2. MATERIALS AND METHODS

2. 1 Tissue culture

2. 1. 1 Isolation of cardiomyocytes from neonatal rat hearts

Neonatal rat ventricular cardiomyocytes were isolated as previously described (Klein et al., 1982) with some modifications. In brief, whole hearts were rapidly removed from 2-to 4-day-old Sprague-Dawley rats (Charles Rivers, Sulzfeld, Germany) and placed in ice-cold Ca^{2+}-and Mg^{2+}-free Hank's buffer salt solution (HBSS), supplemented with 20 mM N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid (HEPES), pH7.4. The hearts were washed two times with HBSS, the atria and aorta were removed and discarded. The ventricles were minced with scissors into 1mm³ fragments, washed again with HBSS for another two times. The tissue was then dissociated in HBSS at 37° by a combination of mechanical agitation and enzymatic digestion with 0.125% trypsin-EDTA (trypsin- ethylendiaminetetraacetic acid). After 15 min, the supernatant was discarded and the tissue fragments were replaced with fresh HBSS. Subsequently, for each of 4 to 5 15 min digestion periods, the liberated cells were collected in 20% ice cold fetal bovine serum (FBS), concentrated by centrifugation at 600×g for 10 min. Cell pellet was resuspended and restored in a DMEM (Dulbecco's modification of Eagle's minimum essential medium) containing 50% FBS (DMEM- FBS 1:1 mixture). The pooled cells from above procedures were collected in one tube and centrifuged again. Cells were then resuspended in DMEM with 20% FBS. Dissociated cells were enriched for cardiomyocytes (CMC) by the technique of differential adhesion: cells were seeded in one plastic culture flask, incubated at 37°C under 5% CO_2 in air to enable nonmyocytes (mainly fibroblasts) to attach to the bottom of the flask. After 1 hour storage, floating cells were collected by decanting and transferred into a fresh flask. The cells were then diluted with fresh DMEM with 20% FBS. CMC at density of 1×10^6/25 cm² culture area were plated, DMEM containing 20% FBS and 100 U/ml penicillin/streptomycine was used for the first 24 h culture. After 24 h, DMEM containing 10% FBS, 100 U/ml penicillin/streptomycine and 0.1 mM Bromodeoxyuridine (BrdU) was used as culture media. BrdU was added to prevent proliferation of the fibroblasts. Every day cells were changed with fresh media. During

culture, over 80% of the cardiomyocytes beat spontaneously on the second day of culture and synchronously in a confluent monolayer after the 4th day of culture. This procedure yielded cultures with more than 90% myocytes, as assessed by microscope observation of cell beating. Experiments were performed on the 4th day of cell culture.

2. 1. 2 Isolation of cardiac fibroblasts from neonatal rat hearts

Highly enriched cultures of cardiac fibroblasts were prepared by 2 passages of cells adhered to the culture flask during the preplating procedure, by 0.025% trypsin-EDTA treatment (see chapter 2.1.1.2 for cell passage procedure). These cells, which divided rapidly, represented cells from cardiac interstitium. After passaging two times, these cultures appeared morphologically homogenous and were presumed to be fibroblasts. Cells were grown in DMEM media added by 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin in 10 cm tissue culture dishes in a humidified atmosphere with 5% CO_2 at 37°C. Every second day cells were changed with fresh media.

2. 1. 3 Rat aortic smooth muscle cell culture

Rat aortic smooth muscle cells were obtained as a kind gift from Dr. Concha Peiró (Madrid, Spain). Smooth muscle cells were grown in Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin in 10 cm tissue culture dishes in a humidified atmosphere with 5% CO_2 at 37°C. Cells grew adherently on the bottom of the dishes. Every second day cells were changed with fresh medium. Cells were used between passage 15 and 20 in all of the studies.

2. 1. 4 Passaging, freezing and thawing of cells

For passaging cells, confluent cells were washed with phosphate buffered saline (PBS) and then treated with 1 ml 0.025% trypsin-EDTA solution in a 10 cm dish for 5 min at 37°C. The treatment with trypsin-EDTA solution lasted less than 10 min because of its cell toxic effect. After trypsinization, the solution was diluted with 5 ml DMEM with 10% FBS and transferred

to a 15 ml centrifuge tube. The cells were collected by centrifugation at 500×*g* for 5 min and resuspended in DMEM with 10% FBS and counted using a hemocytometer (Neubauer, Merck, Berlin Germany) and a distinct number of cells was distributed into a new flask.

For freezing and thawing of cells, confluent cells were washed with PBS and trypsinized as mentioned above. After centrifugation, 1 ml of freezing solution (FBS, DMEM, DMSO (1:8:1,v/v/v)) was added to the cell pellet and the suspension was frozen. The addition of dimethyl sulfoxide (DMSO) avoided crystal occurrences within the cells. Freezing of cells should be a slow process therefor freezing tubes were firstly frozen for 1h at -20° followed by -80° for two days. Then, the cells were stored in liquid nitrogen. On the contrary, the process of thawing should be as fast as possible. The freezing tube was transferred from the liquid nitrogen into a 37°C water bath for 2 min. Defreezed cells were transferred into a 15 ml centrifuge tube, 10 ml of medium was added to the cells, mixed gently and centrifuged at 500×*g* for 5 min. Then, the pellet was resuspended in 12 ml DMEM with 10% FBS and transferred to a 10 cm culture dish. On the next day, the cells were changed with fresh culture media.

