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Effects of different beta adrenoceptor ligands in mice with permanent occlusion of the left anterior descending coronary artery

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1 We have studied the effects of three βAR ligands (carvedilol, alprenolol, and ICI-118,551) with different pharmacological profiles and negative efficacy at the β2AR on cardiac *in vivo*, *in vitro*, biochemical and gene expression parameters in mice with permanent occlusion of the left anterior descending coronary artery.

2 Cardiac *in vivo* parameters were determined using Doppler studies. Mitral-wave E peak velocity (EPV) and aortic peak velocity (AoPV) decreased in the first 2 weeks postocclusion. After 3 weeks of drug treatment, EPV was improved in the carvedilol group to preocclusion values; however, a further reduction in EPV in the alprenolol and control permanent occlusion group was measured and there was no change after ICI-118,551 treatment. AoPV was unchanged between weeks 2 and 5 in all groups.

3 The left atria were isolated to record isometric tension responses to isoprenaline. Permanent occlusion significantly reduced the maximum isoprenaline response to 30% of control and carvedilol increased the maximum response to isoprenaline significantly to 60%.

4 The biochemical and gene expression studies revealed different effects of the three βAR ligands. Most notably, carvedilol reduced gene expression of myosin heavy chain β.

5 These results indicate that chronic treatment with carvedilol is beneficial in a mouse model of myocardial damage resulting from ischaemia. We hypothesise that these beneficial effects of the drug may be because of the negative efficacy at the β2AR, combined with β1AR antagonism.
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Abbreviations: βAR, beta adrenoceptor; AC6, adenylate cyclase 6; ALP, alprenolol; ANF, atrial natriuretic factor; AoPV, aortic peak velocity; CARV, carvedilol; CHF, congestive heart failure; CO PO, control with permanent occlusion; cPKA, catalytic subunit PKA; CTRL, control; EPV, mitral-wave E peak velocity; Gαs, Gαs protein; HIS2, histamine 2 receptor; ICI, ICI-118,551; MHCα, α-myosin-heavy chain; MHCβ, β-myosin-heavy chain; PO, permanent occlusion

Introduction

Congestive heart failure (CHF) is a complex disease characterised by inadequate pump performance of the heart, most commonly caused by ischaemic damage to the myocardium or myocardial infarction, and associated with high morbidity and mortality. In humans, one of the most clinically relevant consequences of nonfatal ischaemic damage to the myocardium is that it often leads to the development of CHF. Despite increased understanding of the pathology of CHF, there is still need for improvement in long-term treatment.

To maintain blood pressure and oxygen supply to the periphery, sympathetic activity is increased in CHF (Brodde, 1991). This increase in sympathetic activity leads to β adrenoceptor (βAR) stimulation in an attempt to maintain inotropy and cardiac output. In the human heart, β1ARs are predominantly responsible for inotropy (Brodde, 1991). In the failing heart, the increased catecholamine levels produce β1AR downregulation and desensitisation, resulting in a decreased responsiveness of the heart to catecholamines (Bristow *et al*., 1982; Brodde *et al*., 1986). The β2AR, although desensitised, is not downregulated (Brodde *et al*., 1986). Consequently, β2ARs may play an increasing role in maintaining inotropy in the failing heart.

Nonetheless, inotropic agents appear to be undesirable for the chronic treatment of heart failure. Inotropic agents such as βAR agonists and phosphodiesterase inhibitors, while acutely beneficial, increase mortality with chronic use (Packer, 1995). However, several recent studies have shown that chronic administration of some βAR antagonists lowers mortality in patients with CHF by more than 30% (Lechat *et al*., 1998).

In the United States, two βAR antagonists, carvedilol (CARV) and metoprolol, are currently approved for the treatment of heart failure (Foody *et al*., 2002). Preliminary studies indicate that CARV may have more beneficial effects than metoprolol in treating heart failure (Gilbert *et al*., 1996; Metra *et al*., 2000; Packer *et al*., 2001). CARV has affinity for

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β1 and β2ARs, while metoprolol is a preferential β1AR antagonist. In addition, because of the relatively low preference of metoprolol for β1ARs (as little as four-fold), it has been suggested that at the doses recommended for treating heart failure, metoprolol may also occupy β2ARs (MERIT-HF, 1999; Brixius *et al*., 2001). Thus, it seems likely that β2AR occupancy will be a desired property. In transgenic mice overexpressing the human β2AR, CARV has been shown to decrease baseline atrial contractility by 40%, demonstrating negative efficacy and inverse agonist activity, which may be relevant in the explanation of its apparent beneficial effect in CHF (Nagaraja *et al*., 1999).

Recent studies have indicated that β2ARs, like many other G protein coupled receptors (GPCRs), can exist in a spontaneously active state (usually termed R*; Milligan *et al*., 1995). Simultaneously, it was found that many, but not all ligands, previously classified as antagonists were capable of inactivating these spontaneously active receptors. These ligands were determined to possess negative efficacy (able to 'turn off' the spontaneously active receptors) and have been termed inverse agonists (Milligan *et al*., 1995). These findings necessitated at least a two-state model in which receptors exist in equilibrium between an inactive state, (R); and the active state, (R*). In this model, agonists bind to and enrich the active R* state, while inverse agonists bind to and enrich the inactive R state. Antagonists do not alter the R-R* equilibrium, but block the effects of agonists and inverse agonists. In this model, both inverse agonists and antagonists can prevent agonist-induced activation of receptors, but inverse agonists differ from antagonists by their ability to inactivate spontaneously active receptors. This means that in a cardiac β2AR receptor system with R*, inverse agonists may have more negative inotropic effects than antagonists may. In summary, β2ARs may play a more significant role in the failing heart, and inotropy (i.e., βAR agonism) is likely to be an undesirable property.

We have therefore investigated the possible role of negative efficacy (or inverse agonist activity) at the β2AR in the beneficial effects of some βAR ligands in a mouse model of ischaemia-induced heart damage caused by permanent occlusion (PO) of the left anterior descending coronary artery. There is some controversy as to the functional relevance of β2ARs in mouse myocardium (see Xiao *et al*., 1995; Rohrer *et al*., 1996) *versus* (Heubach *et al*., 1999; Gong *et al*., 2000). However, both sides present data from radioligand binding studies demonstrating the presence of β2ARs in mouse hearts (Rohrer *et al*., 1996; Heubach *et al*., 1999). Thus, that the protein is present and available for binding by exogenous ligands is well documented. Furthermore, even the groups claiming there is no evidence for a physiologic function of the receptor have shown that the selective β2AR ligand, ICI-118,551 (ICI) may produce effects in mouse myocardium. ICI-118,551 (50 nM) produced a decrease in spontaneously beating mouse right atrium and a decrease in the basal force of contraction of ventricular strips (Heubach *et al*., 1999).

