 7 following MI.



(Fig.9). After myocardial injury caused by MI P53 mRNA levels were determined by RT-PCR. MI-induced myocardial injury led to a gradual upregulation of P53 mRNA levels from day 1 to day 7 after myocardial infarction versus sham-operated animals.

5.4. Change in the expression of apoptotic marker P53 after MI

Western blot analysis demonstrated a single 53-kDa band of P53 protein in the peri-infarct myocardium **(Fig.10)**. The same band was observed in the peri-infarct myocardium at different time points post MI. The approximate molecular mass of the P53 was consistent with the calculated mass based on the molecular sequence and with that previously reported by others **(53).** Densitometry analysis of the 53-kDa band **(Fig. 10)** showed that MI-induced cardiac injury led to an upregulation of P53 from day 1, 3, and 7 to day 14 after MI. The increase of P53 was most significant at day 7 after MI-induced cardiac injury.



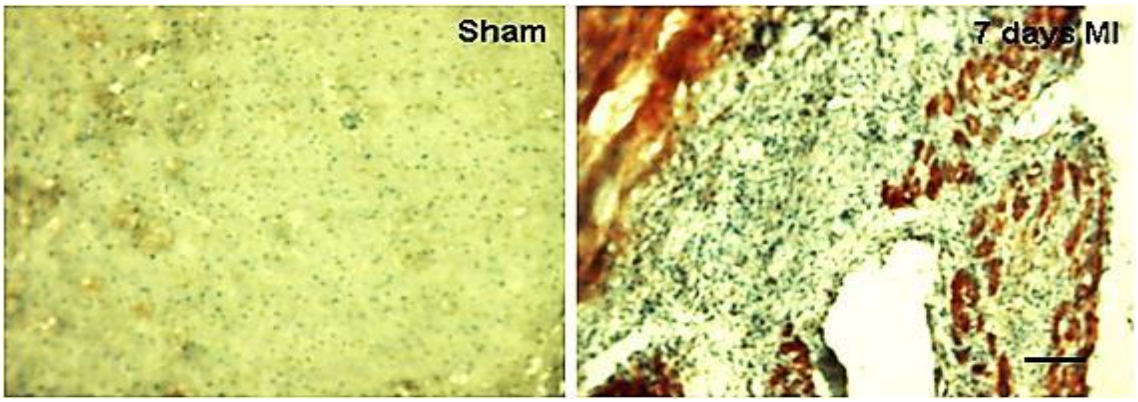
(Fig.10). Western blot analysis of cardiac apoptotic marker P53 after MI.

Relative P53 protein levels were determined by densitometry analysis. Band intensity was calculated by multiplying band areas by their mean optical density. MI led to an increase in protein levels of cardiac P53 from 1, 3 and 7 to 14 days versus sham-operated animals. (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-Kramer test, the asterisks denote significant differences compared to sham-operated animals: $P>0.05$.

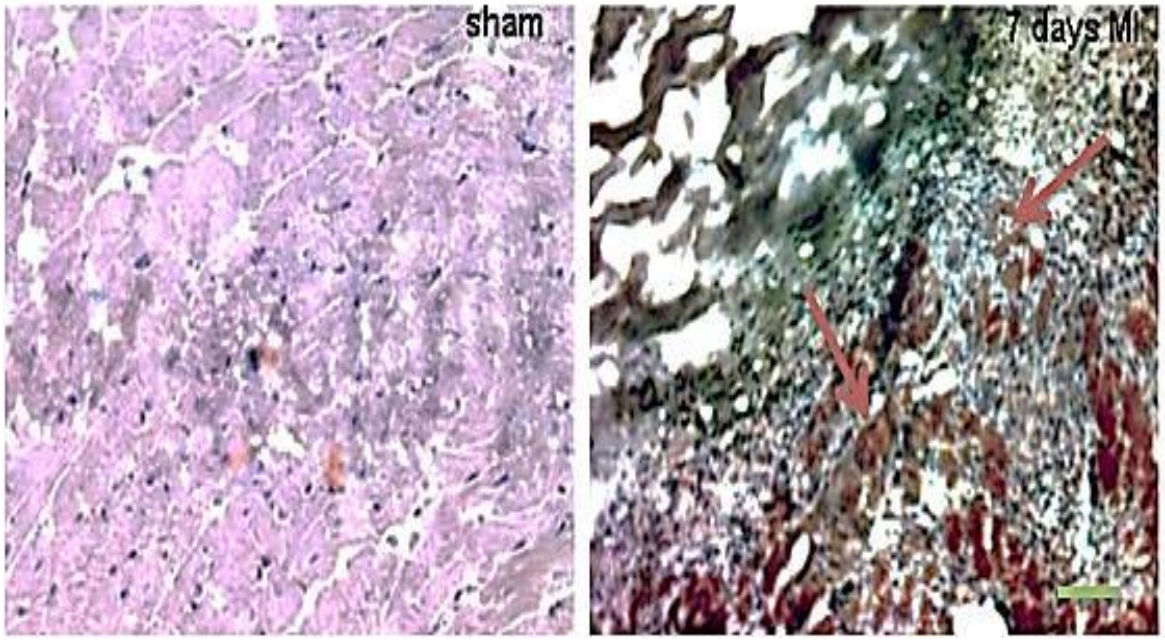
5.5. Immunohistochemical staining of cardiac apoptotic markers during MI

It is well established that apoptosis is an important mechanism in the pathogenesis of myocardial injury after MI**.** We explored the relationship of apoptotic markers with AT1 receptor during MI-induced cardiac injury in order to assess the potential role of AT1 receptor in apoptosis-involved cardiac damage. We assessed the regulation of AT1 receptor in association with the expression of apoptosis markers such as P53, Bax and Caspase-3 at day 7 after experimental MI in rats by immunohistochemistry. We detected a marked upregulation of cardiac AT1 receptor as well as P53 at day 7 after MI **(Fig. 11)**. Immunohistochemical staining revealed abundant and heterogeneous distribution of P53 in cardiomyocytes. The strongest staining of P53 was observed in those cardiomyocytes located in the border-zones of the infarction **(Fig. 11)**.

In contrast, Bax and Caspase-3 were mainly located in the area between the border-zone and necrotic lesion and in the area surrounding the necrotic lesion, respectively **(Fig. 12, 13)**.

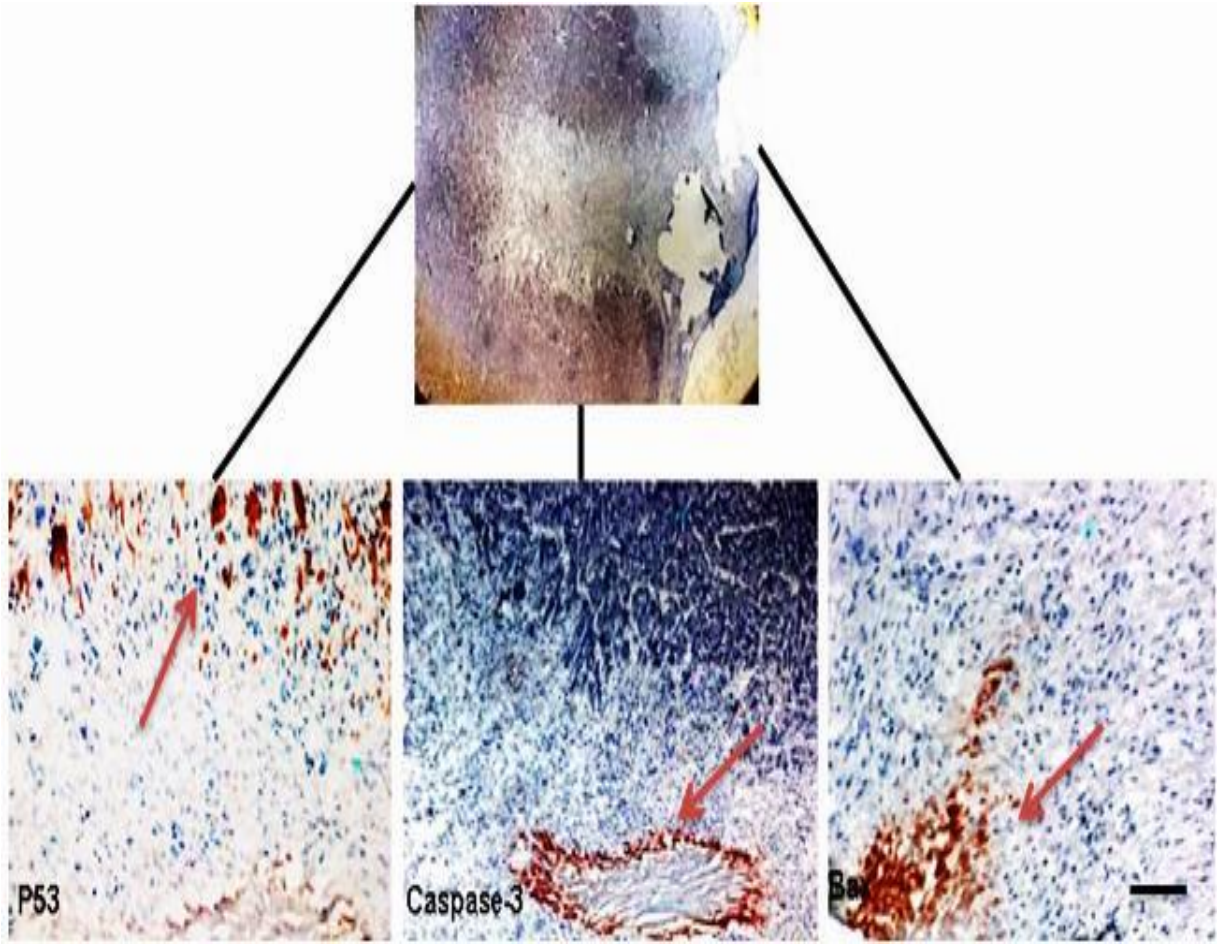


(Fig.11). Immunohistochemical localization of cardiac P53 proteins in rats 7 days after sham-operation (left) or MI (right). Immunostained cardiomyocytes were located within the infarcted myocardium 7 days after MI. P53 signals (brown) were dramatically increased after MI when compared to sham-operated animals. Magnification = 40X, Bar = 20 μM.



(Fig.12). Immunohistochemical staining of cardiac Caspase-3 in rats after sham-operation (left) or MI (right). Immunostained cardiomyocytes were located in the peri-infarct myocardium 7 days after MI. The Caspase-3 signals indicated with brown color (arrow) were dramatically increased after MI compared to sham-operated animals.

Magnification = 40X, Bar = 20μM.



(Fig.13). Immunohistochemical staining of cardiac apoptotic markers like P53, Caspase-3 and Bax in rats after MI. Abundant positive signals of P53 (left) were detected in cardiomyocytes along the border-zones of the infarction. In contrast, Bax (right) and Caspase-3 (middle) were mainly located in the areas surrounding the necrotic lesions.

