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RESEARCH PAPER

Chronic inhibition of fatty acid amide hydrolase by URB597 produces differential effects on cardiac performance in normotensive and hypertensive rats

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BACKGROUND AND PURPOSE

Fatty acid amide hydrolase (FAAH) inhibitors are postulated to possess anti-hypertensive potential, because their acute injection decreases BP in spontaneously hypertensive rats (SHR), partly through normalization of cardiac contractile function. Here, we examined whether the potential hypotensive effect of chronic FAAH inhibition by URB597 in hypertensive rats correlated with changes in cardiac performance.

EXPERIMENTAL APPROACH

Experiments were performed using perfused hearts and left atria isolated from 8- to 10–week-old SHR, age-matched deoxycorticosterone acetate (DOCA)-salt rats and normotensive controls chronically treated with URB597 (1 mg·kg^{-1}) or vehicle.

KEY RESULTS

URB597 decreased BP only in the DOCA-salt rats, along with a reduction of ventricular hypertrophy and diastolic stiffness, determined in hypertension. We also observed normalization of the negative inotropic atrial response to the cannabinoid receptor agonist CP55940. In the SHR model, URB597 normalized (atria) and enhanced (hearts) the positive ino- and chronotropic effects of the β-adrenoceptor agonist isoprenaline respectively. Ventricular CB_1 and CB_2 receptor expression was decreased only in the DOCA-salt model, whereas FAAH expression was reduced in both models. URB597 caused translocation of CB_1 receptor immunoreactivity to the intercalated discs in the hearts of SHR. URB597 increased cardiac diastolic stiffness and modified the ino- and lusitropic effects of isoprenaline in normotensive rats.

CONCLUSION AND IMPLICATIONS

Hypotensive effect of chronic FAAH inhibition depend on the model of hypertension and partly correlate with improved cardiac performance. In normotensive rats, chronic FAAH inhibition produced several side-effects. Thus, the therapeutic potential of these agents should be interpreted cautiously.

Abbreviations

AEA, anandamide; AM3506, 5-(4-hydroxyphenyl)pentanesulfonyl fluoride; CP55940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol; CPP, coronary perfusion pressure; DOCA, deoxycorticosterone acetate; FAAH, fatty acid amide hydrolase; HR, heart rate; LVP, left ventricular pressure; RPP, rate-pressure product; SBP, systolic BP; SHR, spontaneously hypertensive rats; URB694, 6-hydroxy-[1,1′-biphenyl]-3-yl-cyclohexyl-[11C–carbonyl] carbamate; URB597, 3-(3-carbamoylphenyl)phenyl N-cyclohexylcarbamate; WKY, Wistar–Kyoto rats

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Introduction

Hypertensive heart disease, which includes ventricular hypertrophy, contractile dysfunction and their clinical manifestations (arrhythmias and heart failure), is a leading cause of death associated with high BP (Drazner, 2011). The endocannabinoid system is suggested to buffer increases in BP in hypertension. In support of this, the plasma level of the best known endocannabinoid, **anandamide (AEA)**, was higher in hypertensive patients (Engeli *et al.*, 2012) and in spontaneously hypertensive rats (SHR; Li *et al.*, 2009). Moreover, in acute experiments, (1) the **CB_1 receptor**-mediated hypotension elicited by AEA was stronger in anaesthetized SHR than in normotensive controls (Lake *et al.*, 1997; Bátkai *et al.*, 2004); (2) in conscious SHR, AEA decreased BP, but increased it in normotensive controls (Lake *et al.*, 1997); and (3) the systemic acute blockade of **fatty acid amide hydrolase** (FAAH; the key enzyme responsible for AEA degradation) by **URB597** (Bátkai *et al.*, 2004) and AM3506 (Godlewski *et al.*, 2010) has been shown to normalize BP in SHR. Consequently, FAAH inhibitors are postulated to be potential antihypertensive agents (Bátkai *et al.*, 2004; Godlewski *et al.*, 2010). The chronic administration of URB597 to **deoxycorticosterone** acetate (DOCA)-salt hypertensive rats reduced BP in older rats (in a manner partly dependent on vascular changes) and diminished cardiac hypertrophy in younger animals (Baranowska-Kuczko *et al.*, 2016; Toczek *et al.*, 2016).