In this model, we initially chose to test two ligands (carvedilol and alprenolol, ALP) with different degrees of negative efficacy at the β2AR in a mouse myocardial infarction model. At the β2AR, CARV is an inverse agonist and ALP is a neutral antagonist (Bond *et al*., 1995; Nagaraja *et al*., 1999). Both CARV and ALP are also β1AR antagonists. The results of these experiments suggested that negative efficacy and inverse agonist activity at the β2AR were possibly beneficial properties. To further investigate the possible beneficial effects of inverse agonism at the β2AR, we decided to include a third drug, ICI-118,551. Unlike CARV and ALP, ICI-118,551 is highly selective for the β2AR , and ICI-118,551 has the highest degree of negative efficacy and inverse agonist activity at the β2AR (Nagaraja *et al*., 1999). ICI-118,551 would thus provide clues as to the relative benefits of negative efficacy at the βAR and the importance of concomitant blockade of β1AR.

Here, we report on cardiac *in vivo, in vitro,* biochemical and molecular parameters that support negative efficacy at the β2AR combined with the β1AR antagonism as possible desirable drug properties in chronic treatment of CHF.

Methods

Material

ALP was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). ICI-118,551 ((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol), CARV and control (CTRL) chow were gifts from SmithKlineBeecham. Antisera for Gαi protein and Gαq/11 protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antisera for Gαs protein (Gαs) were a gift from Dr Graeme Milligan. Micro-osmotic minipumps (model 2004) were obtained from ALZA Corp. (Palo Alto, CA, U.S.A.). ECL reagent was purchased from Amersham Life Science (Arlington Heights, IL, U.S.A.). Electrophoresis materials were purchased from Bio-Rad (Hercules, CA, U.S.A.). The BCA assay reagent kits were obtained from Pierce Inc. (Rockford, IL, U.S.A.).

The mouse model

The care, treatment and use of all the animals in this study were in accordance with institutionally approved protocols. In 12- to 15-week old, male C57BL/6 mice (Harlan Sprague Dawley, Houston, TX, U.S.A.) with a mean body weight of 26.5 ± 0.27 g, myocardial infarction was induced by PO of the left anterior descending coronary artery. These mice were generated following a previously described protocol (Michael *et al*., 1995) at the DeBakey Heart Center (Houston, TX, U.S.A.) as a service. Mice were anaesthetised by intraperitoneal injection of sodium pentobarbital ($4\,mg\,ml^{-1}$), $10\,\mu l\,g^{-1}$ body weight. After intratracheal intubation, the chest was opened by a vertical cut, lateral to the left or right side of the sternum. The heart was exposed and the left anterior descending coronary artery was ligated using a 7-0 silk suture. After occlusion had been accomplished the chest was closed, and after removal of the tracheal intubation tube the animal was placed under a heat lamp with additional oxygen supply. This procedure resulted in an infarct size of 30% of the left ventricle (Michael *et al*., 1999), which was estimated visually after removing the heart at the end of the treatment period. The mice were allowed to recover for 14 days before drug treatment was started. In the first set of experiments, the mice were randomised into different groups: CTRL with permanent occlusion and no drug treatment (CO PO), CARV, and ALP. Mice without PO and no-drug treatment were used as CTRL.

Weight gain was monitored as a measure of overall well-being for each mouse. In a second set of experiments, 20 mice with PO were randomised into two additional groups of either ICI-118,551/DMSO (ICI) or DMSO (DMSO CO PO) alone.

Drug-treatment protocol

After a 2-week recovery period, drug treatment was started. In a clinical setting, patients undergo a stepwise increasing drug regimen with βAR antagonists to improve tolerance (Packer *et al*., 1996). In our study design, we chose the same stepwise regimen, starting with a very low dose in the first treatment week (2 weeks postocclusion), and increasing the dose during the third week, and again during the fourth week postocclusion. The highest dose was maintained for 2 weeks after which the mice were killed. CARV and ALP were given orally mixed with CTRL chow and the mice had free access to food and water. As a result of the short half-life, ICI was given by continuous infusion using an Alzet minipump (Model 2004) which was attached to coiled Tygon® tubing. The recommended dose of CARV for patients with CHF is 50 mg day^{-1}. The recommended ALP dose for patients with myocardial infarction is 200 mg twice a day (Jurgensen *et al*., 1981). Therefore, we estimated that ALP is roughly six to eight times less potent than CARV and designed the protocol accordingly. CARV was given at 0.4, 1.2 and 2.4 mg g^{-1}of food. ALP was given at 2.4, 7.2 and 14.4 mg g^{-1} of food. It was assumed that all animals ate the same amount of food.

Preparation of the catheter with infusion pump and implantation

Under sterile conditions, a 45 cm long piece Tygon® microbore tubing (i.d. 0.03″, o.d. 0.09″, Fischer Scientific, Pittsburgh, PA) was coiled tightly around a syringe pestle. Subsequently, the coil was immersed in boiling water and then in ice cold water to maintain a diameter of 0.5 cm. At 40 h prior to implantation the pumps were filled with saline under sterile conditions and primed in sterile saline at 37°C. These pumps are designed to deliver 0.25 μl h^{-1} for 36 days after priming for 40 h. At 4 h prior to implantation the catheter coils were filled with different concentrations of ICI solution in DMSO as follows: 42 μl of 11.6 μg μl^{-1}, air bubble, 42 μl of 35 μg μl^{-1}, air bubble, and 84 μl of 70 μg μl^{-1}. The air bubble prevented mixing of the solutions of different concentrations. The catheter coil was connected to the pump, and pump and coil were inserted into the dorsal subcutaneous space of the anaesthetised (pentobarbital sodium 40 mg kg^{-1}) mouse *via* a small midline incision in the lower back, which was then clamped with a stainless steel wound clip (Autoclip®, Becton Dickinson, MD, U.S.A.). All animals were housed in standard plastic cages and were offered food and water *ad libitum*.