2. 1. 5 IL1ß treatment

2.1.5.1 Time course experiments

In the beginning of this study, a pre-experiment was performed. Cardiomyocytes were treated with IL1ß for 12 hours, the expression of B2R mRNA was analysed and showing no significant difference when compared to untreated samples; The B1R mRNA was dramatically upregulated by 4000 pg/ml IL1ß, but exhibiting a high standard deviation among different groups. It was previously reported that in cultured human embryo lung fibroblasts, when cells were treated with 100 pg/ ml IL1ß, the maximum B1R mRNA level was reached at 2 h post-stimulation. At 8 h post-stimulation, the level of B1R mRNA dropped visibly (Zhou et al., 1998). In another *in vitro* study, 6 h incubation of IMR-90 cells with 500 pg/ml IL1ß resulted in a 4 to 6-fold increase in the density of B1 receptors (Phagoo et al., 1999). Based on these findings, in the present study 3 h, 6 h, and 9 h were selected as time points to perform the time course experiment, the effect of IL1ß on the expression of both B1R and B2R mRNA within

this time scope was investigated. For time course experiments, 32 dishes of cells were prepared. Confluent cell cultures were firstly treated with serum free medium. After 12 h cultivation, 8 dishes were taken out from the CO_2-incubator and total RNA were isolated from cells (0h). 12 dishes were changed again with serum free medium. The other 12 dishes of cells were changed with serum free medium but added with IL1ß (400 pg/ml). Subsequently, after 3 h, 6 h, or 9 h of further cultivation, at each time point 4 dishes of cells from each group (with or without IL1ß treatment) were taken out from the CO_2-incubator and total RNA were isolated. For a Schematic diagram of cell treatments see Fig 2.1.

2.1.5.2 Dose dependency experiment

Dosage used in this experiment was set according to clinical researches. It was reported that in the patients early after myocardial infarction, cytokine IL1ß was highly released with a plasma concentration of 422.3 ± 257 pg/ml (Guillen et al., 1995; Blum, 1996). Thus, 400 pg/ml was used as the middle value of IL1ß treatment, other concentrations was set 10 times higher or lower respectively. For dose dependency experiment, 16 dishes of cells were prepared. Confluent cell cultures were firstly treated with serum free medium. After 12 h cultivation, 4 dishes of cells were taken out and total RNA were isolated (0 pg/ml). The other 12 dishes were changed with new serum free medium containing different concentrations of IL1ß (40, 400, or 4000 pg/ml for every 4 dishes) for further culture. After 3 h of cultivation, total RNA were isolated from cells.

Cell treatments

A). ***Cell lines:***

· Primary culture neonatal rat ventricular cardiomyocytes

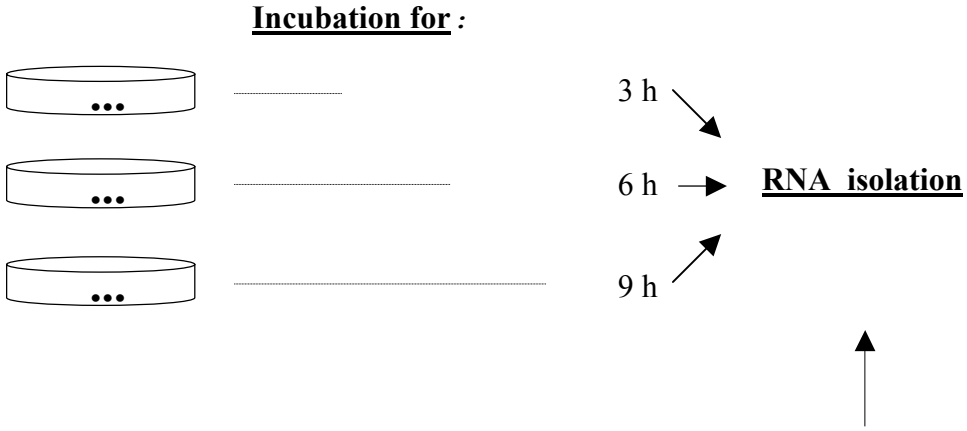
cardiac fibroblasts

· Permanent culture aortic smooth muscle cells

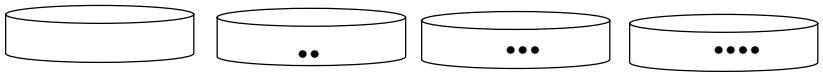
B). ***IL1 ß treatment***:

Cell culture → *12 h serum free medium treatment* → ***RNA isolation***

· Time course experiments**:** *using new DMEM containing 400 pg/ml IL1 ß*



· Dose dependency experiments**:** *using new DMEM containing IL1 ß*



IL1ß: 0 pg/ml 40 pg/ml 400 pg/ml 4000 pg/ml

Fig 2. 1 Schematic diagram of cell treatments

2. 2 Model of myocardial infarction

2. 2. 1 Animals

Experiments were performed in male Sprague-Dawley rats weighing 300-330g (Charles Rivers, Sulzfeld, Germany). The animals were allowed free access to water and standard chow under a 12 h light/dark cycle. The study conformed to the Helsinki Declaration with regard to animal care.

2. 2. 2 Induced myocardial infarction and sham surgery

MI was induced by permanent ligation of the LAD described by Tschöpe et al 1999 (see Immunopharmacology). Briefly, after induction of anesthesia with ketamine (50mg/kg; Parke Davis, Berlin, Germany) and xylasine 2% (5mg/kg; Medistar, Holzwickede, Germany) the rats were intubated and artificially ventilated (Respirator: Ugo basile (Typ 7025), FMI, Seeheim, Germany; respirator rate: 60 breath/min; tidal volume: 6.5 ml/kg (dead space included). Skin and muscles were separated at the left side of the sternum and the third and fourth rib were cut. A rib-spreading chest retractor was inserted, and the LAD was ligated using sterile 6-0 suture material (Ethibond, Ethicon, Norderstedt, Germany) under a stereomicroscope. Left ventricular pressure (LVP) and ECG were monitored continuously during surgery. Successful ligation of the LAD was verified by a drop in LVP and visually by the change in color of the ischemic area. In the rats that underwent sham surgery, ligations were placed beside the LAD. The thoracic cavity was closed by three ligations during inspiration hold, and the muscle and skin layers were sutured. 40% of the animals with MI died within 24h after the procedure.

Three weeks after surgery, rats were again anaesthetised, intubated and artificially ventilated. The thoracic cavity was opened, and the left ventral ribs were partially removed to permit access to the apex of the heart. For messenger RNA (mRNA) analysis of B1R and B2R expression, the hearts were excised and macroscopically separated into left ventricle (LV), right ventricle (RV) and interventricular septum (S), infarcted as well as noninfarcted region of myocardium tissues were isolated and all tissues were rapidly frozen in liquid nitrogen and stored at –80°C.