Cannabinoids have a negative inotropic effect in human (Bonz *et al.*, 2003) and rat atrial muscle (Sterin-Borda *et al.*, 2005) *via* CB_1 receptors and a positive inotropic effect *via* **CB_2 receptors** (Sterin-Borda *et al.*, 2005). Coronary vasodilation is mediated by CB_1 (Wagner *et al.*, 2005) or *via* non-CB_1/CB_2 receptors (Ford *et al.*, 2002). The reduction of increased cardiac contractility in hypertension is suggested to be one of the major components of the antihypertensive action of FAAH inhibitors after their acute injection (Bátkai *et al.*, 2004; Godlewski *et al.*, 2010). Chronic treatment with the FAAH inhibitor URB694 normalized resting heart rate (HR) and prevented the occurrence of arrhythmia in stressed animals (Carnevali *et al.*, 2015a). In FAAH knockout mice, age-related cardiac dysfunction (Bátkai *et al.*, 2007) was decreased, but doxorubicin-induced cardiotoxicity was enhanced (Mukhopadhyay *et al.*, 2011). Thus, the first aim of our study was to examine whether the potential hypotensive effect of chronic FAAH inhibition by **URB597** in hypertensive rats correlated with changes in cardiac performance.

In hypertension, an elevated sympathetic tone alters **β-adrenoceptor**-induced effects (Guyenet, 2006), which might additionally be modified by cannabinoids. There is evidence that cannabinoid agonists **HU210** (Maslov *et al.*, 2004), AEA (Gaskari *et al.*, 2005) and **WIN55212-2** (Liao *et al.*, 2013) can diminish the following responses to the non-selective β-adrenoceptor agonist **isoprenaline**: its positive ino- and/or chronotropic effects and/or stimulation of cAMP production in isolated hearts, ventricular papillary muscle and neonatal cardiomyocytes respectively. Moreover, acute administration of URB694 reduced the occurrence of isoprenaline-induced ventricular tachyarrhythmias (Carnevali *et al.*, 2015b). Therefore, the second aim of our study was to examine the influence of chronic URB597 administration on β-adrenoceptor-mediated effects in cardiac tissues isolated from normotensive and hypertensive rats.

Methods

Animals

All animal care, surgical procedures and experimental protocols were performed in accordance with the European Directive (2010/63/EU) and Polish legislation and were approved by the local Animal Ethics Committee in Białystok (Poland). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). The study was carried out in compliance with the replacement, refinement or reduction (the 3Rs). Male rats were obtained from the Centre for Experimental Medicine of the Medical University of Białystok (Poland). They were housed in collective plastic cages (two rats per cage) with sawdust on the bottom in a temperature-controlled room at 22 ± 1°C under a 12:12 h light–dark cycle with free access to water and standard laboratory food unless otherwise noted. We used age-matched rats with comparable primary (SHR; the most frequently studied genetic hypertensive model) and secondary (DOCA-salt) hypertension in order to distinguish changes induced by hypertension from those related to any one particular hypertension model. We used the DOCA-salt model because a salt-rich diet is one of the main lifestyle factors leading to hypertension.

DOCA-salt hypertension was induced in Wistar rats (6–7 weeks old; initially weighing 260–300 g) as described previously (Toczek *et al.*, 2016). Animals were anaesthetized by i.p. injection of pentobarbital sodium (70 $mg \cdot kg^{-1}$, i.e. ~300 $\mu mol \cdot kg^{-1}$). The right kidney was removed in all rats *via* a right lateral abdominal incision. After 1 week of recovery, hypertension was induced by subcutaneous injections of 11-DOCA (25 $mg \cdot kg^{-1}$, i.e. ~67 $\mu mol \cdot kg^{-1}$; 0.4 $mL \cdot kg^{-1}$) twice weekly for 6 weeks and replacement of drinking water with 1% NaCl solution. Control sham-operated rats (SHAM) received the vehicle for DOCA (N,N-dimethylformamide) twice weekly and drank tap water.

Chronic treatment with URB597

We applied the same protocol and dose of URB597 as reported previously (Toczek *et al.*, 2016) and age-matched DOCA-salt hypertensive rats, in which 2 weeks of URB597 administration was previously reported to decrease BP (Toczek *et al.*, 2016). URB597 (1 $mg \cdot kg^{-1}$, i.e. ~3 $\mu mol \cdot kg^{-1}$; 1 $mL \cdot kg^{-1}$) or its vehicle – a mixture of DMSO, Tween 80 and saline (1:2:7) – was injected i.p. for 14 days, twice daily for the following four groups of rats: hypertensive (1) DOCA-salt (4 weeks after the onset of DOCA-salt administration); (2) 8- to 10–week-old male SHR (270–320 g) and their respective normotensive controls: (3) age-matched SHAM (4 weeks after the onset of vehicle for DOCA-salt administration); and (4) Wistar–Kyoto rats (WKY; 290–390 g). Rats were assigned randomly to the different experimental groups. Before the first dose of URB597 or its vehicle and 12 h after the final dose, systolic BP (SBP) was recorded in conscious rats by the non-invasive tail-cuff method using the Rat Tail Blood

Pressure Monitor from Hugo Sachs Elektronik-Harvard Apparatus (March–Hugstetten, Germany).