In vivo *cardiac parameters*

Cardiac *in vivo* parameters were determined pre and post-occlusion (2, 3, 4, 5, and 6 weeks) using Doppler technique (Hartley *et al*., 1995). Each mouse served as its own CTRL. The mice were anaesthetised with an intraperitoneal injection consisting of a mixture of acepromazine (1.4 mg ml^{-1}), xylazine (8.6 mg ml^{-1}), and ketamine (42.8 mg ml^{-1}) at a dose of 0.5 μl g^{-1} body weight (ALP- and CARV-treated groups) or with pentobarbital at a dose of 40 mg kg^{-1} (ICI-treated group). Subsequently, the mice were placed in a supine position with paws taped to a temperature-controlled laminated plastic board with copper electrodes placed such that the three bipolar limb leads allowed electrocardiogram (ECG) monitoring. A 2 mM diameter 10 MHz Doppler probe was placed below the sternum at the ventricular apex and angled to sense blood flow signals from the aortic outflow and mitral inflow tracks of the left ventricle as indexes of systolic and diastolic ventricular function, respectively (Taffet *et al*., 1996). The probe was connected to a pulsed Doppler instrument and signal processor (DSPW, Indus Instruments, Houston, TX, U.S.A.) with the spectral Doppler signal displayed along with the ECG on a computer monitor in real time (Hartley *et al*., 2000). At each location, the probe position and sample volume depth were adjusted to maximise the Doppler signal from aortic outflow or mitral inflow which were identified by flow direction and timing with respect to the ECG. From each site 2 s of Doppler audio signals were digitised and stored on the computer. The signals were subsequently analysed off-line using a spectrum analyser to obtain the aortic peak velocity (AoPV) and mitral E-wave peak velocity (EPV) from the envelope of the spectrum (Hartley *et al*., 2000).

In addition, ejection fraction was assesed using a radionucleotide ventriculography (Hartley *et al*., 1999).

In vitro *isolated left atrial tension*

At 6 weeks postocclusion the mice were anaesthetised and the hearts were quickly removed and placed in oxygenated modified Kreb's solution (Milano *et al*., 1994). The right atria and ventricles were cut in half and both fractions were immediately frozen in liquid nitrogen for determination of Gαia, Gαq/11, and Gαs amounts and for extraction of total RNA. The left atria were isolated and paced (0.5 ms, voltage at threshold +20% and optimal frequency of 3.2 Hz) essentially as previously described, and isometric tension was recorded (Milano *et al*., 1994). Concentration–response curves to isoprenaline (ISO) were performed. Each response to a concentration of ISO was allowed to plateau prior to addition of the next concentration. The ISO response in control mice without surgery (CTRL) was used to normalise the responses to ISO in the other groups.

Myocardial cytosolic and membrane fraction preparation

The right atria and both ventricles were thawed and minced with a razor blade on ice. The minced tissue was homogenised in 1 ml ice-cold homogenisation buffer (150 mM HEPES pH 7.2, 1 mM DTT, 1 mM PMSF) with 3 × 10 s bursts of a polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 20,000 rpm for 30 min at 4°C. The supernatant was stored as a cytosolic fraction at −20°C until assay. The remaining membrane pellet was resuspended in 1 ml solubilisation buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1.5% digitonin, 10 μg ml^{-1} leupeptin, 20 μg ml^{-1} aprotinin, 25 μg ml^{-1} pepstatin). This resuspension solution was rotated for 1 h at 4°C then centrifuged for 45 min at 4°C and 20,000 rpm. The supernatant was filtered through a 0.45 μM syringe filter (Millex®-HV, Millipore Corp., Bedford, MA, U.S.A.) and stored as the membrane fraction at −20°C until assay.

Total protein estimation

Total protein in the prepared cytosolic or membrane fraction was quantified using BCA assay reagent kit (Pierce Inc., Rockford, IL, U.S.A.) according to the manufacturer's instructions. Briefly, a sample of 10 μl was incubated with the reagent mixture (A : B = 49 : 1). A standard curve was obtained by using a range of concentrations of BSA standard protein treated similarly to test samples. After incubation, the samples were read at 560 nM using a spectrophotometer. The regression equation obtained from the standard curve was used to estimate the quantity of total protein in the cytosolic and membrane fraction of each sample.

G protein level determinations

Western blotting was performed to detect G protein levels using membrane fractions obtained from all groups. After separation on 10% SDS – polyacrylamide minigels (20 μg protein/lane), samples were transferred onto activated PVDF membranes (0.45 μM; Millipore Corp., Bedford, MA, U.S.A.) using a semidry apparatus (Trans-Blot® SD Bio-Rad, Hercules, CA, U.S.A.). The membranes were incubated for 1 h at room temperature in PBS containing 0.01% Tween-20 (PBS-T) and 5 – 10% nonfat dry milk (Bio-Rad, Hercules, CA, U.S.A.). Membranes were incubated overnight at 4°C with primary antibodies raised against Gαi, Gαs, Gαq/11 (titer of 1 : 1000) in PBS-T containing 2.5% nonfat dry milk. After incubation with the primary antibody, membranes were washed three times for 15 min with PBS-T. Horseradish peroxidase-conjugated anti-rabbit secondary antibody at a titer of 1 : 10,000 in PBS-T containing 2.5% nonfat dry milk was added and the membranes were incubated for 1 h at room temperature. After several washes (3 × 15 min) with PBS-T, blots were made visible by chemiluminescence using the ECL Plus Western blotting detection kit (Amersham Life Science Inc., Arlington Heights. IL, U.S.A.) according to the manufacturer's instructions. Purified Gαi, Gαs, and Gαq/11 protein were used as positive controls. In the CARV, ALP, and CO PO groups, bands were quantified using the Quantity One by PDI densitometry system. ICI and DMSO CO PO bands were quantified using NIH Image densitometer reading. In both systems, bands were normalised for loading variability using a nonspecific band that was not altered by treatments.

Quantitative real-time RT – PCR

Quantitative real-time RT – PCR was performed utilising the 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.) (Heid *et al*., 1996; Bustin, 2000). Specific quantitative assays for histamine H2 receptor (HIS2), catalytic subunit PKA (cPKA), Gαs, adenylate cyclase 6 (AC6), atrial natriuretic factor (ANF), α- and β-myosin-heavy chain (MHCα and MHCβ) and β-actin were developed using Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.) following the recommended guidelines based on sequences from Genbank (Table 1). Hearts were powdered on liquid nitrogen and ground on dry ice using a motorised pestle. RNA was extracted from powdered hearts using Trizol reagent. After adding 0.2 vol. of chloroform, the solution was quickly vortexed and centrifuged at 20,000 × *g* for 15 min at 4°C. Total RNA was

Table 1 Summary of primer and probe oligonucleotides and Genbank accession numbers used in the preparation of cDNA for the performance of quantitative real-time RT–PCR