2. 2. 3 Drugs

IL1ß converting enzyme inhibitor (ICEI) HMR 3480, obtained from Aventis AG (Frankfurt am Main, Germany), was dissolved in a water/cremopho (75% : 25%) solution. One day after MI induction, ICEI was given to the rats at 50 mg /kg/day (n=4) per gavage. For the control group (n=4), only water/cremopho solution was administrated to the rats.

2. 3 RNA isolation

2. 3. 1 Isolation of total RNA from cell cultures

Total RNA was isolated from cultured cells using TRIzol TM reagent (Life Technologies, Eggenstein, Germany). Cells were cultured in tissue culture dishes or flasks, with or without reagent treatment. When extracting RNA, culture media was poured off, cells grown in culture dishes or flasks were washed with PBS and then directly added with TRIzol reagent. 1 ml of TRIzol reagent was given per 10 cm of culture dish area. Cells were lysed by pipetting several times. In this procedure the washing steps was minimised to maintain the RNA yield; the volume of reagents was controlled precisely, as an insufficient amount of TRI Reagent might result in contamination of the isolated RNA with DNA. The cell lysate was then transferred into fresh micro centrifuge tubes. For each tube, 200 µl chloroform were added and the solution was mixed by vortexing for 30 s. After 5 min incubation at room temperature, tubes were centrifuged at 10,000×*g* for 15 min at 4°C. The RNA-containing aqueous phase was transferred into a fresh tube. DNA and proteins were discarded which were present in the phenol and at the interface. Pipetting of any of the white cloudy interface was avoided since it would contain DNA. Then 500 µl isopropanol was added to the tubes to precipitate RNA. Solution was mixed by shaking or vortexing, then centrifuged at 10,000×*g* 4°C for 10 min. The precipitated RNA was washed using 1 ml 75% ice-cold ethanol. After vortexing and subsequent centrifugation at 4°C, RNA products were dried in room temperature for 15 min. Over drying was avoided for it would cause RNA hard to dissolve in water. Obtained RNA pellet was resuspended in 30 µl diethylpyrocarbonate (DEPC) treated water. Heating to 55°C

and shaking would help resuspend the pellet. The concentration and purity of the RNA samples were determined by spectrophotometry at 260 and 280 nm (Spectrophotometer DU 640i, Beckman, California, U.S.A). For this purpose the samples were diluted in order to loose as little as possible. The extinction ratio at 260 nm/280 nm provides information about the purity of the RNA and should be within 1.8 to 2.0. RNA samples were directly immersed in liquid nitrogen and then stored at -80 °C.

2. 3. 2 Isolation of total RNA from tissues

Tissue total RNA was isolated also using TRIzol TM reagent (Life Technologies, Eggenstein, Germany). Heart LV, RV, or S from the animals was homogenized in 1 ml of TRIzol TM reagent per 10–100 mg of tissue using an Ultra-Turrax T25 homogenizer. The homogenate was held for 8-10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform was added and the solution was thoroughly mixed by inversion for 30 seconds. Tubes were stored at room temperature for another 3 min. The homogenate was centrifuged at 4°C 10,000×g for 30 min, and solution in the tubes was separated. The upper phase was transferred into a fresh tube without disturbing the interphase. 0.5 ml of isopropanol was added to each tube and solution was vortexed. After 10 min storage at room temperature, tubes were centrifuged at 10,000×g, 4°C for another 10 min. Supernatant was poured off and the obtained RNA pellet was washed with 1 ml of 75% ice-cold ethanol, briefly dried for 15–20 min, then dissolved in 150 ml of DEPC-treated water. Heating to 55°C and shaking helped to resuspend the pellet. The concentration and purity of the RNA samples were determined by spectrophotometry at 260 and 280 nm. RNA samples were directly immersed in liquid nitrogen and then stored at -80 °C.

2. 4 RNase protection assay

Expression of Bradykinin B1R and B2R mRNA in tissues or in cultured cells was evaluated using the Ribonuclease Protection Assay (RPA) method. Detailed procedures are described as below. A schematic overview of the RPA procedure is shown in figure 2.2.