Magnification = 20X, Bar = 40μM.

5.6. Localization of cardiac AT1 receptor with apoptotic marker P53 after MI

To study the co-localization of cardiac AT1 receptor with P53 proteins 7 days after MI - induced cardiac injury, we performed a double immunofluorescence staining of cardiac AT1 receptor and apoptotic marker P53. We observed that in the border-zones of the infarction a co-expression of AT1 receptor and P53 protein in the cardiomyocytes 7 days after MI **(Fig. 14)**.

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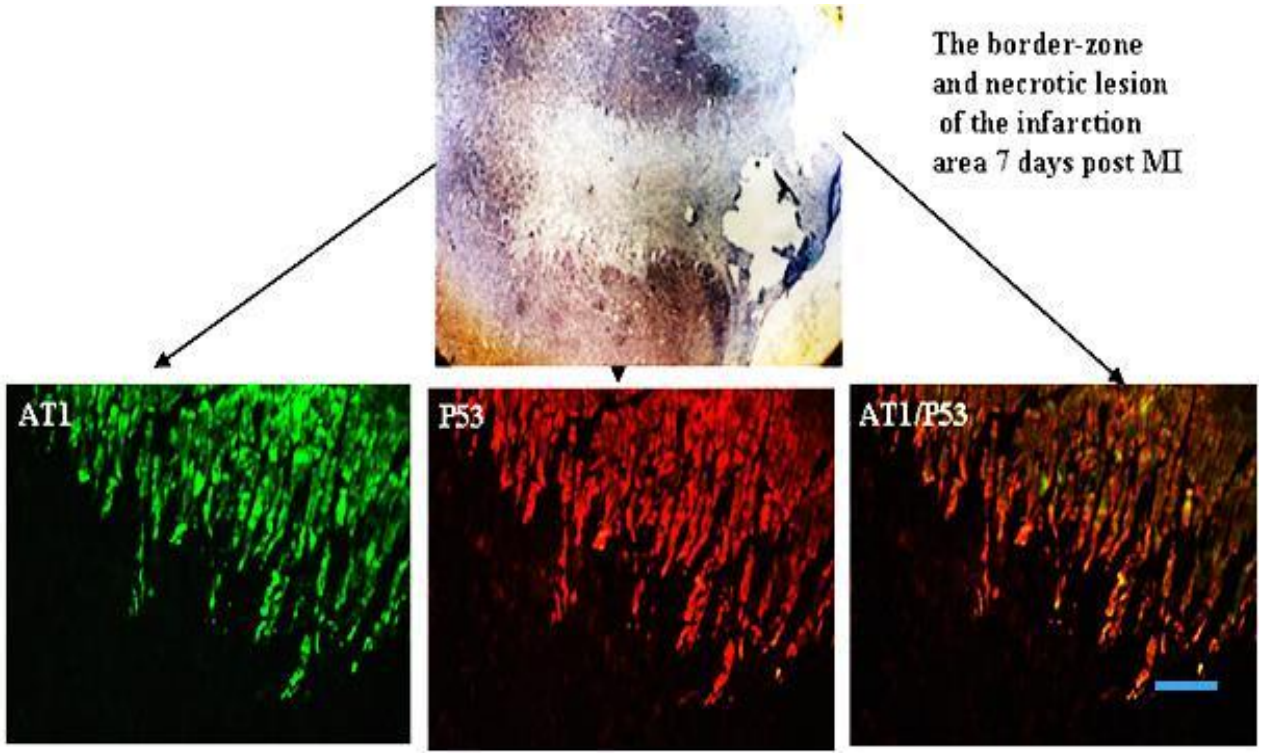
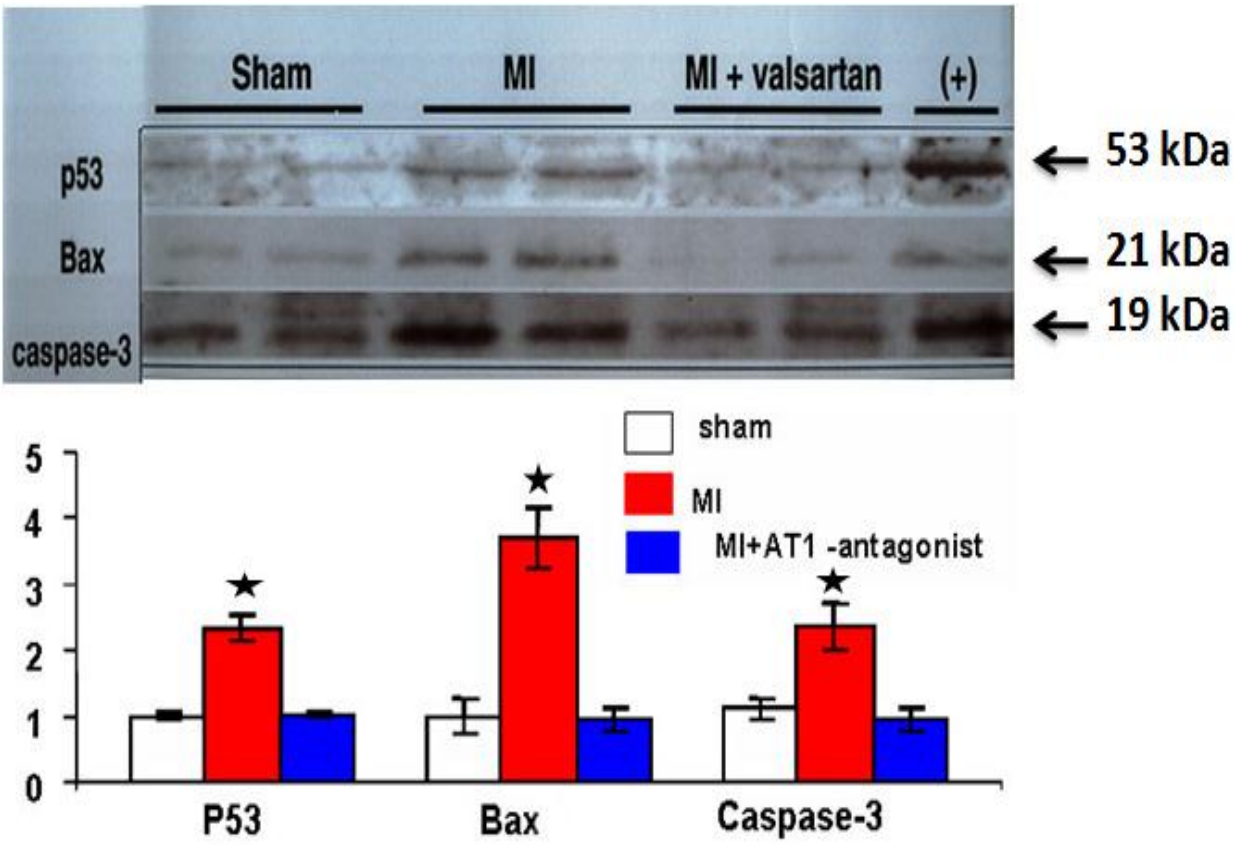


(Fig.14). Double immunofluorescence staining of cardiac AT1 receptor and P53, 7 days after MI that AT1 receptor (left) was co-localized with P53 (middle) in cardiomyocytes in the border-zones of the infarction. Magnification = 20X, Bar = 40μM.

5.7. The role of AT1 receptor in cardiac apoptosis in rats with MI

These experiments was designed to evaluate the cardiac role of AT1 receptor by AT1 blocker valsartan (Val) versus AT2 blocker PD123319 (MI+PD) in cardiac apoptosis in rats with MI. Treatment with AT1 receptor blocker suppressed MI-induced upregulation of cardiac apoptosis markers including P53 and Caspase-3 **(Fig. 15)**. Interestingly, we found that blocking AT2 receptor did not influence the levels of apoptosis markers involving P53 and Caspase-3.

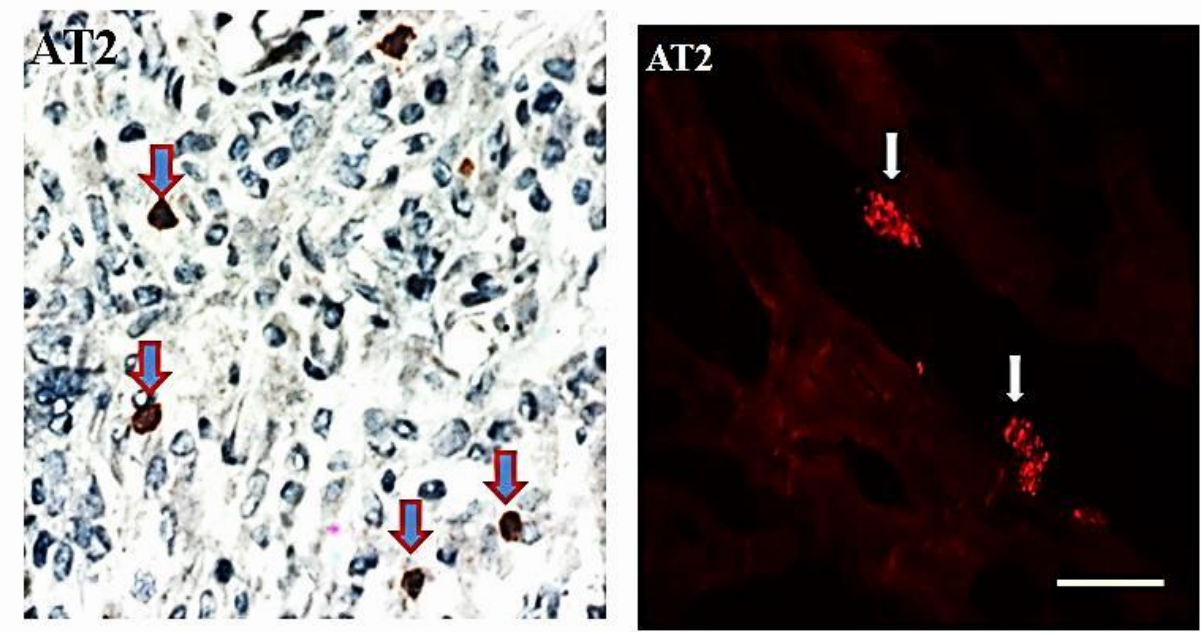


(Fig.15). Western blot analysis of cardiac apoptotic markers including P53 and caspase-3 after treatment with AT1 receptor blocker valsartan (MI+Val) or AT2 receptor blocker PD123319 (MI+PD) in rats with MI.