Gene	*Forward primer*	*Reverse primer*	*Probe oligonucleotide*	*Accession no.*
HIS2	CCTGGCTGTCAGCTTGAATC	CCAGGGACACAATGAAGCA	FAM TCGGCTCCGCAGTCTGACCAAT	NM_008286
cPKA	AGTACTTGGCCCCCGAGATTA	ACTCCGAGAGCCCACCAGT	FAM CCTGAGCAAAGGCTACAACAAGGCTGT	NM_008854
Gαs	CAACTTCCACATGTTCGATGTG	ACATCATTGAAGCACTGGATCC	FAM AGCGCGATGAACGCCGCA	NM_010310
AC6	GCCTCAGCCTGCTTACGTG	GTGTCGGTTACACACCACCAT	FAM CCTGCTGACCTGTGCCTCTGTCCTT	NM_007405
ANF	GTGCGGTGTCCAACACAGA	TTCTACCGGCATCTTCTCCTC	FAM CTGATGGATTTCAAGAACCTGCTAGACCACC	K02781
MHCα	ACTACGCCTTCGTCTCTCAGG	TATCAGTGGCCAAGAGCTCCT	FAM AGAGGTGTCCGTGGCCTCCATTGA	NM_010856
MHCβ	AGGGTGGCAAAGTCACTGCT	CATCACCTGGTCCTCCTTCA	FAM AGTCACCGTCTTGCCATTCTCCGTCT	AY056464

HIS2, Histamine2 receptor; cPKA, catalytic subunit of PKA; Gαs, Gαs protein; AC6, adenylate cyclase 6; ANF, atrial natriuretic factor; MHCα, myosin heavy chain α; and (MHCβ), myosin heavy chain β.

extracted and purified using a RNeasy® Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.). RNA quality and concentrations were determined spectrophotometrically at 260 and 280 nM. cDNA was synthesised in 10 μl total volume by the addition of 6 μl well^{-1} RT master mix consisting of (final concentrations): 400 nM assay-specific reverse primer, 500 μM deoxynucleotides, Superscript II buffer, DTT and 10 U Superscript II reverse transcriptase (Life Technologies, Rockville, MD, U.S.A.), to a 7700 96-well plate followed by a 4 μl volume of sample (25 ng μl^{-1}). Each sample was measured in triplicate plus a CTRL without reverse transcriptase. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log range and a no-template CTRL. Plates were covered with Biofilm A (MJR) and incubated in a thermocycler (MJR, Waltham, MA, U.S.A.) for 30 min at 50°C followed by 72°C for 10 min. Subsequently, 40 μl of a PCR master mix (400 nM forward and reverse primers, 100 nM fluorogenic probe, 3 mM $MgCl_2$, and 200 μM deoxynucleotides, PCR buffer and 1.25 U Taq polymerase (Life Technologies, Rockville, MD, U.S.A.)) was added directly to each well of the cDNA plate. RT master mixes and all RNA samples were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan U.S., Research Triangle Park, NC, U.S.A.); PCR master mixes were pipetted utilising a Biomek 2000 robotic workstation (Beckman, Fullerton, CA, U.S.A.). Each assembled plate was then capped and run in the 7700 using the following cycling conditions: 95°C, 1 min; 40 cycles of 95°C, 12 s and 60°C, 1 min. The resulting data were analysed using SDS software (Applied Biosystems, Foster City, CA, U.S.A.) with TAMRA as the reference dye.

Synthetic DNA oligos used as standards (sDNA) encompassed exactly the entire 5′–3′ amplicon for the assay (Biosource International, Camarillo, CA, U.S.A.). It has been shown for several assays that *in vitro* transcribed RNA amplicon standards (sRNA) and sDNA standards have the same PCR efficiency when the reactions are performed as described above.

Owing to the inherent inaccuracies in quantitating total RNA by absorbance, the amount of RNA added to an RT–PCR from each sample was more accurately determined by measuring the β-actin transcript levels in each sample. The final data were normalised to β-actin and are presented as the molecules of transcript/molecules of β-actin × 100 (% β-actin).

Statistical analysis

Data are shown as mean ± s.e.m. (standard error of the mean). Student's *t*-test was applied to compare two groups. ANOVA was applied to compare more than two groups and Games–Howell's correction was used as a *post hoc* test. $P<0.05$ was considered significant.

Results

In vivo *cardiac parameters*

The coronary occlusion resulted in an infarction of 30% of the left ventricle (Michael *et al*., 1999) (data not shown). Doppler studies were used to measure mitral EPV and AoPV in all groups at preocclusion, 2 weeks postocclusion and 5 weeks postocclusion and percent changes from baseline were calculated. Baseline values were not significantly different among any of the groups (Table 2).

In all groups, mitral EPV decreased in the first 2 weeks postocclusion (CO PO 88 ± 1.6%, CARV 95 ± 4.5%, ALP 87 ± 2% and ICI 93 ± 4.8% of preocclusion values) with no significant difference among the treatment groups (Table 2 and Figure 1a). Mean EPV at 2 weeks postocclusion was significantly decreased from preocclusion values. Mean AoPV was also significantly reduced at 2 weeks postocclusion. When analysed for each group (though treatment had not yet commenced), AoPV was either reduced or unchanged in all groups at 2 weeks postocclusion (CO PO 82 ± 2.8%, CARV 92 ± 5.2%, ALP 90 ± 2.5% and ICI 103 ± 8% of preocclusion values).

After 3 weeks of drug treatment (5 weeks postinfarct) EPV was improved back to the preocclusion values (100 ± 7.3%) in the CARV group, while there was no change in the ICI (92 ± 6.5%) or a further reduction in the ALP and CO PO group (79 ± 3.2% and 77 ± 9.8%, respectively) (Figure 1a). The improvement in the CARV group was significant compared to the CO PO and ALP group. EPV was not altered in the ICI group during drug treatment. AoPV had no further decrease between weeks 2 and 5 (CARV 94 ± 5%, ALP 93 ± 7%, ICI 88 ± 7% and CO PO 82 ± 7% of preocclusion values) (Figure 1b).

There was no change in heart rate in all the groups at 2 or 5 weeks postocclusion (data not shown).

Table 2 Summary of cardiac *in vivo* parameters 2 and 5 weeks after permanent occlusion (PO) collected from mice during drug treatment using Doppler technique. Values are expressed as mean ± s.e.m.

Parameters	*CARV* n=*11*		*ALP* n=*9*		*ICI 118,551* n=*10*		*CO PO* n=*6*	
	2-week PO	*5-week PO*	*2-week PO*	*5-week PO*	*2-week PO*	*5-week PO*	*2-week PO*	*5-week PO*
EPV (% of pre)	95 ± 4.5	100 ± 7.3	87 ± 2.0**	79 ± 3.2*,**	93 ± 4.8	92 ± 6.5	88 ± 1.6**	77 ± 9.8*
(EPV preoccl.; cm s^{-1})	(75.4 ± 2.9)		(80.3 ± 1.8)		(64.6 ± 2.3)		(83.2 ± 2.6)	
AoPV (% of pre)	92 ± 5.2	94 ± 5.2	90 ± 2.5	93 ± 5.0	103 ± 8.0	88 ± 7.3	82 ± 2.8**	82 ± 7.5
(AoPV preoccl.; cm s^{-1})	(109.4 ± 5.7)		(108.3 ± 4.7)		(107.4 ± 3.2)		(119.8 ± 6.2)	
BW (% of pre)	95 ± 1.9**	101 ± 1.5	94 ± 1.5**	94 ± 1.7*, **	116 ± 2.5**	125 ± 2.2**	97 ± 1.3	100 ± 1.8
(BW preoccl.; g)	(27.5 ± 0.5)		(26.2 ± 0.4)		(23.7 ± 0.5)		(25.5 ± 0.4)	

The values represent the percent change of preocclusion (preoccl.) values, which are listed in parentheses: EPV, cm s^{-1}; AoPV, cm s^{-1}; BW, g. *$P<0.05$ *versus* CARV (ANOVA). **$P<0.05$ *versus* preocclusion values (paired *t*-test).