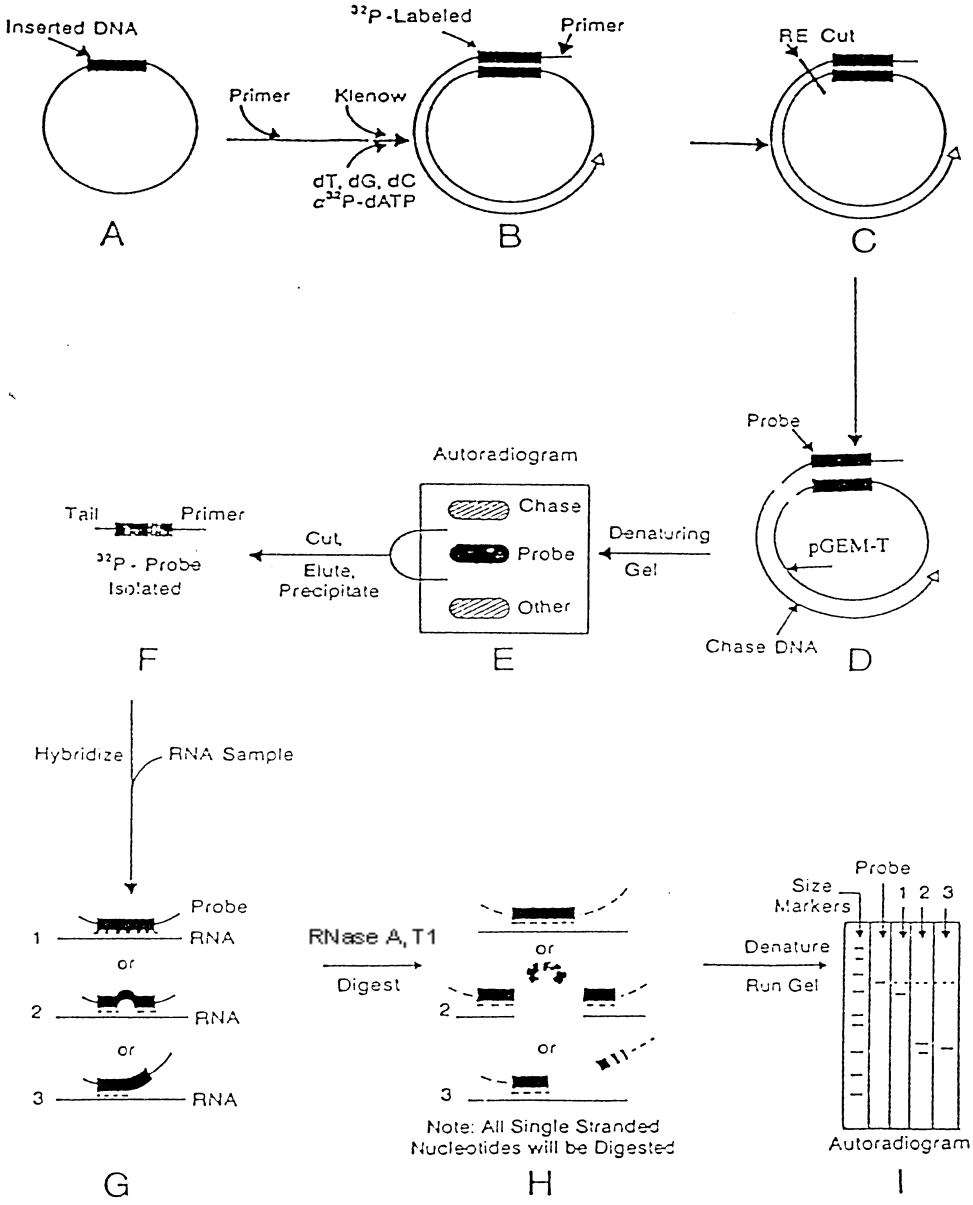


Fig 2.2 Schematic diagram of RNase protection assay. A) pGEM-T vector with inserted DNA of interest is isolated. B) A second strand, with radiolabelled nucleotides, is synthesised extending from the primer region. C) The product is isolated on the denaturing gel. E) From the autoradiographed gel, the radiolabelled probe, complementary to the DNA of interest, is identified. F) The probe is isolated from the gel. G) The probe is hybridised with RNA sample. H) The hybridised product is subjected to RNase A /T1 digestion: all single strand RNA will be digested. I) The sample is run on a denaturing polyacylamide gel to determine the size of nondigested (protected) hybridised pieces.

2. 4. 1 Generation of ^{32}P-radiolabeled anti-sense RNA probes

Synthesis of complementary DNA

To conduct RNase protection assay, specific anti-sense probes for the B1R and B2R, as well as inner controls GAPDH (glyceraldehyde-3-phophate dehydrogenase) or rL32 (50S ribosomal protein L32) were constructed. In detail, total RNA was isolated from rat heart tissues since both kinin receptors are expressed in the myocardium. In a 0.5 ml Eppendorf tube, approximately 3 μg RNA was added with 1 unit ribonuclease-free DNase (Roche Diagnostics GmbH, Mannheim, Germany), in presence of 5 mmol/L $MgCl_2$ and 1 unit RNase inhibitor, incubated at 37°C for 45 minutes to remove genomic DNA. The RNA sample was then transcribed by the technique of RT-PCR (reverse transcription-polymerase chain reaction). 10×PCR buffer (0.5 M KCl and 0.1 M Tris-HCl, pH8.3), in the presence of 5 mmol $MgCl_2$, 1 mmol/L dNTPs, 0.1 U/μl RNase inhibitor, 2.5 umol/L random primers, and 0.1 U/μl cloned Moloney murine leukemia virus reverse transcriptase (GiBco Life technology GmbH, Germany) were added and mixed. For annealing of primers with the template, the tubes were incubated at 20°C for 10 minutes, then tubes were incubated at 42°C for 60 minutes to allow the synthesis of the first strand of cDNA. Followed by denaturation at 95°C for 5 minutes and then cooling at 4°C for 5 minutes. Obtained cDNA was stored at 4°C.

Polymerase chain reaction

The cDNA regions of rat B1R and B2R, as well as inner controls GAPDH or rL32 genes were amplified by the technique of PCR. In a 0.2 ml tube, 50 pmol of the primers, 4.5 μl of 10×PCR buffer, 2.4 μl of 10 mM dNTP, 1.8 μl of 25 mM $MgCl_2$, and 0.5 U of Bio Therm^{TM} DNA polymerase (*Rapidozym* GmbH, Berlin, Germany) were added, and finally sterile water was added to a total volume of 45 μl. The forward and reverse primers for B1R were 5'-CTC CCA AGA CAG CAG TCA CC-3' and 5'-CAG ATA GTG ATG ACG AAC C-3', respectively. The forward and reverse primers for B2R were 5'-GAG AGT GAG TAG TAC TGT TGG-3' and 5'-GC AGT TGA CCT CTG AAA AGG-3', respectively. Thirty four cycles proceeded as follows: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 40 seconds. The PCR products were detected on an 2% agarose gel (for gel

ingredients see chapter 2. 5. 1). To run gel electrophoresis, 10 μl fragments were mixed with 1 μl 10×DNA loading buffer (containing Glycerol 50%, Bromphenol Blue 0.4% and Xylene Cyanol 0.4%. MBI, Fermentas Inc, MD, USA) and placed into the gel slots, 1×TAE (Tris acetate EDTA buffer) was used as electrophoresis buffer, ϕ X174 DNA/*Bsu*RI and λDNA/*Eco*RI+*Hin*dIII (MBI, Fermentas Inc, MD, USA) were used as DNA molecular weight markers to evaluate the size of the fragments. Electrophoresis was performed at 100 V and 20 mA for 30 min. In the gel, DNA molecules could interact with EB (ethidium bromid) and were detectable under ultra violet light. By comparison with molecular weight markers, bands with the right size were assumed as target fragments.