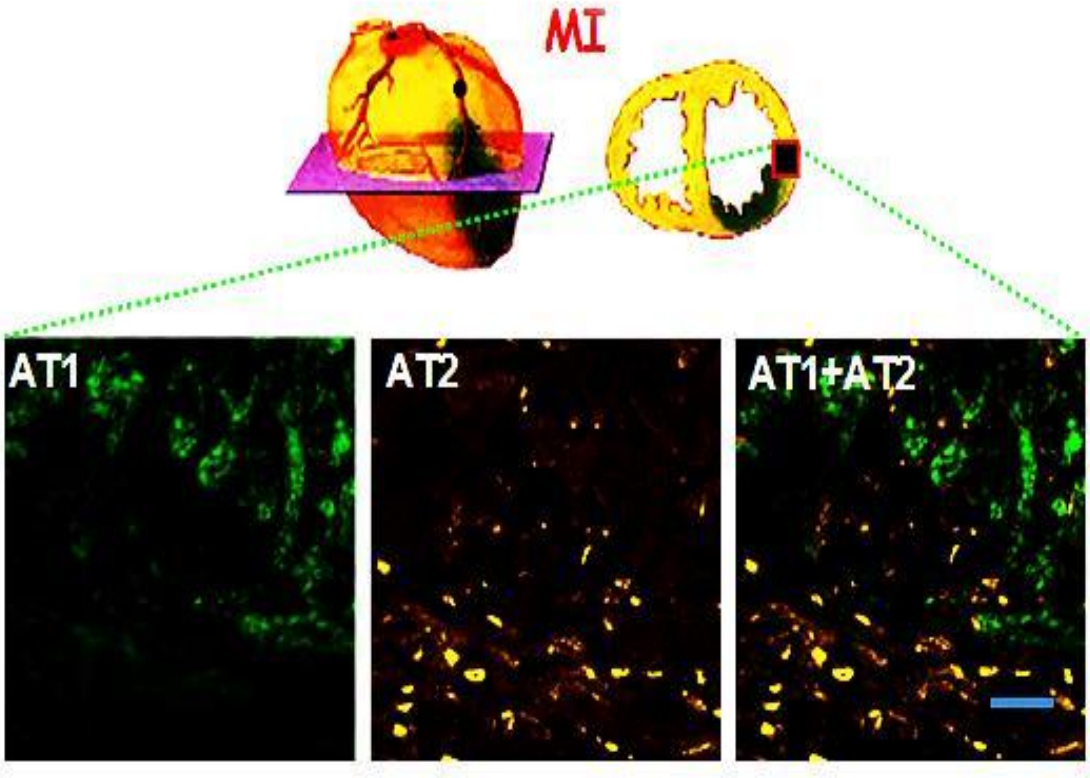
The protein levels of P53, Bax and caspase-3 were determined by densitometric analysis. Band intensity was calculated by multiplying band areas by their mean optical density. The protein levels of cardiac P53 and Bax were increased in rats after MI versus sham-operated animals. AT1 receptor blocker (valsartan) abolished MI induced upregulation of cardiac P53 and Bax proteins. (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-Kramer test, the asterisks denote significant differences compared to sham-operated animals: P>0.05.

5.8. AT2 receptor expression in cardiomyocytes after MI

Our immunohistochemical studies showed a dramatic increase of AT2 receptor in small infiltrating non-cardiomyocytes cells at 7 days after MI-induced cardiac injury **(Fig. 16)**. Furthermore, double immunofluorescence staining demonstrated a differential cellular distribution of cardiac AT1 and AT2 receptors at 7 days after MI-induced cardiac injury. Consistently, our experiments showed expression of AT1 receptor immunoraectivity in cardiomyocytes. But AT2 receptor expression was detectable in non-cardiomyocytes probably including immune cells such as infiltrating macrophages within the peri-infarct myocardium 7 days after MI-induced cardiac injury **(Fig. 17).**



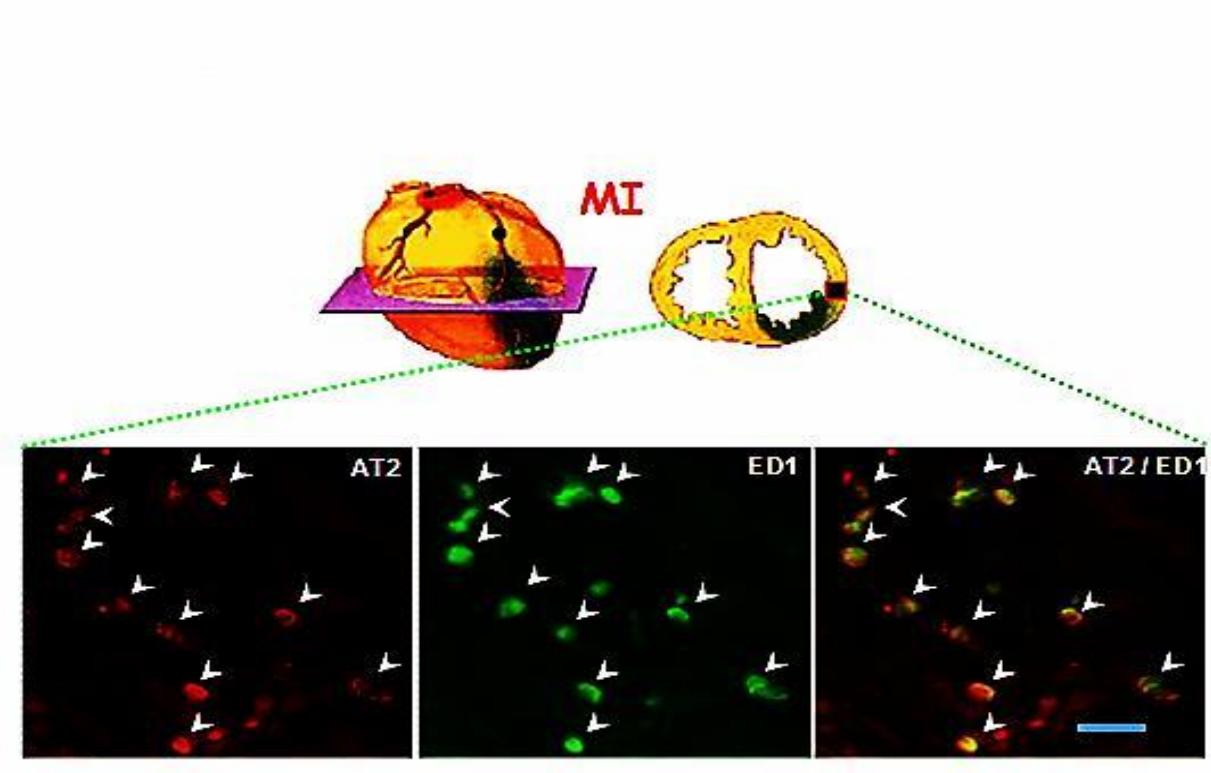
(Fig.16). Immunohistochemical staining of cardiac AT2 receptor in peri-infarct myocardium at 7 days after MI showed that AT2 receptor is localized in small infiltrating non-cardio myocytes dark brown immunoreactivity (left) or red immunofluorescence (right). Magnification = 40X, Bar = 20 μM.



(Fig.17). Double immunofluorescence staining of peri-infarct myocardium at 7 days after MI showing differential cellular distribution of AT1 receptor (green immunofluorescence, arrow head) (left) and AT2 receptor (yellow immunofluorescence, arrow head) (middle) in peri-infarct myocardium. The representative immunofluorescence staining on control heart cryo-section shows that AT1 receptor signals are localized in cardiomyocytes and AT2 receptor was concentrated in small non-cardiomyocytes. Magnification = 40X, Bar = 20 μM.

5.9. Localization of AT2 receptor with macrophage marker ED1 during MI

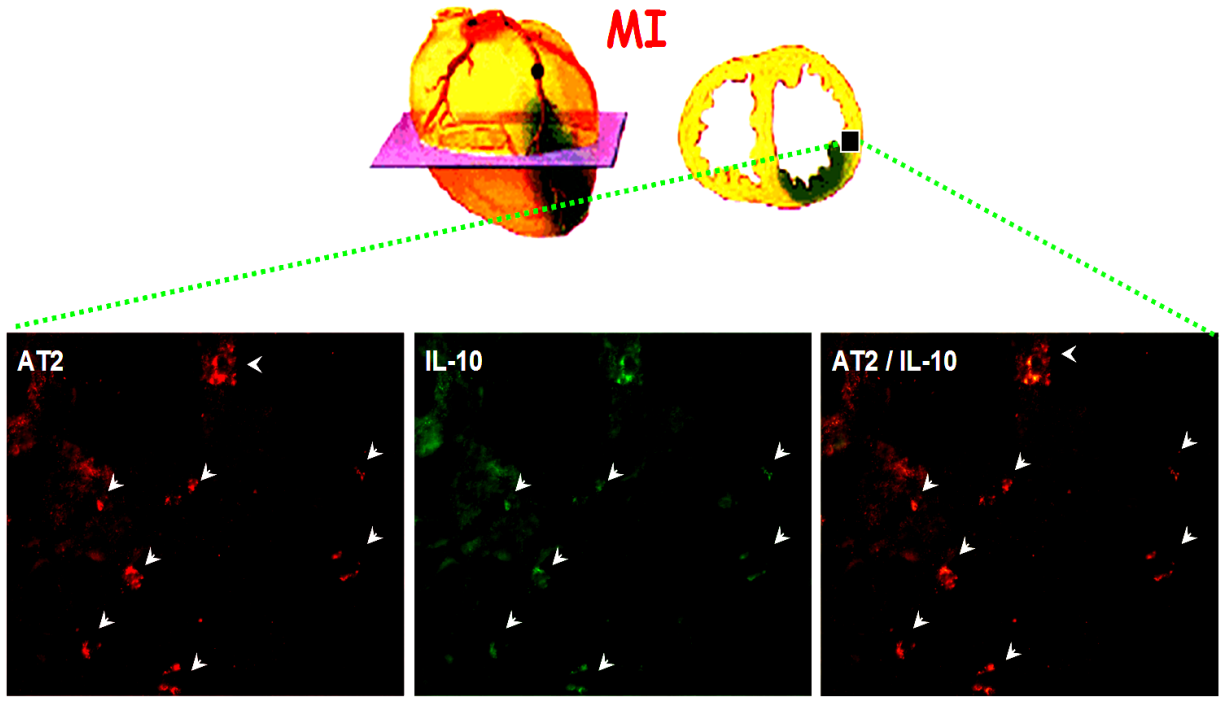
We are able to show that AT2 receptor expressed in non-cardiomyocytes 7 days after MI-induced cardiac injury. Therefore, we further examined weather these non-cardiomyocytes expressing AT2 receptor are macrophages by investigating the co-localization of macrophage marker ED1 with AT2 receptor in the peri-infarct myocardium 7 days after MI. Double immunofluorescence staining clearly demonstrated that AT2 receptor were expressed in ED1 positive cells indicating that cardiac AT2 receptor are localized in infiltrating macrophages 7 days after MI **(Fig.18)**.