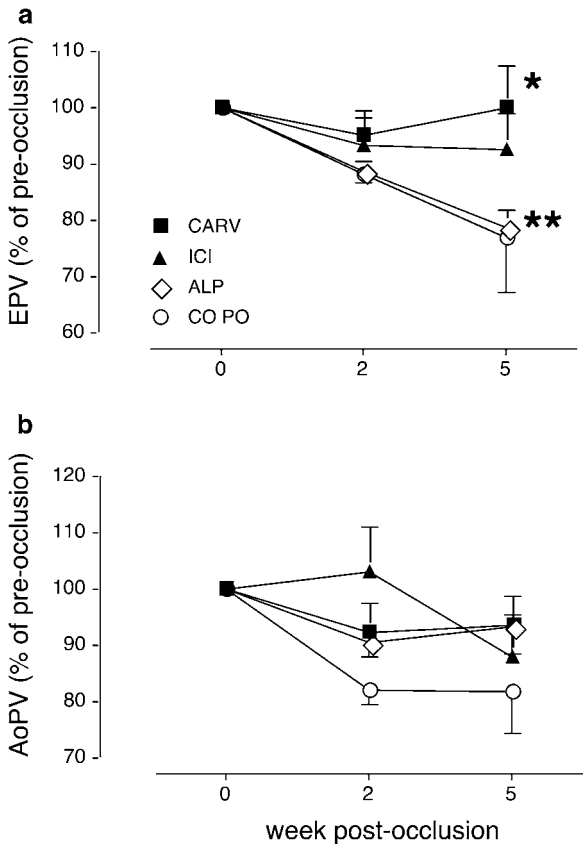

Figure 1 Hemodynamic function in mice subjected to permanent occlusion of the left anterior descending coronary artery. Doppler studies were used to measure: (a) mitral EPV and (b) AoPV in all animal groups at preocclusion, 2-week and 5-week postocclusion. Drug treatment was started 2 weeks following permanent occlusion. Data are percent of preocclusion values and are expressed as mean ± s.e.m. No-drug treatment-permanent occlusion (CO PO) ($n=14$), alprenolol (ALP) 2.4 – 7.2 mg g^{-1} food ($n=9$), carvedilol (CARV) 0.4 – 2.4 mg g^{-1} food ($n=11$) and ICI-118,551 (ICI) 11.6 – 70 μg μl^{-1} ($n=10$). *$P<0.05$ CARV 5-week postocclusion *versus* ALP and CO PO 5-week postocclusion (unpaired *t*-test). **$P<0.05$ ALP 5-week postocclusion *versus* ALP preocclusion value (paired *t*-test).

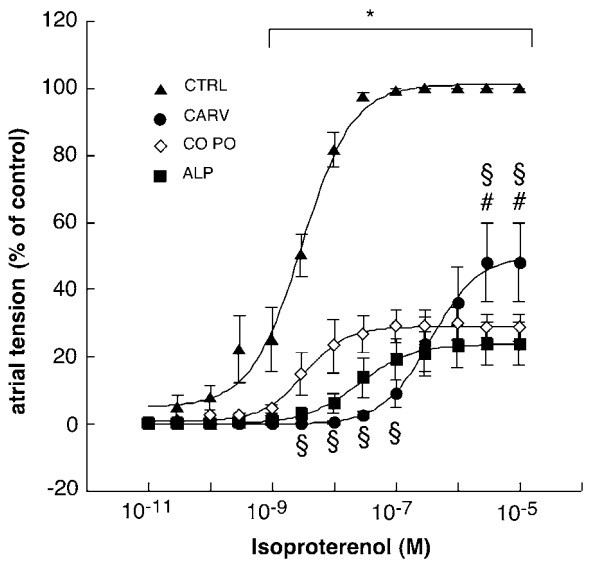

Figure 2 At 6-week postocclusion (after 4 weeks of drug treatment) mice were killed, left atria were isolated and placed in oxygenated modified Kreb's solution. The atria were paced (0.5 ms, voltage at threshold +20%, and optimal frequency of 3.2 Hz) and isometric tension was recorded. Cumulative responses to increasing concentrations of isoprenaline were measured. Data are normalised to CTRL values. CTRL ($n=5$; EC50: 2.5×10^{-9}), CARV ($n=9$; EC50: 5.4×10^{-7}), ALP ($n=9$; EC50: 2.5×10^{-8}), CO PO ($n=6$; EC50: 3.3×10^{-9}). Data are expressed as mean ± s.e.m. *$P<0.05$ *versus* all other groups (ANOVA); $^{\S}P<0.05$ *versus* CTRL (ANOVA); $^{\#}P<0.05$ *versus* ALP (ANOVA).

In vitro *isolated left atrial tension*

We performed isoprenaline (ISO) concentration – response curves on the isolated left atria of mice from the treatment groups and from untreated mice without surgery (CTRL) (Figure 2). The baseline left atrial tension was not different among the groups (CTRL 127.3 ± 29.5 mg; CO PO 110.9 ± 36.5 mg; CARV 184.4 ± 15.4 mg; ALP 169.1 ± 27 mg). For technical reasons (equipment failure), we were unable to record baseline atrial tension for the ICI-118,551 group. The maximal response to ISO of CTRL was set as 100% (347.8 ± 44.7 mg) and responses of all groups were calculated as percentage of CTRL (Figure 2). In CO PO mice, the response to ISO was significantly reduced to approximately 30% of response of CTRL ($P<0.05$). In CARV and ALP mice, the response to ISO was shifted to the right (EC50: 2.5×10^{-9}, 3.3×10^{-9}, 5.4×10^{-7} and 2.5×10^{-8} for CTRL, CO PO, CARV, and ALP, respectively). In the group treated with CARV the maximum response to ISO was significantly increased, compared to CO PO, to 60% of response of CTRL ($P<0.05$, unpaired *t*-test). Mice in the ALP-treatment group did not show any improvement and their maximal response to ISO was similar to CO PO.