Ligation and transfomation

An aliquot of the PCR product was firstly analyzed on an agarose gel before using it in the ligation reaction. The target PCR fragment was then extracted from agarose with an Ultrafree-DA system (Millipore Corp. USA). Briefly, 30 μl of PCR product were run on an 2% agarose gel. After 30 min electrophoresis at 100 V and 20 mA, distinct individual bands were separated and could be detected under ultraviolet light. Using a razor blade, the slice of agarose containing target bands were precisely cut out from the gel. In this procedure, exposure to ultraviolet light was minimized in order to avoid the formation of pyrimidine dimers. The DNA containing gel slice was then placed into Gel Nebulizer of the tube, spinned at 5,000×*g* for 10 minutes. Extruded DNA would pass through the microporous membrane after centrifugation and collected in the filtrate vial of the system. The DNA containing liquid was then transferred on a special membrane filter (VM 0.05 μm, Millipore, Ireland), which was placed on sterile water. After 10 min storage at room temperature, the DNA containing solution was collected into a fresh tube and was ready for cloning. For ligation, the pGEM T vector system (Promega, Madison, WI, USA) was used. In 0.5 ml microfuge tubes, 3 μl PCR products, 1 μl pGEM-T vector (45 ng/μl), 1 μl T4 DNA ligase (3 units/μl) and 5 μl 2×ligation buffer (containing 60 mM Tris-HCL, 20 mM $MgCl_2$, 20 mM DTT [dithiothreitol], 2 mM ATP [adenosine triphosphate], 10% polyethylene glyco, pH 7.8) was added and mixed by pipetting. In another tube, control insert DNA (4 ng/μl) was used for standard positive background. Ligation was carried out by overnight incubation at 4°C. Generally, a longer incubation time would increase the number of transformants.

To do transformation, two LB (L-Broth growth media for bacteria, for ingredients see chapter 2.6.1, all from Becton Dickinson, MD, USA) plates containing ampicillin (SERVA, Electrophoresis GmbH, Heidelberg, Germany), IPTG and X-Gal (isopropyl-beta-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-ß-D-galactoside; for ingredients see chapter 2.6.1) were prepared for each ligation reaction, all of the 10 µl ligation mixture was transferred into a 1.5 ml sterile microcentrifuge tube on ice. Tubes of frozen JM109 High Efficiency Competent Cells (Promega, Madison, WI USA) were removed from –80°C storage and placed in an ice bath until just thawed (about 5 minutes). 50 µl of cells were carefully transferred into each tube. In this process excessive pipetting was avoided, as the cells were very fragile. Cells and ligation were mixed by gently pipetting. Tubes were then placed on ice for 30 minutes, followed by heating at 37°C for 30 seconds and cooling on ice for 3 minutes (heat and cool shock). In each tube, 800 µl of 37°C heated LB medium was added and mixed by gentle pipetting, foam was avoided. Tubes were then incubated at 37°C with shaking for 50 min. For cell seeding, 135 µl of each transformation culture was plated onto duplicate LB plates with ampicillin/IPTG/X-Gal and well spreaded. Cells were grown on the plates by overnight (16-24 hours) incubation at 37°C. Using this method, approximately 20- 50 colonies per plate could be seen in the experiments, and generally the white colonies contained the target inserts.

Mini / Maxi DNA extraction

Recombinant plasmid DNA was extracted by Mini and/or Maxi DNA extracting steps. For Mini preparations, 3 ml aliquot of LB media containing 100 µg/ml ampicilin were placed into a 15 ml sterile culture tube. Using a yellow pipette tip, distinct white single colonies (plasmid containing cells) were picked into the media-containing tube. Then cells were grown by incubation at 37°C with 250 rpm shaking (Shaker Swip, Edmund Büler, Martinsried, Germany). After 16-18 hours incubation, 1.5 ml of the cell solution was transferred into a 1.5 ml tube and centrifuged. The supernatant was poured off, obtained pellet was re-suspended in GTE buffer (containing 50 mM glucose, 25 mM Tris/HCl, 10 mM EDTA, pH 8.0). Solution was vortexed and then added with 10% RNase A (Ribonuclease A. Serva, Heidelberg, Germany). Cells were lysed by 100 mM NaOH, 0.5% SDS (sodium dodecyl sulfate). Then

solution was neutralized by 1.7 M ammonium acid, pH 5.5, followed by centrifugation at 4°C 10,000×*g* for 15 min to pellet the cellular debris, protein, precipitated SDS and chromosomal DNA. The supernatant was centrifuged again and the pellet was discarded. Obtained supernatant was then transferred into a fresh 1.5 ml tube. To precipitate the DNA, 500 µl isopropanol was added and solution was mixed by vortexing. Tubes were then centrifuged at 10,000×*g* for 30 min to collect DNA. Precipitated DNA was washed with 1 ml ice-cold 70% ethanol, dried, and then dissolved in 30 µl 1×TE (Tris-EDTA) buffer. An aliquot of the DNA sample was quantified spectrophotometrically by absorbance at 260 nm and 280 nm; the quality was analysed by electrophoresis. Extracted DNA samples were stored at 4°C.

For Maxi DNA preparation, the JETSTAR KITs (Genomed, Bad Oeymhausen Germany) were used. In brief, 1.5 ml of cells containing desired plasmid (determined by sequencing, see below) were grown in 250 ml LB/ampicilin media. After overnight incubation at 37°C with shaking, cells were collected by centrifugation at 5,000rpm for 10 min. Then, cells were resuspended in 10ml of E1 solution (containing 50 Mm Tris, 10 mM EDTA, pH 8.0), followed by addition of E2 solution (containing 200 mM NaOH, 1% SDS). Solution was mixed by gentle inversion. After storage at room temperature for 5 min, 10 ml E3 solution was added (containing 3.1 M potassium acetate, pH 5.5) and solution was gently mixed. The mixture was then centrifuged at 5,000 rpm for 30 min. The supernatant was centrifuged again and the pellet was discarded. Obtained supernatant was applied onto the JETSTAR column (which were equilibrated by applying 30 ml E4 solution) by decanting, the liquid (lysate) would run through the column. Then column was then washed with 60 ml solution E5 and allowed to empty by gravity flow. To elute DNA, 15 ml solution E6 was applied onto the column and liquid was collected, then 0.7 volumes of isopropanol was added to precipitate the DNA. The mixture was centrifuged at 4°C and 6000 rpm for 30 min. Obtained plasmid DNA was washed with 70% ice-cold ethanol and recentrifuged. The pellet was dried for 20 min and then dissolved in 50-300 µl 1×TE buffer. Obtained DNA samples were stored at 4°C.