(Fig.18). Double immunofluorescence staining of AT2 receptor and macrophage marker ED1 in the peri-infarct myocardium 7 days after MI-induced cardiac injury. Upregulated AT2 receptor (red immunofluorescence, arrow head) (left) are located in ED1 positive macrophages (green immunofluorescence, arrow head) (middle) right panel is the merged image. Magnification = 40X, Bar = 20 μM.

5.10. Localization of AT2 receptor and IL-10 after MI

Because we observed an increase in ED1 positive macrophages containing AT2 receptor, we extended our experiments to investigate the possible involvement of pro-inflammatory cytokines IL-10 synthesized by ED1 positive macrophages during MI-induced cardiac injury. Double-immunofluorescence staining of AT2 receptor and IL-10 showed a co-localization of AT2 receptor with IL-10 in the peri-infarct myocardium 7 days after MI-induced cardiac injury **(Fig.19)**.

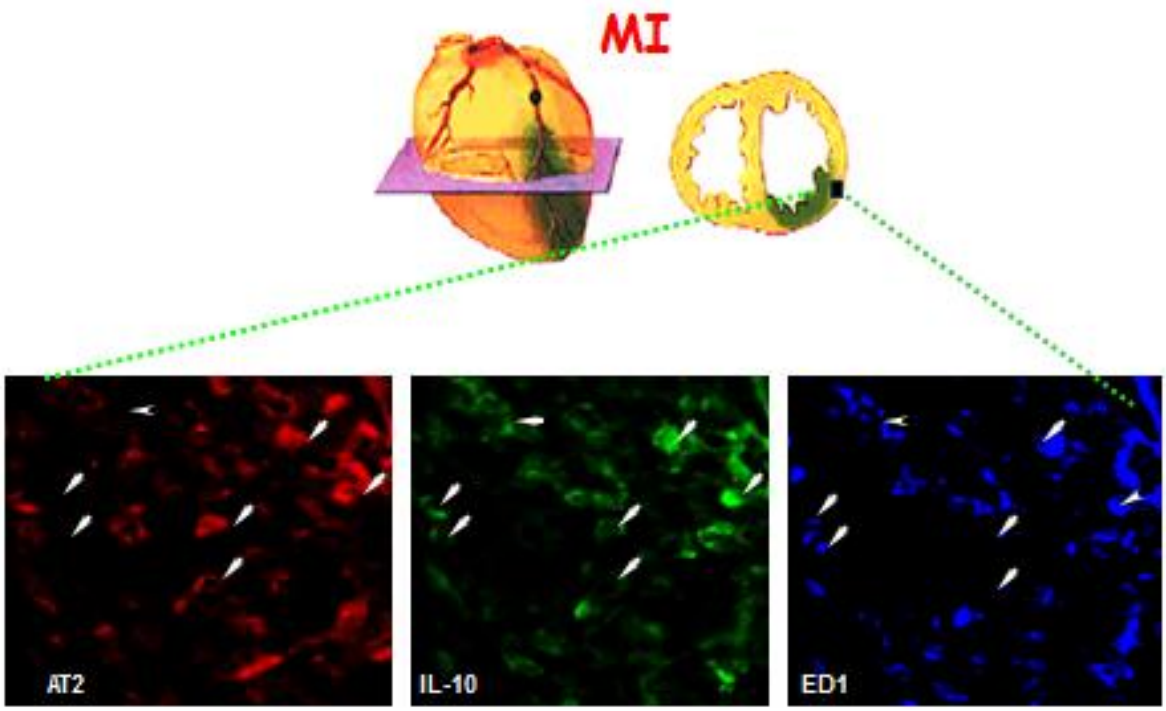


(Fig.19). Double immunofluorescence staining of AT2 receptor and IL-10 in the peri-infarct myocardium 7 days after MI. Upregulated AT2 receptor (red immunofluorescence, arrow head) (left) are located in IL-10 positive cells (green immunofluorescence, arrow head) (middle) and double staining is indicated in the merged image (right).

Magnification = 40X, Bar = 20 μM.

5.11. Triple immunofluorescence staining of AT2 receptor and IL-10 with ED1 during MI

Because we detected co-localization of AT2 receptor with IL-10 or ED1 during MI, we extended our experiments to perform a triple immunofluorescence staining of AT2 receptor, IL-10 and ED1. Indeed ED1+ macrophages infiltrated cardiomyocytes expressed both AT2 receptor and IL-10, 7 days after MI-induced cardiac injury **(Fig.20)**.

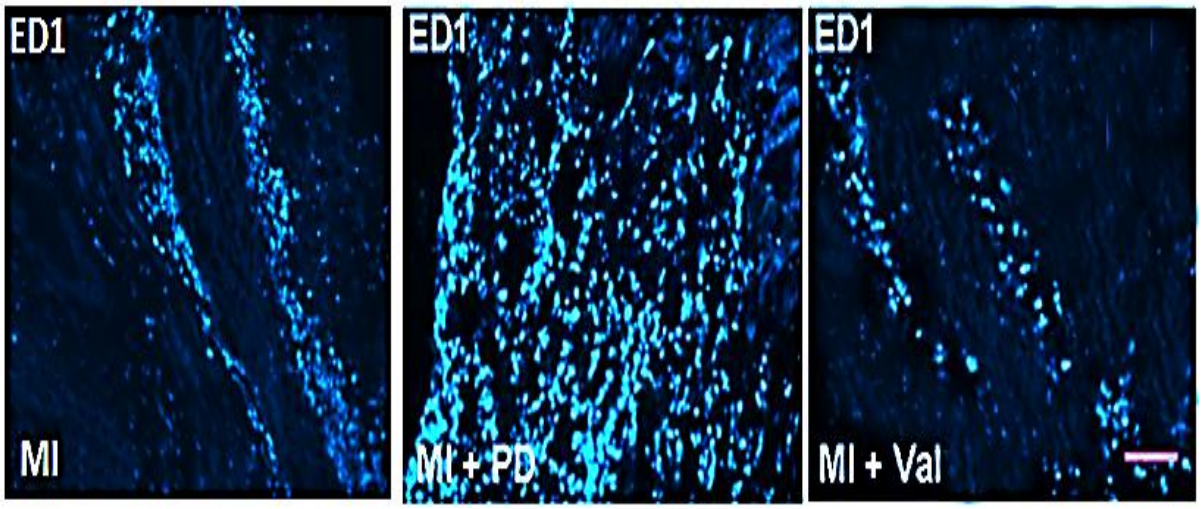


(Fig.20). Triple-immunofluorescence staining of AT2 receptor, IL-10 and ED1 in the peri-infarct myocardium 7 days after MI. Upregulated AT2 receptor (red immunofluorescence; arrow) (left) are located at ED1 positive macrophage (blue immunofluorescence; arrow) (right) expressing IL-10 (green immunofluorescence; arrow) (middle).

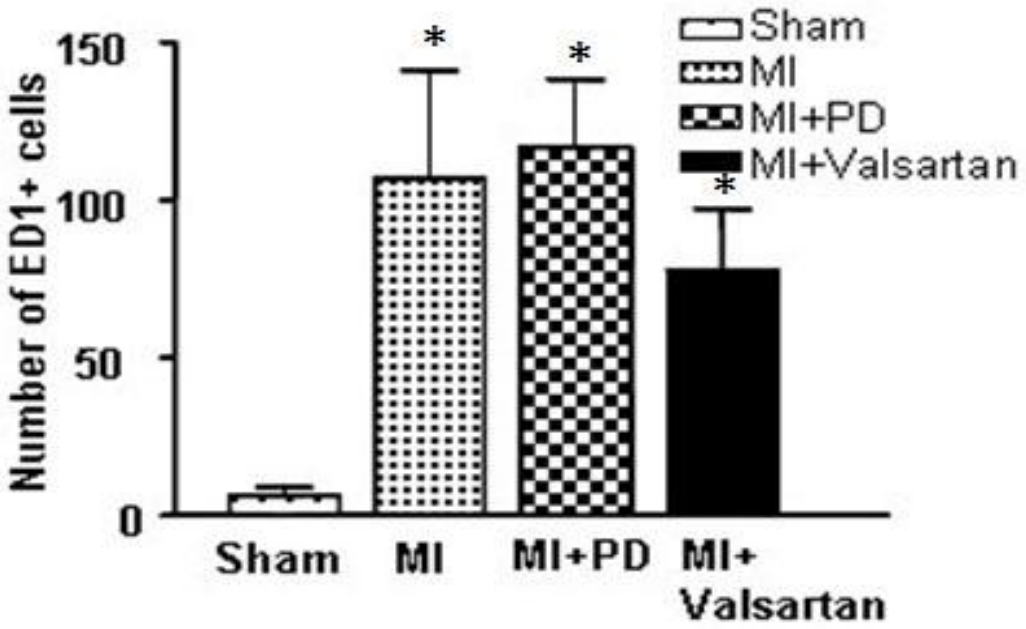
Magnification = 40X, Bar = 10 μM.

5.12. The role of AT1 or AT2 receptor in cardiac inflammatory reaction in rats with MI

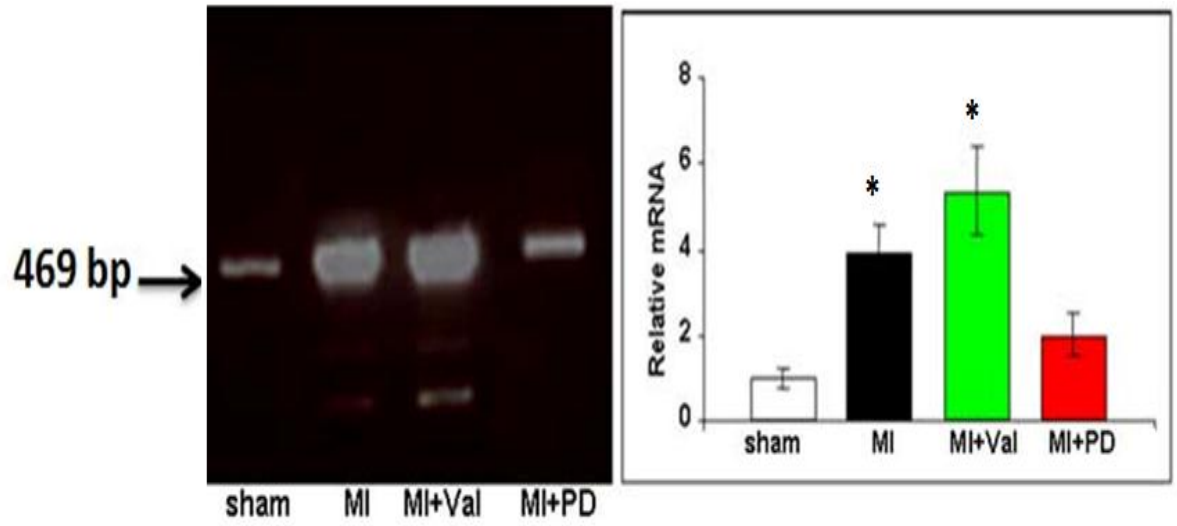
To evaluate the possible role of AT1and AT2 receptors in MI-induced cardiac injury in rats, the AT1 receptor blocker valsartan (MI+Val) or AT2 receptor antagonist PD123319 (MI+PD) were used for treatment. Our results showed that AT1 receptor blockage by valsartan treatment reduced the number of ED1+ macrophages infiltrating cardiomyocytes 7 days after MI. The number of cardiac ED1+ macrophages which was greater in MI than in sham could be reduced by AT1 receptor blocker (MI+VAL) but not by AT2 receptor blocker PD (MI+PD) **(Fig. 21 and 22)**. In addition, we were able to show upregulation of IL-10 as determined by increased levels of mRNA and protein after MI compared to the control. Moreover, this upregulation of IL-10 after MI was reversed by AT2 receptor blocker treatment but not with AT1 receptor blocker Valsartan **(Fig. 23, 24)**. Finally, we are able to show that infarct area was significantly increased in MI compared to control rat and this increase can be reversed by AT1 receptor blocker treatment but not with AT2 receptor blocker PD (MI+PD) **(Fig. 25, 26)**.