Quantification of G protein

Gαi, Gαs, Gαq/11 protein levels in the myocardial membrane fraction of ventricle and the right atria were quantified using Western blotting (Table 3). Bands representing either Gαi, Gαs, or Gαq/11 were normalised for loading variability to a nonspecific band on the Gαq/11 blot at approximately 75 kDa that was not altered by drug treatment. In all the groups, there were no changes in Gαs and Gαq/11 protein levels (Table 3). However, Gαi protein levels were increased in the CO PO group by a factor of 2.7 compared to CTRL (Table 3). Following drug treatment with ALP and CARV, Gαi protein levels were significantly reduced to the same level as CTRL protein levels (Table 3). ICI and DMSO CO PO both tended to increase Gαi levels compared to CTRL, but this increase was not significant (Table 3).

Effects of PO and drug treatment on mRNA levels of selected genes with a known role in cardiac physiology

Following 6 weeks of PO, total RNA was extracted from hearts of all treatment groups. Eight genes were investigated using quantitative RT – PCR (Heid *et al*., 1996; Bustin, 2000). β-actin levels were unchanged in all groups and all data were normalised to β-Actin (expressed as percent of β-actin mRNA

Table 3 Western immunoblotting GαI, Gαs and Gq/11 protein levels following 4 weeks drug treatment (6 weeks postocclusion)

Mean o.d. (arb. Units)	*CARV* n=7	*ALP* n=9	*CO PO* n=6	*CTRL* n=8
GαI	1.8±0.57**	2.3±0.52**	4.6±1.3s*	1.7±0.41
Gαs	1.5±0.43	2.4±0.66	1.7±0.34	2.1±0.58
Gαq/11	0.88±0.22	0.96±0.16	1.0±0.33	0.89±0.13
	ICI n=6	DMSO CO PO n=4	CTRL n=6	
Gαi	21.6±5.4	19.4±8.6	5.3±2.4	
Gαs	0.78±0.33	0.29±0.25	0.13±0.06	
Gαq/11	2.5±0.94	1.5±0.13	0.86±0.16	

Data are expressed as mean optical density readings in arbitrary units. Bands were normalised to an unaltered nonspecific band on each blot. Data represent mean±s.e.m. of four to nine protein homogenates. Gαi protein level for CO PO and DMSO CO PO were consistently three to four-fold higher than CTRL. *$P<0.01$ *versus* CTRL (ANOVA). **$P<0.05$ *versus* CO PO (ANOVA).

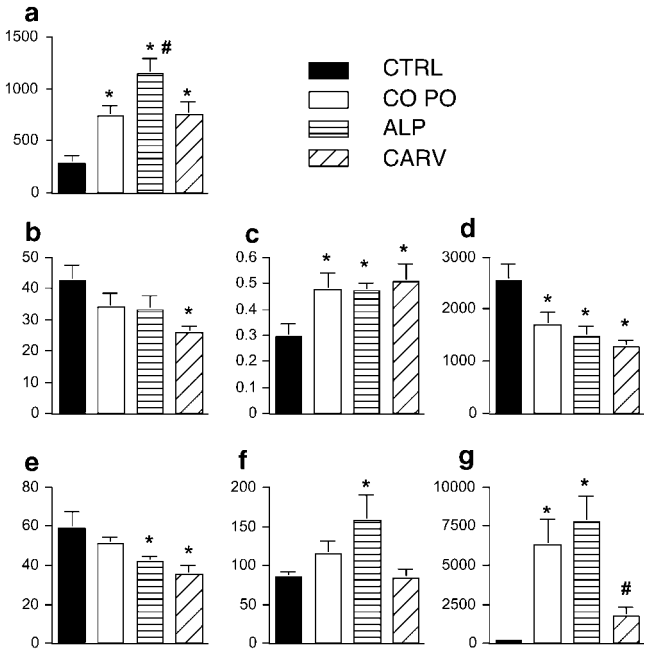

Figure 3 Effect of permanent occlusion and drug treatment on mRNA levels in myocardial tissue using quantitative RT-PCR. RNA was isolated from myocardial tissue using trizol/chloroform extraction and purified using the RNeasy® Mini Kit. mRNA was quantified and normalised to β-actin as a housekeeping gene and graphed as molecules of transcript % β-actin^{-1}. Data are expressed as mean±s.e.m. Seven transcripts were measured: (a) atrial natriuretic factor (ANF), (b) catalytic subunit of PKA (cPKA), (c) histamine2 receptor (HIS2), (d) myosin heavy chain α (MHCα), (e) Gαs protein (Gαs), (f) adenylate cyclase 6 (AC6) and (g) myosin heavy chain β (MHCβ). *$P<0.05$ *vs* CTRL (ANOVA); #$P<0.05$ *vs* control permanent occlusion (CO PO) (ANOVA). Note that alprenolol and carvedilol had differential effects on three transcripts (ANF, AC6 and MHCβ; $P<0.05$, ANOVA).

levels). The other genes investigated were: AC6, cPKA, Gαs, histamine 2 receptor (HIS2), ANF, MHCα and MHCβ.

Compared to CTRL, PO significantly increased the mRNA levels for ANF (by a factor of 2.6), HIS2 (by a factor of 1.6) and MHCβ (by a factor of 43) (Figure 3a, c, and g, respectively). In contrast, PO significantly reduced MHCα mRNA levels (Figure 3d).

Compared with CO PO, treatment with ALP had no effect on mRNA levels of cPKA, HIS2, MHCα, Gαs, or MHCβ (Figure 3b, c, d, e, and g, respectively). ALP treatment enhanced the effect of PO on the mRNA levels of ANF and AC6 ($P<0.005$ *versus* CTRL). The increase in mRNA levels for ANF in ALP-treated mice was significantly higher than both the CO PO and CARV group. Treatment with ALP decreased the Gsα mRNA levels further (70% of CTRL).

Compared with CO PO, treatment with CARV had no effect on gene expression of any of the genes tested except MHCβ. CARV significantly reduced mRNA levels for MHCβ compared to CO PO bringing mRNA levels closer to CTRL values. CARV treatment also reduced cPKA and Gαs mRNA levels significantly when compared to CTRL ($P<0.005$ *versus* CTRL).

The effects of ICI on mRNA levels were evaluated by comparing the ICI-treated mice to DMSO CO PO. When compared to DMSO CO PO there was no difference in mRNA levels for all seven genes investigated (data not shown). However, when compared to CO PO, DMSO CO PO alone significantly reduced mRNA levels of MHCβ to near CTRL values (6275±1626% of β-actin and 323±82% of β-actin for CO PO and DMSO CO PO, respectively; and 147±33% of β-actin for CTRL). Furthermore, DMSO CO PO significantly decreased AC6 mRNA levels compared to the CO PO group (53±8.5% of β-actin and 115±17% of β-actin for DMSO CO PO and CO PO, respectively).