Plasmid linearisation and DNA elution

The plasmid extracted by Mini preparations should contain the cDNA fragment of interest, which was determined by restriction endonucleases (RE) digestion and subsequent DNA

sequencing. For RE digestion, in 0.5 ml tubes, 7 µl plasmid DNA, 1µl 10×SuRE/Cut buffer and 1 µl of appropriate RE were mixed in a clean 0.5 ml tube. Digestion was carried out by incubating the tube at 37°C for 1 h. After this, products were run on an 2% agarose gel at 100 V and 20 mA for 30 min. By comparing with DNA molecular weight markers, the length of the fragments were detected. Then, the inserted fragments of the plasmids extracted from the colonies were verified by sequencing (Invitek, Berlin, Germany). For Plasmid linearisation, appropriate RE was used (RE and buffers used in this part see table 2.1, all from Roche, Diagnostics GmbH, Mannheim, Germany). Linearisation was performed by incubation of DNA, RE and buffer mixture at 37°C for 1 h as described above. For DNA elution, the products were run on an 2% agarose gel at 100 V and 20 mA for 30 min. Under ultra violet light, bands of interest were precisely cut out from the gel and placed into a prepared blue pipette tip, which was inserted in an 1.5 ml tube, inside the tip a piece of cotton was stuffed. The gel containing blue tip was fast frozen with liquid nitrogen, then placed in the tube and centrifuged by 10,000×*g* at room temperature. Liquid that collected at the bottom of the tube was transferred into fresh tubes, added with the same volume of phenol and chloroform/isoamylalcohol (24:1), and was vortexed and centrifuged. The upper phase was added with double amount of 99% ethanol and 1/10 amount of 3 M sodium acetate. Tubes were stored at –20°C for 30 min and centrifuged again. The collected DNA pellet was washed with 300 µl ice cold 70% ethanol, dried, and finally dissolved in 1×TE buffer. Quality and concentration of the eluted DNA were determined as described above.

Probe radiolabelling

Radiolabelled anti-sense RNA probes were generated by *in vitro* transcription. In a sterile 1.5 ml microfuge tube, following reagents (all from Promega, Madison, WI USA) were added: 5 µl 5×transcription buffer, 1 µl 0.1M DTT (dithiothreitol), 3 µl 2.5mM NTP's (A, C, G), 1 µl RNasin (RNase inhibitor, 25U/ul), 5 ul of 100 uM cold UTP, 1 µl of 1 µg/µl linearized DNA template, 5 µl of 10 µCi/ul ^{32}P-UTP (800Ci/mmol) (Dupont NEN - #NEG507X), and 1µl RNA polymerase SP6 or T7. Total volume was added with DEPC-H_2O to 25 µl. The transcription was conducted by incubation at 37°C for 1 hour. Then, 2 µl of DNase I (1unit /µl) was added and solution was incubated at 37°C for 20 minutes to remove DNA. The reactions (RNA probes) were separated by polyacrylamide gel electrophoresis. In brief, the

mixture was added with 20- µl gel-loading buffer (containing 95% formamide, 0.025% xylene cyanol, 18 mM EDTA, and 0.025% SDS), heated at 90°C for 5 min, then cooled at 4°C for 3 min. Electrophoresis was conducted using a 6% polyacrylamide gel containing 10 M urea (gel ingredients see chapter 2. 6. 1). After gel running at 300 V 50 mA for 90 min, the gel was removed from the apparatus and exposed on a FUJI phosphor plate for 20 seconds. Images were acquired using the FUJIFILM BAS1500 Phosphor-Imager system (Fuji, Tokyo, Japan). Autoradiographic signals were processed using a Tina software (2.09 raytest; Straubenhardt, Germany). Bands of interest were located at the autoradiographic picture. The obtained picture, which had the same size as the polyacrylamide gel, was placed under the gel. With a razor, the slices of gel containing anti-sense RNA probes were carefully cut out and placed in new microfuge tubes. 350 µl elution buffer (containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS) were added into and mixed by vortexing. Then tubes were incubated overnight at 37°C.

	RnB1R	***RnB2R***	***IL1ß***	***GAPDH***	***rL32***
Restriction Enzyme	Not I/ PstI	Not I/ PstI	Apa I/ Sac II	HindIII	Xba I
SuRE/Cut Buffers	H	H	L	M	H
Transcription Enzyme	T7	T7	SP6	T7	T7

Table 2.1 Enzymes and buffers used for the preparation of anti-sense RNA probes

2. 4. 2 Hybridisation analysis of mRNA

The amount of B1R and B2R mRNA isolated from heart tissues or cultured cells were estimated using the RPA II Ribonuclease Protection Assay Kit (Ambion, Austin, TX, USA). Assays were performed according to the manufactures instructions. Figure 2.3 shows a schematic overview of the procedure. Firstly, the concentration of total RNA and its quality were determined by UV spectrophotometry at 260/280 nm and electrophoresis on 2% agarose