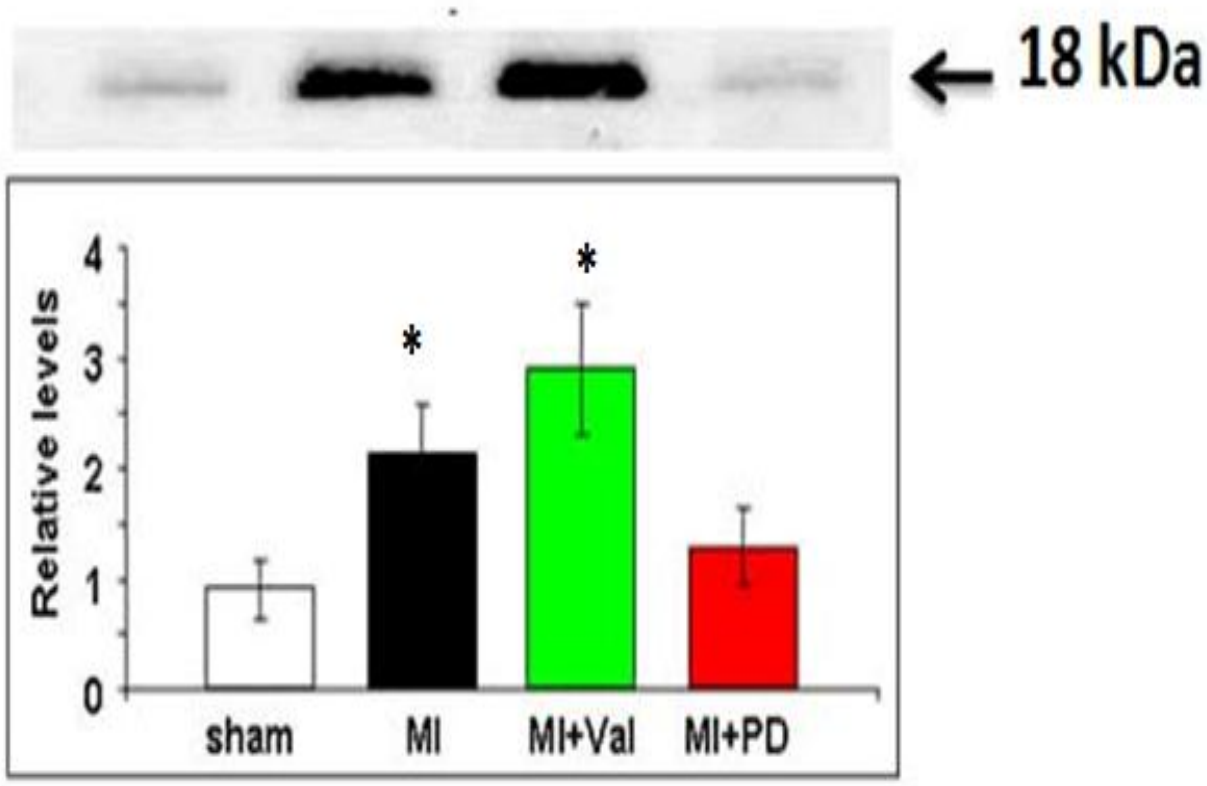
(Fig.21). The effect of AT1 receptor blockage on the number of ED1+ macrophages infiltrating cardiomyocytes were evaluated 7 days after MI. ED1+ macrophages 7 infiltrating cardiomyocytes days after MI in rats treated with saline (MI), AT1 receptor blocker Val (MI +Val) or AT2 receptor antagonist PD (MI+PD). The number of ED1+ macrophages infiltrating cardiomyocytes after MI (left) was apparently reduced by AT1 receptor blocker treatment (MI+Val) (middle) but not with AT2 receptor blocker PD (MI+PD) (middle). Magnification = 20X, Bar = 40 μM.



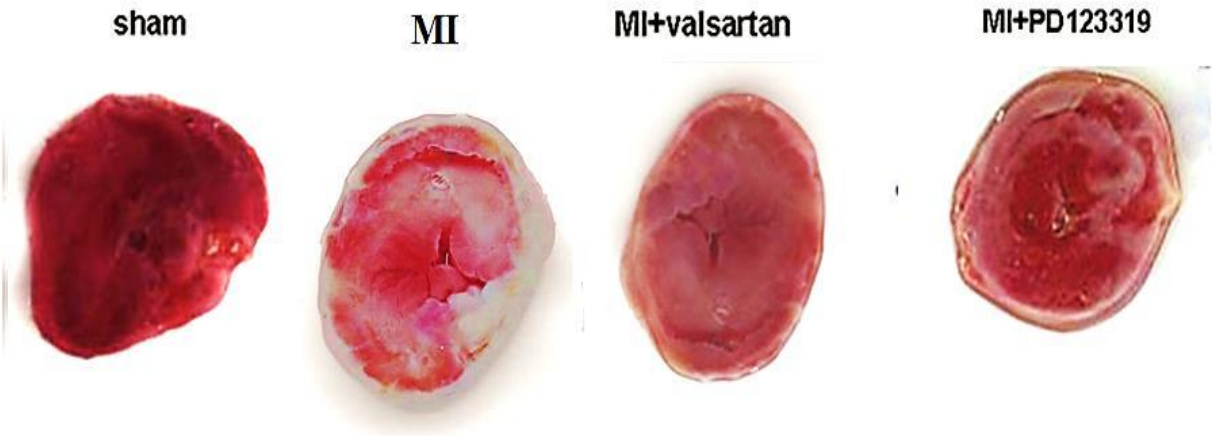
(Fig.22). The effects of AT1 receptor blockage on the number of ED1+ macrophages infiltrating cardiomyocytes were evaluated 7 days after MI. The number of ED1+ macrophages infiltrating cardiomyocytes 7 days after MI in rats treated with saline (MI), AT1 receptor blocker Val (MI +Val) or AT2 receptor antagonist PD (MI+PD) versus sham-operated animals was evaluated. The number of ED1+ macrophages infiltrating cardiomyocytes was significantly increased in MI versus sham-operated animals and this increase was significantly reversed by AT1 receptor blocker treatment but not with AT2 receptor blocker PD (MI+PD) (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-Kramer test, the asterisks denote significant differences compared to sham-operated animals and MI +Val: $P>0.05$.



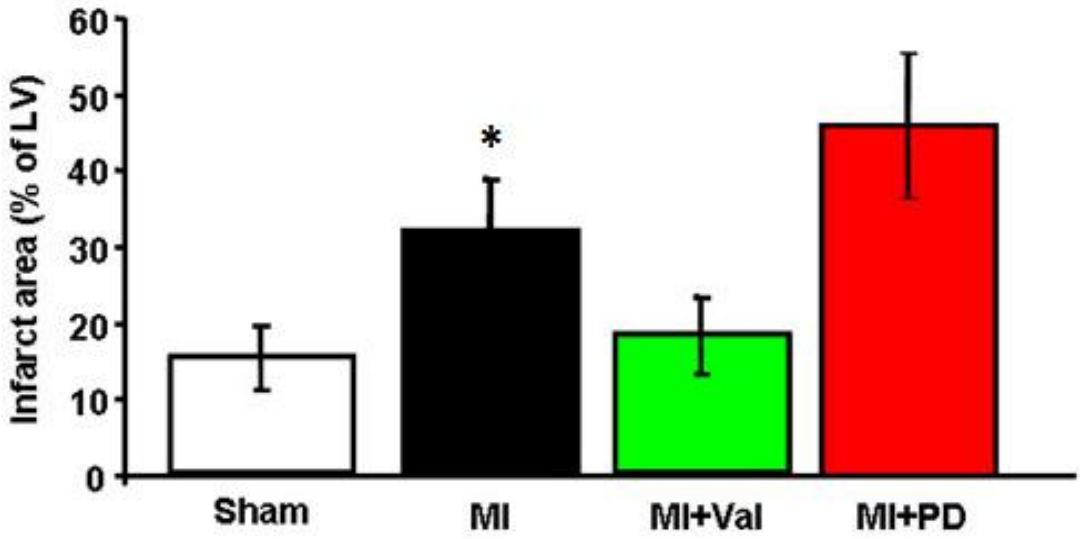
(Fig.23). AT2 receptor Blocker inhibits MI induced upregulation of cardiac IL-10 mRNA level in rats with MI. Cardiac IL-10 mRNA in rats with MI treated with saline (MI) or AT1 receptor blocker valsartan (MI+Val) or AT2 receptor blocker PD123319 (MI+PD) and control rats with sham-operation was measured by RT-PCR. IL-10 mRNA level was significantly increased in MI compared to sham-operated animals and this increase was significantly reversed by AT2 receptor blocker PD123319 treatment but not by AT1 receptor blocker valsartan (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-Kramer test, the asterisks denote significant differences compared to sham-operated animals and MI+PD: $P>0.05$.



(Fig.24). AT2 receptor blockade inhibits MI-induced upregulation of cardiac IL-10 production. protein was analyzed by western blot in rats with MI treated with saline (MI) or AT1receptor blocker valsartan (MI+Val) or AT2 receptor blocker PD123319 (MI+PD) versus sham-operated animals. IL-10 expression is greater in MI than in sham and this can be abolished by AT2 receptor blocker treatment but not with AT1 receptor blocker (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-kramer test, the asterisks denote significant differences compared to sham-operated animals and MI+PD: $P>0.05$.