Discussion

CHF is a complex disease associated with high morbidity and mortality. Loss of functional myocardial muscle secondary to ischaemia is the leading cause of heart failure. The addition of a new therapeutic approach, using β adrenoceptor (βAR) antagonists has attracted much attention to possible mechanisms of these previously contraindicated compounds. Also, βAR antagonists improve survival postinfarct in part by decreasing CHF (Gilbert *et al*., 1996).

We used mice with a PO of the left anterior descending coronary artery to investigate possible mechanism(s) of three βAR ligands, CARV, ALP, and ICI-118,551 (ICI). The mice exhibited several symptoms and characteristics of heart failure such as decreased physical activity, decreased atrial contractility to isoprenaline, elevated Gαi protein levels and increased

ANF expression, but did not exhibit other signs of failure such as weight loss or increased mortality (Gould *et al*., 2002).

The three different βAR ligands differ significantly in their pharmacological profile. CARV is a nonselective β1/β2AR ligand, being an inverse agonist with negative efficacy at the β2AR (Nagaraja *et al*., 1999). In addition, CARV also has documented α1AR-antagonist properties and possesses antioxidant properties (Ruffolo *et al*., 1990; Yue *et al*., 1998). ALP acts as a neutral antagonist showing zero negative efficacy in TG4 mice and has β1/β2/β3AR antagonist properties (Blue *et al*., 1990; Bond *et al*., 1995). ICI-118,551, a highly preferential β2AR ligand has been shown to have the highest negative efficacy and inverse agonist activity in the TG4 mouse model (Bond *et al*., 1995). Thus, these three ligands have varying degrees of negative efficacy at the β2AR and were chosen for these experiments based on this characteristic.

In vivo *cardiac parameters*

Using Doppler studies we have measured *in vivo* cardiac function in mice with PO treated with the three βAR ligands. Heart rate did not change in any of the groups. This might be because of the anaesthesia during the Doppler measurements, which lowers heart rate. In mice treated with CARV the EPV, an index of diastolic function (Taffet *et al*., 1996; Zile & Brutsaert, 2002), was improved over time, suggesting that CARV reverses some of the deleterious effects of myocardial infarction induced by PO of the left anterior descending coronary artery. This may result in a more efficient diastole and improved lusitropy and chamber filling. The improvement was also noticeable in the increased physical activity levels of the CARV mice (the mice were more difficult to catch) compared to ALP-treated or-nontreated (CO PO) mice (observations made independently by three scientists experienced in murine models). In contrast to CARV, the mice treated with ALP did not show any improvement in diastolic function, but rather paralleled the worsening in EPV of the CO PO group. In these two groups, diastolic function decreased further to 80% of preocclusion values by the end of 5 weeks. Abnormalities in diastolic function have been reported to be present in all patients with symptomatic heart failure, and diastolic heart failure, alone or in combination with systolic heart failure and increases the mortality and morbidity for patients significantly (Zile & Brutsaert, 2002). Thus, the results of the experiments comparing CARV and ALP suggested that negative efficacy at the β2AR may be a desirable property. We tested this hypothesis by using ICI; however, in the ICI-treated mice, diastolic function was not altered.

A possible explanation for the *in vivo* EPV results comes from the different pharmacological characteristics of ALP, CARV and ICI. ALP was ineffective compared to CARV in improving diastolic function. Two differences between ALP and CARV are that ALP possesses some positive efficacy at the β1AR (Blue *et al*., 1990), while CARV does not, and that CARV has negative efficacy and is an inverse agonist at the β2AR, while ALP is a neutral antagonist (Bond *et al*., 1995). Together, these results suggest that negative efficacy at the β2AR may have alleviated some of the deleterious effects of PO. Since ICI did not alter diastolic function, these results also suggest that the beneficial effect was not solely because of the negative efficacy at the β2AR but that β1-AR blockade was also necessary. ICI possesses greater negative efficacy than CARV at the β2AR (Bond *et al*., 1995), but was not as beneficial as CARV at restoring EPV, suggesting β1AR antagonism or inverse agonism may be required for full beneficial effects. Consistent with this notion is that metoprolol, one of the two approved 'beta-blockers' for the treatment of heart failure, is a preferential β1AR ligand that has also been shown to possess negative efficacy in failing human myocardium (Maack *et al*., 2000). This negative efficacy is likely to be due to inverse agonism at the β2AR for at least two reasons. First, metoprolol exhibits only a four to six fold preference for β1AR *versus* β2AR in human myocardium (Brixius *et al*., 2001), therefore binding at the β2AR is likely. Second, the β1AR has less spontaneous activity relative to the β2AR making inverse agonist effects more likely to be mediated via the β2AR (Zhou *et al*., 2000). The importance of negative efficacy at the β2AR as a beneficial influence in the treatment of heart failure is further supported by the failure of two other 'beta-blockers'. Celiprolol, a β1AR antagonist and a β2AR agonist and bucindolol, a β1AR and β2AR neutral antagonist have shown no beneficial effects in the treatment of heart failure patients (Witchitz *et al*., 2000; Investigators, 2001), suggesting that neither positive efficacy or zero efficacy at the β2AR is helpful in reducing mortality. In summary, these observations suggest that positive efficacy at the β1AR is detrimental, and/or negative efficacy at the β2AR is beneficial together with β1AR blockade.

In addition to EPV, we measured (AoPV), an index of systolic function. Our data revealed that AoPV decreased significantly within two weeks after PO. However, during the period of the drug treatment there was no change in AoPV. These results are consistent with previous work showing that mice of this age group (2 – 3 months) with PO exhibit a significant drop in AoPV within the first 2 weeks, but AoPV does not decrease further in the next 5 – 6 months (Michael *et al*., 1999).

In vitro *isolated left atrial tension*

At 6 weeks after PO the mice were killed, and the *in vitro* contractility of isolated left atria was measured. Although baseline contractility was not affected, PO decreased the contractile response to ISO. The ISO response in CO PO group was reduced by 70% compared to a CTRL group, indicating a severe impairment of the βAR system. This observation is consistent with other models of chronic βAR activation including heart failure (Harding *et al*., 1992; Brodde *et al*., 1998; Litwin *et al*., 1999). In both ALP- and CARV-treated groups, the response curve to ISO was shifted to the right, indicating that the βARs were blocked by the drugs. CARV treatment partially restored the impaired isoprenaline response though the concentrations needed were higher. However, the role of an apparent restoration of the impaired βAR signalling as a mechanism of the beneficial effects of the drug in heart failure is difficult to reconcile given the large rightward shifts produced by the drug.