gel. Samples that had 260/280 ratio more than 1.8 and no signs of degradation based on agarose electrophoresis were used for the RNase assay. The radioactivity of the radiolabeled probes was measured using a Beckman LS 1701 Coulter (UK). In an 1.5 ml sterile eppendorf tube, 60,000 cpm of [^{32}P]UTP-labeled cRNA probes, 15–20 µg of investigated RNA samples, 10 µl 5M ammoniumacetate, and 250 µl ethanol were added and mixed. Finally, DEPC-H_2O was added to a total volume of 100 µl. Additionally, two yeast RNA samples were used as a system control. Mixture was stored at -20°C for 1 h then centrifuged at 10,000×g 4°C for 30 min. The supernatant was discarded and as much liquid as possible was carefully removed without disturbing the pellet. Pellet was shortly dried and resuspended by vortexing in 20 µl Hybridization Buffer containing 80% formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4) and 1 mM EDTA. The hybridization was performed by overnight incubation of the tubes at 42°C. The mixed RNA contained in the reaction was digested with a mixture of RNase A and T1 (final concentrations 2.50 units/ml and 10,000 units/ml respectively) at 37°C for 55 min (except for one of the yeast RNA). After this, RNase was inactivated by adding 350 µl inactivation buffer. After RNase treatment, all of single RNA in the tubes would be degraded. RNA was then precipitated by storage at –20°C for 30 min and subsequent centrifugation at 4°C 10,000×g for 20 min. Collected RNA pellet was resuspended in 8 µl loading buffer (24 µl for undigested yeast RNA) containing 80% formamide. The protected (undigested) fragments were separated on a 6% polyacrylamide gel containing 10 M urea. After 2 h electrophoresis at 300 V and 50 mA, the gel was dried using the Gel-dryer D62 (Biometra, Göttingen, Germany), then subjected to autoradiography by exposure on storage phosphor screens for up to 24 h. Images were acquired using the FUJIFILM BAS1500 Phosphor-Imager system (Fuji Tokyo, Japan). Using Tina software, quantitative analysis was performed by measuring the intensity of the interesting bands normalised by the intensity of housekeeping gene GAPDH or rL32. The intensity of the bands was directly proportional to the amount of protected mRNA samples, thus the expression of B1R and B2R mRNA by tissues or cultured cells were evaluated.

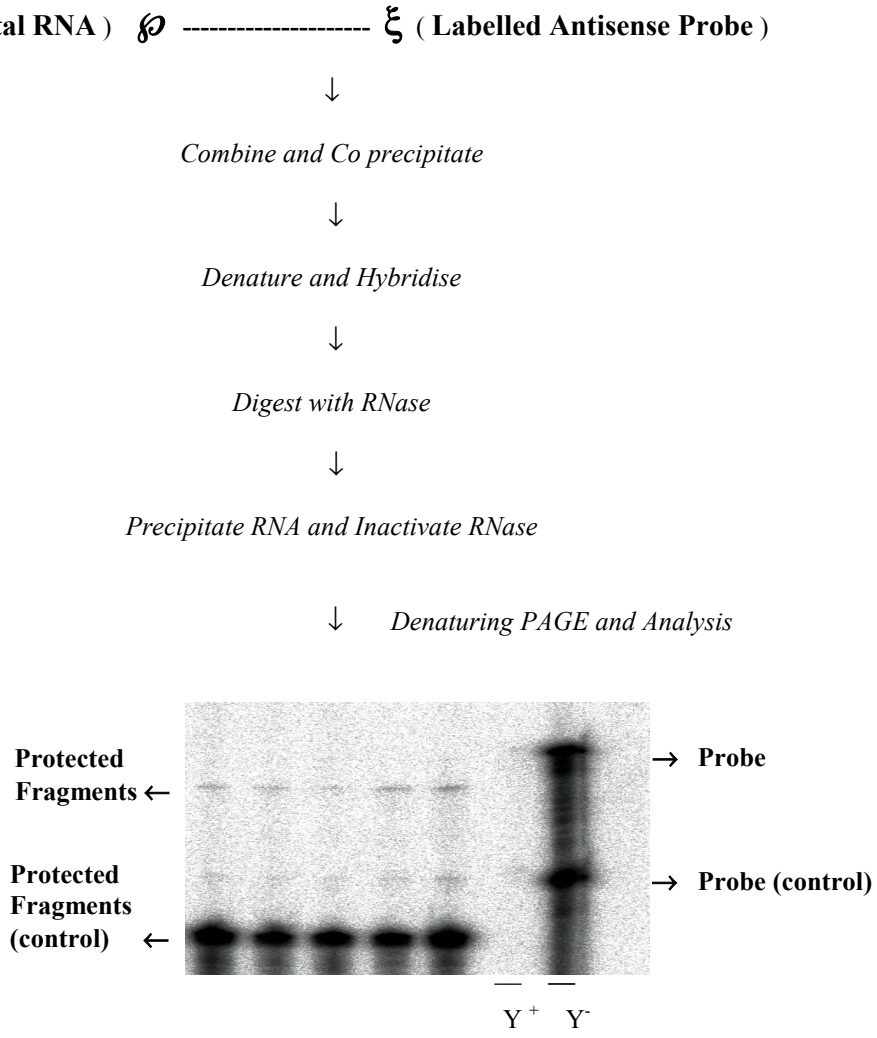


Fig 2.3 Schematic diagram of hybridisation analysis of mRNA procedures in RNase protection assay. Y^+, yeast RNA with RNase digestion; Y^-, yeast RNA without RNase digestion.

2. 5 Materials

2. 5. 1 Solutions and chemicals

cell culture media

In cell culture, Dulbecco's modification of Eagle's minimum essential medium (DMEM) was used as standard culture media (Gibco, Karlsruhe, Germany), some other ingredients were added according to the experimental requirements.

cardiomyocytes media:

DMEM (4500mg/l glucose)	500 ml
FBS	55 ml (10%)
BrdU (50 mM)	1 ml (0.1 mM)
sodium pyruvate (100mM)	5 ml
penicillin (100 IU/ml) and streptomycin (100 µg/ml)	6 ml

cardiac fibroblasts media:

DMEM (4500mg/l glucose)	500 ml
FBS	55 ml (10%)
sodium pyruvate (100mM)	5 ml
penicillin (100 IU/ml)and streptomycin (100 µg/ml)	6 ml

smooth muscle cells media:

DMEM (1000mg/l glucose)	500 ml
FBS	55 ml (10%)
sodium pyruvate (100mM)	5 ml
penicillin (100 IU/ml) and streptomycin (100 µg/ml)	6 ml

serum free media for CMC and FBC:

DMEM (4500mg/l glucose)	500 ml
sodium pyruvate (100mM)	5 ml
penicillin (100 IU/ml) and streptomycin (100 µg/ml)	6 ml

serum free media for SMC:

DMEM (1000mg/l glucose)	500 ml
sodium pyruvate (100mM)	5 ml
penicillin (100 IU/ml) and streptomycin (100 µg/ml)	6 ml

cell treatment solution

freezing solution	FBS, DMEM,DMSO (1:8:1,v/v/v)	
trypsin-EDTA-solution	HBSS	100 ml
	trypsin	0.05 g
	EDTA	0.02 g
10×PBS buffer	H_2O	1 l
	NaCl	80 g
	KCl	2 g
	Na_2HPO_4	2 g pH 7.2

gel and gel electrophoresis buffer

1% agarose gel	agarose	1.5 g
	1×TAE buffer	150 ml
	EB	15 µl (500 µg/ml)
6% polyacrylamid gel	H_2O	24.5 ml
	urea	28.8 g (10 M)
	10×TBE	6 ml
	40 % acrylamide/bisacrylamide (19:1)	7.5 ml
	APS	480 µl
	TEMED	64 µl

50×TAE buffer	H_2O	1 l
	Tris-base	242 g
	Na-acetat	68 g
	EDTA	18.5 g pH 7.8
10×TBE buffer	H_2O	1 l
	Tris-base	162 g
	Boric acid	27.5 g
	EDTA	9.3 g

transformation, mini/maxi plasmid DNA extraction reagents

GTE buffer	H_2O	1 l
	glucose	4.50 g
	EDTA	1.86 g
	Tris-HCl	1.97 g pH 8.0
LB-Medium	NaOH (2 M)	1.78 ml
	Trpton	10 g
	Yeast-extract	5 g
	NaCl	5 g
X-Gal/IPTG-plate	LB-Medium	1 l
	Agar	15 g (1.5%)
	ampicillin	100 µg/ml
	X-Gal	800 µl (50 mg X-Gal/ 800 ul DMSO)
	IPTG	200 ul (200mg IPTG/ 1ml H_2O)
10×TE buffer	H_2O	1 l
	Tris-base	12.1 g
	EDTA	3.722 g pH 7.4

Sources of materials

cell culture flasks, dishes	Nunc ,Wiesbaden, Germany
pipettes	Pipetman, Gilson, France
DMEM	Gibco BRL, Paisley, Scotland
DMSO	Sigma, Stenheim, Germany
HBSS	Seromed-Biochrom, Berlin, Germany
HBSS	Gibco BRL, Paisley, Scotland
FBS	Seromed-Biochrom, Berlin, Germany
penicillin/streptomycine	Seromed-Biochrom, Berlin, Germany
trypsin-EDTA	Sigma, Stenheim, Germany
bromodeoxyuridine (BrdU)	Sigma, Stenheim, Germany
glucose	Sigma, Stenheim, Germany
SDS	Sigma, Stenheim, Germany
EDTA	Sigma, Stenheim, Germany
agarose	Gibco BRL, Paisley, Scotland
X-Gal	AppliChem, Darmstadt, Germany
IPTG	Sigma, Stenheim, Germany
urea	Sigma, Stenheim, Germany
APS	Sigma, Stenheim, Germany
interleukin 1ß	Sigma, Missouri, USA
^{32}P-UTP	NEN Dupont, Boston, MA, USA

2. 5. 2 Equipments

Centrifuges	Centrigude 5403, Eppendorf, Hamburg, Germany
	Centrigude 5415 D, Eppendorf, Hamburg, Germany
	Megafuge 1.0, Hanau, Germany
Centriguge tubes	Nunc, Wiesbaden, Germany
Densitometer	Model GS-700, imaging densitometer, Bio-Rad, Munich, Germany
CO_2 –incubator	BB16, Heraeus, Hanau, Germany
Dispersing system	ULTRA-TURRAX-Antrieb T25 B, IKA Werk, Staufen, Germany
Electrophoresis-apparatus	GNA 200, Pharmacia Biotech, Freiburg, Germany
	Agagel Minin, Biometra, Göttingen, Germany
Electrophoresis-power	Mini Power PACK P20, Biometra, Göttingen, Germany
	Standard Power Pack P26, Biometra, Göttingen, Germany
Darkroomhood	BioDocAnalyze, Biometra, Göttingen, Germany
Gel-dryer	D62, Biometra, Göttingen, Germany
Heating bath	GFL 1083, Burgwedel, Germany
Magnetic stirrer	Modell MR 3001, Heidolf, Germany
Mixer	Thermomixer compact, Eppendorf, Hamburg, Germany
	Thermomixer comfort, Eppendorf, Hamburg, Germany
Microbiological safety cabinet	HERAsafe HS18/2, Hanau, Germany
Microscope	Leica DMIL, Wetzlar, Germany
Precision balance	SBC 21 Semi-micro Balance / SBA laboratory Balance Heiligenstadt, Germany
Radiocoulter	Beckman coulter LS 1701 Glenrothes, Scotland, UK
Shaker	Mini-shaker VF2, IKA, Düsseldorf, Germany
Thermal cycler	Mastercycler personal, Eppendorf, Hamburg, Germany
	Mastercycler gradient, Eppendorf, Hamburg, Germany
UV-photometer	Spectrophotometer DU 640i, Beckman, California, U.S.A

2. 6 Experimental design

in vitro*:* neonatal cardiac myocytes, fibroblasts and aortic vascular smooth muscle cells were cultured. After 12 h serum free medium treatment, total RNA was isolated. Using RPA method, the amounts of B1R and B2R mRNA from these cell-RNA samples were investigated, basic expression of B1R and B2R mRNA was analysed for each cell system. To evaluate the influence of cytokine IL1ß on the expression of B1R and B2R mRNA in different cell types, cells were firstly treated with serum free medium, then, new media containing IL1ß was used. Cells were treated with IL1ß using different concentrations or for varying times. After treatment, total RNA was isolated from cells and the amounts of B1R and B2R mRNA were investigated.

In vivo**:** This study was conducted to characterize whether an inhibition of IL1ß production during myocardial infarction can affect the release of kinin receptors in the heart. Using a rat model, myocardial infarction was introduced by coronary artery ligation. Since the beginning of MI induction rats were treated with the IL1ß converting enzyme inhibitor HMR 3480. 3 weeks after coronary artery ligation, the hearts were excised and the left ventricles were separated. Total RNA was isolated from the infarcted ventricular myocardium, the amounts of B1R and B2R mRNA were analysed using RPA method.

2. 7 Statistic analysis

Comparisons among different groups were assessed by analysis of variance (ANOVA) followed by T- test. The criterion for statistical significance was set as $P<0.05$. Numerical values are given as means ± SE.