(Fig.25). Sagittal sections through adult rat heart showing the infarct area in left ventricle of sham-operated animals (sham) or MI rats treated with saline (MI) , AT1 receptor blocker valsartan (MI +Val). Infarct area is greater in MI than that in sham-operated animals and this can be reduced by AT1 receptor blocker (MI +Val) treatment but not with AT2 receptor blocker (MI+PD).



(Fig.26). AT1 receptor blocker Val treatment reversed enlarged infarct size after MI. Infarct area measured in ventricles from 7 days MI rats treated with saline (MI), AT1 receptor blocker Val (MI+Val) or AT2 receptor blocker antagonist PD (MI+PD) versus sham-operated animals. Infarct area is significantly increased in MI than in sham and this significantly reversed compared to sham-operated animals by AT1 receptor blocker treatment but not with AT2 receptor blocker PD (MI+PD). (n= 6). In the statistical analysis using one-way ANOVA followed by multiple pair-wise comparisons of geometric means with alpha-adjustment by Tukey-kramer test, the asterisks denote significant differences compared to sham-operated animals and MI +Val: $P>0.05$.

6. Discussion

MI commonly known as a heart attack occurs when the blood supply to part of the heart is interrupted. This is most commonly due to occlusion (blockage) of a coronary artery. The resulting ischemia (restriction in blood supply) and oxygen shortage can cause damage and/or death (infarction) of heart muscle tissue (myocardium), if left untreated for a sufficient period.

6.1. The possible role of AT1 receptors during experimental MI-induced cardiac injury

In the present study, we used RT-PCR to investigate the changes in AT1 receptor mRNA at 1 day, 7 days and 14 days after experimental MI-induced cardiac injury. Interestingly, RT-PCR analysis revealed that cardiomyocytes AT1 receptor mRNA levels were gradually increased at time intervals from 1 day, 7 days to 14 days after experimental MI-induced cardiac injury. The upregulation of AT1 receptor reflected by the increase in mRNA approach were prominent at 7 days after MI with little change at the end of our experiments (14 days after MI). Therefore, we performed all our experiments including prevention therapy of AT1 receptor mediated MI-induced cardiac injury at 7 days after MI. Consistently with our PCR results, double immunofluorescence staining demonstrated that AT1 receptor immunoraectivity expressed in cardiomyocytes within rat ventricle walls 7 days after MI-induced cardiac injury. The upregulation of AT1 receptor observed in the present study are consistent with the previous observation that the AT1 receptor is the predominant receptor in the adult heart and is considered to be the major mediator of the Ang II induced effects in the cardiovascular system **(52).** After the induction of MI by coronary ligation in rats, a time-dependent increase of AT1 and AT2 receptors mRNA levels in the heart is observed during the acute phase of MI **(11).** It was shown that both AT1 and AT2 receptors gene expression was markedly increased at 30 min and peaked at 24h post MI.

It is well established that apoptosis plays a critical role in normal development and in pathology in a wide variety of tissues **(89)**. Indeed, apoptosis is recognized as one of the mechanisms of cardiomyocyte loss in cardiac ischemia reperfusion injury, MI, and vascular wall remodeling. In vitro studies have demonstrated the capacity of Ang II to induce apoptosis in both neonatal and adult cardiomyocytes via activation of AT1 and AT2 receptors **(53, 68)**. Cardiomyocyte apoptosis may thus contribute to the remodeling that occurs in the transition from compensated hypertrophy to heart failure in hypertensive heart disease **(90,91)**. However, the molecular mechanisms resulting in cardiomyocyte apoptosis after exposure to Ang II remain largely unknown. In the present study, we tested the hypothesis that stimulation of the MI-induced cardiac injury in rat heart by AT1 receptor is accompanied by apoptosis. We delineated the role of AT1 receptors and changes in Bax, Bcl-2, and Caspase 3 activation involved in AT1 receptor-associated apoptosis. Our present study using RT-PCR analysis revealed that cardiomyocytes P53 mRNA levels gradually increased at times intervals from 1 day, 7 days to 14 days after experimental MI-induced cardiac injury and this upregulation was prominent at 7 days. Therefore, we performed all our experiments including prevention therapy of AT1 receptor mediated MI-induced cardiac injury at 7 days after MI. Consistently, we detected a single 53-kDa band corresponding to apoptotic marker P53 in the adult rat ventricle wall using Western blot analysis. Moreover, densitometry analysis of the 53-kDa band demonstrated that MI-induced cardiac injury induced gradual upregulation P53 from 1, 3, and 7 to 14 days versus control animals. Our findings are in agreement with the previous studies by Zhu et al, **(11)** investigated the apoptotic development and apoptotic-related gene expression after MI at different time points using PCR. The authors observed that Bax gene expression was increased at 12 h after MI and peaked at 24 h. Also, Kobara et al., **(92)** showed that apoptotic markers such as P53 were increased in MI using Western blot and immunohistochemical techniques.

It is well established that apoptosis is an important mechanism in the pathogenesis of myocardial injury after MI**.** We aimed to define the relationship of apoptotic markers with AT1 receptors during MI-induced cardiac injury in order to confirm that apoptotic injury is an integral component of AT1 receptor mediated cardiac damage. We have assessed the regulation of AT1 receptor with specific regard with the association with the expression of apoptosis markers such as P53, Bax and Caspase-3 at 7 days after experimental MI in rats by immunohistochemistry. Interestingly, we observed a marked upregulation of cardiac AT1 receptors as well as P53 protein on day 7 after experimental MI. Immunohistochemical staining revealed abundant and heterogeneous distribution of P53 in the cardiomyocytes. Moreover, double immunofluorescence staining of AT1 receptor with apoptotic marker P53 demonstrated a co-localization of P53 protein and AT1 receptor in the same rat injured cardiomyocytes 7 days after MI-induced cardiac injury. These present finding received strong support from the previous studies by Kossmehl et al., **(93)** showed that P53 proteins were increased in MI hearts and further elevated by Ang II suggesting that AT1 receptor P53 plays a key role in the pathogenesis of cardiac diseases such as MI. In this study, we also investigated whether another apoptotic marker Bax and Caspase-3 were upregulated during MI-induced cardiac injury. In deed our results showed that Bax and Caspase-3 were mainly located in the area between the border-zone and necrotic lesion and the area surrounding the necrotic lesion, respectively. Our findings are consistent with the previous studies by Zhu et al., investigated the apoptotic development and apoptotic-related gene expression after MI at different time points using PCR. The authors observed that Bax and Caspase-3 genes expression was increased at 12 h after MI and peaked at 24 h. Also, Kobara et al., **(92)** showed that apoptotic markers such as Bax and Caspase-3 were increased in MI using Western blot and immunohistochemical techniques. Although little is known of the exact molecular mechanisms controlling apoptosis in cardiac muscle, it has been suggested that

members of the Bcl-2 family may be general mediators of apoptosis **(94).** The Bcl-2 gene family consists of more than 15 members. They can be classified as anti-death or pro-death. Bcl-2 is a prototype for an anti-death or survival factor, whereas Bax accelerates the apoptotic process. Over expressed Bax also counters the death repressor activity of Bcl-2 **(95,96)**. These proteins appear to dimerize with themselves or each other through Bcl-2 homology domains and thereby determine the susceptibility of the cell to induction of apoptosis. Thus the ratio of Bax to Bcl-2 determines death or survival after an apoptotic stimulus. Additional factors involved in apoptotic regulation include cysteine proteases belonging to the ICE/CPP32 family, now called Caspase-3, also referred to as CPP32, YAMA, or Apo pain **(97)**.

Caspase-3 is expressed in cells as an inactive 32-kDa precursor. During apoptosis, the 32-kDa Caspase-3 proenzyme is first cleaved to release a 12-kDa fragment and an inactive, intermediate 20-kDa cleavage product consisting of a 3-kDa pro domain plus a 17-kDa subunit. Removal of the 3-kDa pro peptide from the 20-kDa peptide generates the 17-kDa mature, active form associated with Caspase-3 activity **(98,99)**. One molecular mediator of hypoxia-induced apoptosis is the tumor suppressor P53.

P53 is a transcription factor that affects cell cycle arrest or apoptosis in response to a variety of genotoxic and physical stresses **(100,101)**. Apoptosis may be elicited, in part, by P53 stimulated transcription of the pro apoptotic gene Bax 3 **(102,103,104)**. Protein levels of P53 increase significantly in response to oxidative stress **(105)** and hypoxia **(35).** It has been shown that, P53 is induced by hypoxia in cultured neonatal cardiomyocytes and overexpression of P53 in normoxic myocytes leads to apoptosis **(106)**. Apoptosis may play an important role in cardiac remodelling after MI.

6.2. The AT1 receptor mediate apoptosis after MI

In order to evaluate the influence of AT1 receptor blocker Valsartan versus AT2 receptor blocker PD (MI+PD) on the apoptotic markers such as P53, Bax and Caspase-3 and infarct area in rats with MI, we used western blot and morphological techniques. Interestingly, we are able to show that upregulated P53, Bax and Caspase-3 as indicated with increased levels of P53, Bax and Caspase-3 proteins in MI compared to control can be reversed to that of control by AT1 receptor blocker treatment but not with AT2 receptor blocker PD. Accordingly, P53 is likely to play a regulatory role in ischemia-related cardiomyocyte death as well. Matsusaka et al., **(107)** examined the effects of target deletion of the P53 gene on post-MI hearts. They conclude that P53 is involved in cardiac rupture after MI, probably via the induction of a pro apoptotic pathway. Interestingly, the inhibition of P53 may be a potentially useful therapeutic strategy to manage post-MI patients. Indeed, Nakajima et al., **(108)** suggest that antagonization of P193 and P53 activity relaxes the otherwise stringent regulation of cardiomyocyte cell cycle re-entry in the injured adult heart. In accord with our present findings showing the reduction of apoptotic markers by AT1 receptor blockage, Kanamori et al., **(109)** showed that blockade of AT1 receptor signalling attenuates heart failure following MI, perhaps through reduction of fibrosis in the non-infarcted myocardium. The authors showed that ten days post-MI, apoptosis among granulation tissue cells was significantly suppressed in the Olmesartan-treated hearts, where expression of Fas, Bax, and Caspase-3 were all significantly attenuated. They also suggested that Olmesartan exerts a negative regulatory effect on the alternate pathway downstream of Fas, Bax, procaspase-3 and proposed that the anti-apoptotic effect is important mechanism for an AT1 receptor blocker in improving post-MI ventricular remodelling, as well as its anti-fibrotic effect. Fortuno et al., **(110)** studied the effect of AT1 receptor blockade by Losartan on cardiac apoptosis in Ang II induced hypertension.

The authors showed that protein expression of AT1 and AT2 receptors accompanied with an increased rate of Bax and Caspase-3 mediated apoptosis was significantly increased in Ang II-infused rats compared with control rats. Moreover, the authors added that AT1 receptor antagonist Losartan treatment was able normalized apoptosis, the level of Bax, Caspase-3 activity, and AT1 receptors. Ang II stimulation of AT1 receptor in the heart in vivo is associated with an increased rate of apoptosis without major hemodynamic consequences. Bax and Caspase-3 are involved in the apoptotic signaling pathway in this experimental paradigm.