Downstream of β adrenoceptors: quantification of G protein levels

βARs elicit their lusitropic response mainly through the Gαs/cAMP signalling pathway (Steinberg, 1999). In addition, β2AR also couple to Gαi (Xiao *et al*., 1999) which has been

shown to be increased by 30–40% in patients with dilated cardiomyopathy (Neumann *et al*., 1988; Feldman *et al*., 1989; Bohm *et al*., 1990). Indeed, there is recent evidence that ICI-118,551 may actually function as an 'agonist' and traffic the β2AR to a preferential coupling with Gαi (Gong *et al*., 2002). There is some evidence that Gαi might activate protein phosphatase and subsequently induce a lusitropic response (Steinberg, 1999). This cAMP-independent pathway might play a more important role in the context of CHF, where β1AR are downregulated and the β2AR have a proportionally more pronounced role in eliciting contractility. In patients with CHF, metoprolol significantly reduced ventricular Gαi after 3 months of treatment (Sigmund *et al*., 1996). In our experiments, we also found an increase in Gαi in mice with PO. Both ALP and CARV reduced Gαi levels in whole myocardium preparations, while only CARV restored diastolic function. This observation would suggest that reduction of Gαi levels was not enough to confer the beneficial effect observed *in vivo*.

In our studies and in failing human hearts, protein levels of Gαq were not altered (Takeishi *et al*., 2000), although the Gαq signalling pathway has been reported to be activated (Bowling *et al*., 1999). It is possible that changes in Gαq may be found only in the ischaemic area in parallel to regional changes in βAR (Gu *et al*., 1998), and that by using whole myocardium preparations these changes may be too small to be detected by Western blots.

Effects of myocardial infarction and drug treatment on mRNA levels of selected genes with a known role in cardiac physiology

mRNA levels from seven selected genes were quantified, that is, AC6, cPKA, Gαs, HIS2, ANF, MHCα and MHCβ. On the transcriptional level, major effects of CHF on Gαs or cPKA levels were not observed. Although both appeared to be downregulated, the differences were not significant. It is noteworthy however, that compared to CO PO, CARV further decreased Gαs expression levels, suggesting a possible redirection of the signalling pathway away from Gαs towards Gαi. We did not measure any effects of ICI on any of the selected genes when compared to DMSO CO PO. However, since DMSO CO PO alone exhibited changes in RNA levels compared to CO PO, these results are difficult to interpret. It is possible that ICI effects were masked by DMSO effects, and therefore not quantifiable.

Histamine exhibits its inotropic effect through the activation of the Gαs/cAMP pathway (Brodde *et al*., 1992) and in patients with dilated cardiomyopathy and in ischaemic cardiomyopathy, both the positive inotropic effects in isolated paced ventricles and the activation of adenylate cyclase by histamine were significantly reduced (Brodde *et al*., 1998). In transgenic mice overexpressing cardiac-specific human β2AR (TG35), we recently observed that 14-day treatment with different βAR ligands (CARV, ALP and ICI-118,551) restored the positive inotropic response to histamine in isolated paced left atria (Liu *et al*., 2002). In our mice with PO, we measured a 50% increase in HIS2 mRNA compared to CTRL. Therefore, we speculate that the HIS2 receptor may indeed be upregulated, and as a consequence, positive inotropic effects mediated through HIS2 may play a more prominent role in our model of CHF.

In the mammalian heart two isoforms of myosin heavy chain are expressed, with MHCβ being the predominant foetal isoform (for review see Weiss & Leinwand, 1996). In humans, MHCβ remains the predominant form in the ventricles, whereas in small rodents an isoform shift from MHCβ to MHCα is observed postnatal, resulting in a predominance of MHCα. In mice, thyroid depletion, aging, cardiomyopathy and pressure overload have been shown to increase MHCβ and decrease MHCα (Weiss & Leinwand, 1996). This increase in MHCβ has a significant impact on cardiac performance, as has been shown recently in mice overexpressing cardiac-specific MHCβ in moderate levels (12% of total MHC) (Tardiff *et al*., 2000). These mice had a substantial decrease in Ca^{2+}-activated myofibrillar ATPase activity and a significant decrease in systolic function (Tardiff *et al*., 2000). In our experiment, MHCβ mRNA levels in mice with PO were increased 40-fold compared to CTRL, which is consistent with other investigations (Depre *et al*., 1998; Lowes *et al*., 2002). We saw a significant decrease in MHCβ mRNA in CARV-treated mice by a factor of three compared to CO PO, whereas ALP had no such effect. Although it is not clear whether this effect was a direct or indirect consequence of the CARV treatment, this result suggests that the expression levels of certain genes mirror *in vivo* parameters. The observed reduction of MHCβ in CHF mice may be an additional reason why CARV has been shown to be more beneficial than other βAR ligands in the treatment of CHF.

ANF protein levels and gene expression have been shown to be upregulated in different animal models of CHF (Poulos *et al*., 1996; De Boer *et al*., 2001; Luchner *et al*., 2001), and in patients with CHF (Arendt *et al*., 1986). Furthermore, in patients with CHF there is a positive correlation between severity of the disease and ANF mRNA levels (De Boer *et al*., 2001). In clinical trials with CHF patients, improved cardiac function under therapy with angiotensin-converting enzyme inhibitors is paralleled by decreased plasma levels of natriuretic peptides (van Veldhuisen *et al*., 1998; Brunner-La Rocca *et al*., 1999), indicating that plasma levels of natriuretic peptides are a reliable biochemical marker for severity of heart failure. In our experiments, we observed an upregulation in ANF mRNA in CO PO mice compared to CTRL mice. Interestingly, we observed a significant additional increase in ANF mRNA in mice treated with ALP. These results correlate well with the *in vivo* data, where cardiac diastolic function decreased under ALP treatment. Like other βAR antagonists, CARV treatment increased plasma natriuretic peptide levels and cardiac mRNA expression in rat myocardia (Ohta *et al*., 2000). However, in patients and in rodents with a dilated cardiomyopathy and elevated plasma atrial natriuretic peptide levels, CARV-treatment is able to reduce plasma natriuretic peptide levels and improve cardiac function (Fujimura *et al*., 2000; Watanabe *et al*., 2000). In our experiments, cardiac ANF mRNA levels in the CARV treated group are increased compared to CTRL, but are not different from CO PO. We conclude, that although CARV increases ANF mRNA levels in healthy rat myocardia, it seems not to affect already increased mRNA levels in ischaemic myocardia. However, we do not know whether CARV lowered plasma ANF levels in our set of experiments.

In summary, we have examined the effects of three ligands with varying degrees of negative efficacy at the β2AR. The

cumulative results of investigating biochemical, isolated tissue, *in vivo* and gene expression data suggest that negative efficacy at the β2AR may be a beneficial property when combined with β1AR blockade.

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