Finally, we are able to show that infarct area is markedly increased in MI compared to control rat and this increase can be reversed by AT1 receptor blocker treatment but not with AT2 receptor blocker PD (MI+PD). These present findings are consistent with the previous studies by Kanamori et al., **(109)** a demonstrated that after MI induction in mice, treatment with the AT1 receptor blocker Olmesartan, beginning on the third day post-MI, significantly improved survival (94%) 4 weeks post-MI, compared with saline (53%) and hydralazine (73%). The authors observed also that Olmesartan-treated mice also showed significant attenuation of left ventricular dilatation and dysfunction, as well as significantly greater infarct wall thickness, although the absolute size of the infarct scar was unchanged. In addition, significantly greater numbers of nonmyocytes (mainly vascular cells and myofibroblasts) were present within the infarct scar in Olmesartan-treated hearts. Our present results are also in agreement with the previous studies by Leri et al., **(53)** showed that physical forces such as stretch activate apoptosis and gene expression, of AT1 receptor, P53, and Bax increased and Bcl-2 decreased in stretched myocytes. Additionally, the AT1 receptor blocker, losartan, abolished apoptosis in stretched cardiomyocytes.

6.3. The possible role of AT2 receptor during experimental MI-induced cardiac injury

Our present study using RT-PCR analysis demonstrated gradual upregulation of AT2 receptor mRNA in ventricle wall from 1 day, 3 days, and 7 days till 14 days after experimental MI-induced cardiac injury. Consistently, our immunohistochemical studies showed a dramatic increase in AT2 receptor immunoreactivity on non cardiomyocyte structures resembling immuocytes infiltrating adult rat ventricle wall 7 days after myocardial infarction-induced cardiac injury. Furthermore, double immunofluorescence staining demonstrated a differential cellular regulation of AT1 and AT2 receptors immunoreactivity in adult rat ventricle wall 7 days after MI-induced cardiac injury. Consistently, our experiments showed expression of AT1 receptor immunoraectivity in cardiomyocytes but AT2 receptor was expressed in non cardiomyocytes such as immune cells including macrophages within rat ventricle walls 7 days after MI-induced cardiac injury. Our findings are consistent with the previous observation that the AT1 receptor is the predominant receptor in the adult heart and is considered to be the major mediator of the Ang-II induced effects in the cardiovascular system **(52).** In contrast, AT2 receptor activation has been reported to inhibit cell proliferation induce differentiation **(18)**, apoptosis **(56)** and regeneration **(69)**.

Ang II has been implicated in ventricular remodelling after MI. After the induction of MI by coronary ligation in rats, a time-dependent increase of AT1 and AT2 receptors mRNA levels in the heart is observed during the acute phase of MI **(11)** It was shown that both AT1 and AT2 receptors gene expression was markedly increased at 30 min and peaked at 24h post MI. The data demonstrated that cardiac left ventricular AT1 and AT2 receptors genes expression are transiently increased after MI and the increased angiotensin II receptor density may serve as an enhancer of the acute cardiac effects of angiotensin peptides post MI and be instrumental in initiating the remodelling phase. Recent investigation, using a single-cell reverse transcriptase-polymerase chain reaction, demonstrated that approximately 40% of

adult rat cardiomyocytes expresses AT1 receptor and approximately 10% expressed AT2 receptor **(14)** These proportions were unchanged after the induction of acute myocardial infarction at day 1. At day 7 after infarct, however, AT2 receptors were expressed in 50% of cardiomyocytes. Accumulating evidence suggests that the inflammatory response is a key component of the structural deterioration associated with post-MI LV remodelling **(63), (111, 112)**. The progressive extension of border zone myocardium to normally perfused myocardium during the chronic phase of MI leads to late-phase HF following MI **(113).** In consistent with our present finding, the inflammatory cells, macrophages are present in highest numbers and for longest period in the border zone and infarcted regions **(114).** Macrophages play a central role in the pathogenesis of fibrosis, thus it is plausible that persistent macrophage infiltration might contribute to the expansion of myocardial fibrosis in the border zone region, leading to progressive LV dysfunction. Therefore, we aimed to examine weather non myocardial structures containing AT2 receptor are macrophages in order to study the possible role of macrophage in AT2 receptor mediating MI-induced cardiac injury and weather there is correlation between upregulation of AT2 receptor and number of ED1 infiltrating rat ventricle wall during MI-induced cardiac injury. We performed these experiments especially at 7 days because the upregulation of AT2 receptor reached a prominent value at this time point. Indeed, double immunofluorescence staining clearly demonstrated that AT2 receptor expressed on ED1 positive cells indicating that AT2 receptor upregulated on macrophages infiltrating rat ventricle walls 7 days after MI-induced cardiac injury. The inflammation in cardiovascular diseases is associated with the activation of a variety of cells including lymphocytes, monocyte/macrophage, endothelial cells, smooth muscle cells, and cardiomyocytes, which express and secrete pro inflammatory cytokines and chemokines **(63).** These cytokines can modulate cardiac function and cardiovascular remodelling.

Various cytokines were increased after MI, and the increases of TGF-, MIP-1, IP-10, and MCP-1 were more prominent in wild mice compared with Ang II type1a mice. These results suggest that Ang II is involved in production of various cytokines after MI, which induce post-infarcted cardiac remodelling including impaired cardiac function and increased fibrosis **(115).** Therefore, we extended our experiments to investigate the possible involvement of macrophage expressing cytokines such as IL-10 in myocardial infarction-induced cardiac injury. Double immunofluorescence staining clearly demonstrated that IL-10 expressed on immune cells indicating that IL-10 upregulated on macrophages infiltrating rat ventricle walls 7 days after MI-induced cardiac injury. Since we able to show an increase in ED1 positive macrophages containing AT2 receptor, we extended our experiments to investigated the possible involvement of cytokines IL-10 synthesized by ED1 positive macrophage in MI-induced cardiac injury. Indeed, triple immunofluorescence staining showed that ED1 positive macrophage infiltrating rat ventricle walls 7 days after myocardial infarction-induced cardiac injury express AT2 receptor and cytokine IL-10. The release of an anti-inflammatory cytokine IL-10 is important in cardiac injury. IL-10 may play a protective role by suppressing the production of pro inflammatory cytokines **(116).** IL-10 also exerts its cardio protective function by inhibiting neutrophil-endothelial interaction **(117).** Recently, Curato et al., **(118)** identified the non-cytotoxic and IL-10 producing CD8, AT2 receptor, T cell population, increasing after ischemic heart injury. Moreover, the authors demonstrated that AT2 receptor activation enhances cardio protective CD8 + AT2 receptor + T cells and IL-10 production in the infarcted myocardium confirming the protective role of AT2 receptor-during cardiac infraction by reducing inflammatory injury in the heart.

6.4. The effect of MI-induced cardiac injury mediated by upregulated AT2 receptor after MI

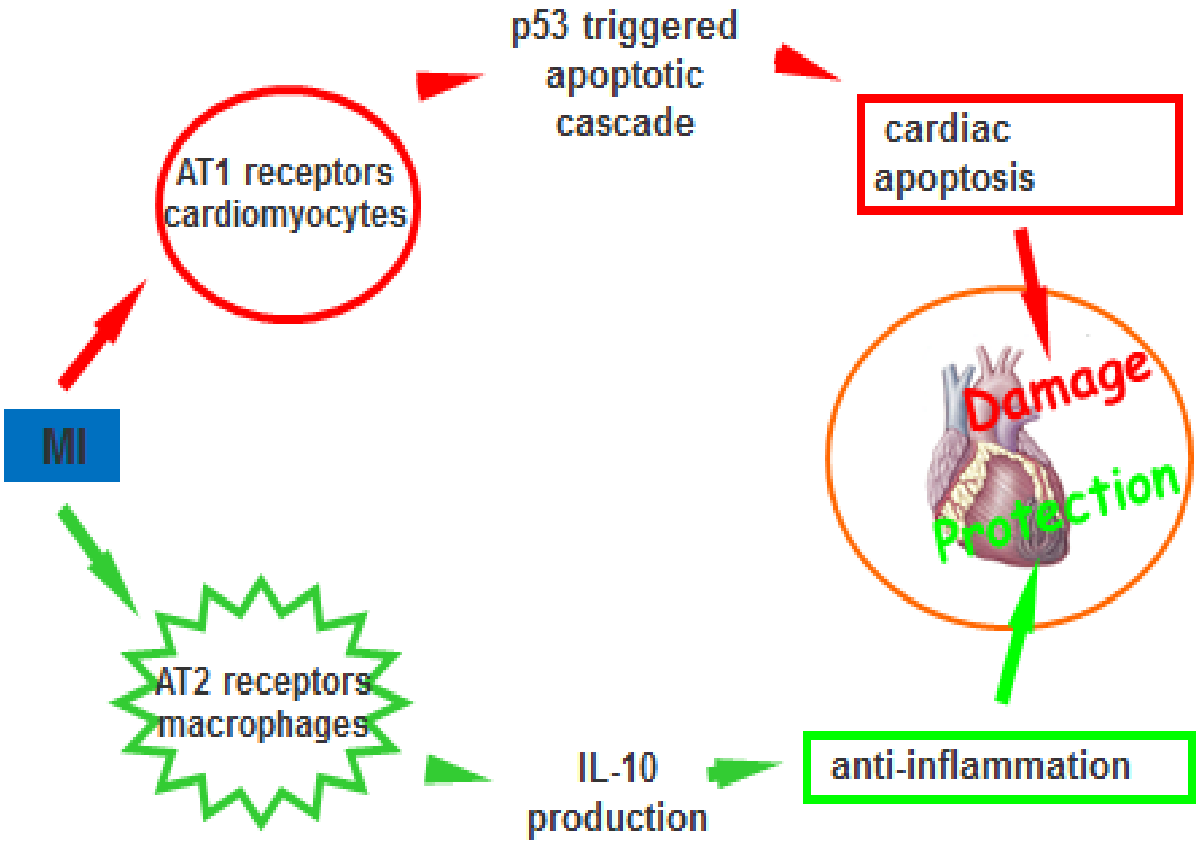
In the present study we evaluated the influence of AT1 receptor blocker Valsartan (Val) versus AT2 receptor antagonist PD123319 (PD) on the number of macrophages, IL-10 mRNA, IL-10 protein, IL-1ß , IL-2 and infarct area in rats with MI using different techniques such as western blot, PCR and immunohistochemical techniques. Interestingly, AT1 receptor but not AT2 receptor blockage reduces the number of cardiac ED1 macrophages infiltrating adult rat ventricle wall after 7 days of MI-induced myocardial injury. The prevention effects of MI-induced cardiac injury mediated by upregulated AT2 receptor by AT1 receptor blocker Valsartan is consistent with the previous study by kohno et al., **(119)** showing the reduction macrophage infiltration in the border zone of rat with MI, resulting in less myocardial fibrosis.

AT1 receptor plays a critical role in inflammatory cell infiltration, cytokine production, and neovascularization in infarcted hearts **(115).** In deed, we are able to show that upregulated IL-10 as reflected with increased levels of mRNA and protein in MI compared to control can be reversed to that of control by AT2 receptor blocker treatment but not with AT1 receptor blocker Valsartan. In contrast to the prevention of IL-10 and IL-1ß upregulation by blocking AT2 and AT1 receptors respectively, IL-2 expression remains unchanged in MI.

7. Conclusion

Our main aim was to investigate the cellular regulation of cardiac AT1 and AT2 receptors and their potential roles in response to MI in rats.

In the first part we showed that upregulation of AT1 receptor on cardiomyocytes in response to MI in rats was concomitant with enhanced expression of apoptotic factors such as P53, Bax and Caspase-3 on cardiomayocytes. AT1 receptor blocker treatment suppressed MI induced upregulation of cardiac apoptosis markers including P53, Bax and caspase-3 **(Fig. 15 and 16)**. Interestingly, we found that blocking AT2 receptor increase but blocking AT1 receptor suppressed MI-induced upregulation of cardiac apoptosis markers including P53, Bax and Caspase-3, reduce Enlarged Infarct Size and consequently prevent myocardial injury after MI. These results indicate that over expressed AT1 receptors may participate in triggering cardiomyocyte apoptosis by inducing P53 mediated apoptotic cascade after experimental MI **(Fig 10).** In the second part we found that AT2 receptors are mainly localized in infiltrated ED1 macrophages expressed IL-10 production in response to MI-induced myocardial injury. Interestingly, AT1 receptor blockage by specific blocker VAL treatment to rat with MI inhibit inflammatory reaction as indicated with reversed the increased number of ED1 macrophages infiltrating cardiomyocytes, upregulation in cardiac IL-10 and enlarged infarct area in MI. In contrast, AT2 receptor blocker PD treatment to rats with MI have no effect on inflammatory reaction as indicated with no change in the increased number of ED1 macrophages infiltrating cardiomyocytes, upregulation in cardiac IL-10 and enlarged infarct area in MI. These results indicate that blocking upregulation of AT1 receptor after MI-induced myocardial injury protects against inflammation-induced cardiac injury by IL-10 Production in ED1 macrophages.



(Fig. 27). Overexpressed AT1 receptors participate in triggering cardiomyocyte apoptosis by inducing P53-mediated apoptotic cascade after experimental MI.

8. Summary

Firstly, the study was designed to assess the role of the angiotensin AT1 receptor subtype 1 (AT1) and receptor subtype 2 (AT2) in apoptosis and inflammatory processes following myocardial infarction in vivo, as the data available suggests that Angiotensin II (Ang II) induces apoptosis via AT1 and AT2 receptors.

Ang II, the most important effector peptide of the renin-angiotensin system, mediates its effects through two main receptor subtypes, namely AT1 and AT2 receptors. Data from ex vivo studies indicates that the AT2 receptor mediates apoptotic effects. We have previously shown that both AT1 and AT2 are expressed in adult rat cardiomyocytes and in response to myocardial infarction-induced cardiac injury. However, it is currently unknown whether angiotensin receptors mediate apoptosis in cardiomyocytes in vivo. We have assessed the regulation of the AT1 receptor in association with the expression of apoptosis markers such as P53, Bax and Caspase-3 on day 1, day 3, day 7 and day 14 following experimental myocardial infarction in rats by Western Blot and/or immunohistochemistry. In addition, the colocalization of angiotensin receptors and apoptosis or cardiomyocyte markers were evaluated by immunofluorescence double labeling. We detected a marked upregulation of the cardiac AT1 receptor as well as P53 protein on day 7 after experimental myocardial infarction. Immunohistochemical staining revealed abundant and heterogeneous distribution of P53, Bax and Caspase-3 in the myocardium. The strongest staining of P53 was observed among injured cardiomyocytes at the border zone of the infarction. By contrast, Bax and Caspase-3 were mainly located in the area between the border zone and the necrotic lesion and the area surrounding the necrotic lesion, respectively. Also, we demonstrated a marked upregulation of cardiac AT2 receptors co-localized with ED1 positive macrophages infiltrating injured cardiomyocytes around the infarct areas.

Furthermore, our triple immunofluorescence labeling demonstrated a colocalization of AT2 receptors with IL-10 within ED1-positive macrophages infiltrating injured cardiomyocytes, suggesting a possible role of IL-10 and AT2 receptors in the inflammatory development after myocardial infarction.

Secondly, the study sought to investigate the role of AT1 and AT2 receptors in apoptosis and inflammation following myocardial infarction. We therefore investigated the influence of a specific AT1 and AT2 receptor antagonists on apoptotic markers, cytokine production and infarct size of the heart after myocardial infarction. Notably, AT1 receptor inactivation by the specific blocker valsartan treatment in rats with myocardial infarction suppressed myocardial infarction-induced upregulation of cardiac apoptosis markers including p53, Bax and Caspase-3, inhibited inflammatory reaction as indicated with reversed the increased number of ED1 macrophages infiltrating cardiomyocytes, upregulation in cardiac IL-10 and enlarged infarct area in myocardial infarction. In contrast, no effect on apoptotic process, inflammatory reaction or enlarged infarct area in myocardial infarction was observed in rats treated with PD to block AT2 receptor.

8.1. Objectives

1. Investigation of apoptosis and inflammatory reaction in association with the expression of AT1 and AT2 receptors in rats following acute myocardial infarction.

2. Comparison of the effects of the specific AT1 receptor antagonist (valsartan) and the AT2 receptor antagonist (PD123319) administrated at different doses on apoptosis, inflammatory reaction, cardiac function and infarct size in rats following acute myocardial infarction.

9. Zusammenfassung

Die Rolle von Angiotensin AT1 und AT2 Rezeptoren in der Entwicklung von Apoptose und Entzündung nach einem experimentellen Myokardinfarkt

Die Studie wurde konzipiert, um die Rolle des Angiotensin AT1 Rezeptor-Subtyp 1 (AT1) und Subtyp 2 (AT2) Rezeptor in der Apoptose und im Entzündungsprozess nach einem Myokardinfarkt in vivo zu bewerten, da Veröffentlichungen darauf hinwiesen, dass Angiotensin II (Ang II) Apoptose via AT1 und AT2 Rezeptoren veranlasst. Ang II, das wichtigste Effektor-Peptid des Renin-Angiotensin-Systems, übt seine Wirkung durch zwei Rezeptor-Subtypen aus, nämlich den AT1 und den AT2 Rezeptor. Daten von ex vivo Studien wiesen darauf hin, dass AT2 Rezeptoren einen apoptotischen Effekt haben. Es ist bereits nachgewiesen, dass sowohl AT1 Rezeptoren als auch AT2 Rezeptoren in Kardiomyozyten von adulten Ratten und als Antwort auf Myokardinfarkt induzierte Herzschädigung exprimiert werden. Es ist jedoch zurzeit noch unbekannt, ob Angiotensin Rezeptoren in vivo eine Apoptose in Kardiomyozyten auslösen. Wir haben die Regulation von AT1 Rezeptoren in Verbindung mit der Expression von Apoptosemarkern, wie P53, Bax und Caspase-3, am Tag 1, Tag 3, Tag 7 und Tag 14 nach einem experimentellen Myokardinfarkt in Ratten mittels Western-Blot und/oder Immunhistochemie untersucht. Darüber hinaus wurden die Co-Lokalisierung von Angiotensin Rezeptoren und Apoptose- bzw. Kardiomyozytenmarkern durch Immunfluoreszenz Doppelmarkierung ausgewertet. Wir fanden eine deutliche Hochregulierung von Herz AT1 Rezeptoren als auch P53 Protein am Tag 7 nach einem experimentellen Myokardinfarkt. Immunhistochemische Färbung ergab reichliche und heterogene Verteilung von P53, Bax und Caspase-3 im Myokard. Die stärkste Färbung von P53 wurde in verletzten Herzmuskelzellen an den Grenzzonen des Infarkts beobachtet. Im Gegensatz dazu wurden Bax und Caspase-3 hauptsächlich im Bereich zwischen der

Grenzzone und der nekrotischen Läsion beziehungsweise der Umgebung der nekrotischen Läsionen festgestellt. Außerdem haben wir eine deutliche Hochregulierung des AT2 Rezeptors am Herzen mit ED1 positiven Makrophagen nachgewiesen, die verletzte Kardiomyozyten um die Infarktbereiche infiltrieren. Darüber hinaus zeigte eine Dreifach-Immunfluoreszenzfärbung eine Co-Lokalisation von AT2 Rezeptor mit IL-10 innerhalb der ED1 positiven Makrophagen, die ebenfalls verletzte Herzmuskelzellen infiltrieren. Dies könnte auf eine mögliche Rolle von IL-10 und AT2 Rezeptoren in der Entzündungsentwicklung nach einem Myokardinfarkt hinweisen.

Des Weiteren wollten wir in dieser Studie die Rolle der AT1 und AT2 Rezeptoren in der Apoptose und Entzündung nach Myokardinfarkt hinterfragen. Wir untersuchten daher den Einfluss von spezifischen AT1 und AT2 Rezeptor Antagonisten auf apoptotische Marker, Zytokin Produktion und die Infarktgröße von Herzen nach Myokardinfarkt. Wichtig ist zudem, dass die AT1 Rezeptor Inaktivierung bei Ratten mit Myokardinfarkt durch die Behandlung mit dem spezifischen Blocker Valsartan auch die Myokardinfarkt-induzierte Hochregulation der Herzapoptosemarker einschließlich P53, Bax und Caspase-3, unterdrückte. Die Behandlungen inhibierten außerdem die entzündliche Reaktion wie beschrieben mit einer Umkehr der erhöhten Anzahl von Kardiomyozyten infiltrierenden ED1 Makrophagen und einer Hochregulierung des kardialen IL-10 und eines vergrößerten Infarktbereichs in MI. Im Gegensatz dazu wurde keine Wirkung auf den apoptotischen Prozess, die entzündliche Reaktion oder den vergrößerten Infarktbereich in Myokardinfarkt bei Ratten beobachtet, die mit PD behandelt wurden um den AT2 Rezeptor zu blockieren.

10. References

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13. Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass die Dissertation von mir selbst und ohne die unzulässige Hilfe Dritter verfasst wurde, auch in Teilen keine Kopie anderer Arbeiten darstellt und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind.

Berlin, den10.04.2014

Kamal Elkhrbash