Isolated Langendorff heart preparation

Twelve hours after the final dose of URB597 (or its vehicle), rats were anaesthetized with 300 μmol·kg^{-1} pentobarbital sodium i.p. and then injected with 500 IU heparin i.p. (Polfa, Warsaw, Poland). Hearts were rapidly excised, weighed and immediately mounted in a Langendorff system (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). Hearts were perfused at a constant flow rate (12 mL·min^{-1}) with oxygenated (95% O_2 and 5% CO_2) Krebs–Henseleit solution of the following composition (mM): NaCl 118.0, KCl 4.8, $CaCl_2$ 1.8, $NaHCO_3$ 24.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.0, Na-pyruvate 5.0, EDTA 0.03 (pH 7.4; 37°C). A distilled water filled latex balloon connected to a pressure transducer and inflated to increase the left ventricular end-diastolic pressure to approximately 8–10 mmHg was inserted into the left ventricle through the mitral valve to measure the left ventricular pressure (LVP). Another pressure transducer located just above the aorta recorded coronary perfusion pressure (CPP). Hearts were allowed to beat spontaneously. After a 5 min stabilization period, increments in balloon volume (using a 50 mL Hamilton syringe connected to the fluid-filled pressure transduction circuit) were applied to the heart at 0, 5, 10, 15, 20 and 30 mmHg, and left ventricular end-diastolic pressure was recorded. Myocardial diastolic stiffness was calculated as the diastolic stiffness constant (κ, dimensionless), the slope of the linear relation between tangent elastic modulus (E, dyne·cm^{-2}) and stress (σ, dyne/cm^2) as previously described (Loch *et al.*, 2007).

Heart parameters were continuously monitored using the ISOHEART^R software. Preparations were allowed to stabilize for 25 min. Two hearts (one from WKY URB597 and one from SHAM URB597 group) with persistent arrhythmias and poor contractility (LVP < 75 mmHg) were excluded from the study. After the stabilization period, increasing concentrations of isoprenaline (0.01 nM – 1 μM) were infused by a peristaltic pump (Ascor, Warsaw, Poland) at one hundredth of the current flow rate into the coronary arteries until a plateau was reached; concentrations referred to the final concentrations in the heart. Measurements recorded after the initial equilibration period were considered as the baseline values. The cardiac performance of the Langendorff-perfused heart was evaluated from HR, LVP (which is an index of contractile activity) and the rate-pressure product (RPP: HR × LVP, mmHg × beats·min^{-1}), which is an index of cardiac work (Angelone *et al.*, 2008), the maximum rate of positive $+(LVdP/dt)_{max}$ (inotropism) or negative $-(LVdP/dt)_{max}$ (lusitropism) changes in LVP and CPP as an index of coronary dilation. All parameters are expressed as Δ changes from baseline before addition of the first concentration of isoprenaline. After experiments, the left atrium and the right and left ventricle with septum were weighed, and their weights were expressed as a ratio of tissue weight (mg) to total body weight (g).

Isolated left atria

The left atria were dissected before the isolated heart perfusions (see above). They were weighed and mounted in 10 mL organ baths containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1, $NaHCO_3$ 29, $NaHPO_4x12H_20$ 1, $CaCl_2$ 2.25, glucose 10, Na-pyruvate 5, EDTA 0.04 (pH 7.4; 37°C). Atria were continuously stimulated electrically with square wave pulses (just over threshold, 5 ms duration, 2 Hz) using a pair of platinum electrodes. Contractions were recorded using an isometric force transducer (PIM 100RE, Bio-Sys-Tech, Białystok, Poland). Tissue was allowed to equilibrate for 60 min. When a stable amplitude of contractions had been reached, the cannabinoid receptor agonist **CP55940** (1 nM – 30 μM) was cumulatively added to the atria. Atria were then washed several times and sensitized by a brief exposure to 5×10^{-6} M isoprenaline (Dincer *et al.*, 2000). When steady basal values were obtained again, concentration–response curves for isoprenaline (0.01 nM – 3 μM) were constructed. The initial contractile forces (in mN) of atria in normotensive SHAM and WKY measured before the first concentration of **CP55940** were as follows: 2.4 ± 0.1 (n = 11) and 2.5 ± 0.1 (n = 10), respectively, and before the first concentration of isoprenaline were the same 2.4 ± 0.1 for SHAM (n = 10) and WKY (n = 9). Hypertension and URB597 did not affect the basal values of this parameter (data not shown). Agonist responses were measured as the decrease or increase in the basal force (expressed as % of basal values) of the left atrium.

Western blots

Routine Western blotting procedure was used to examine protein expression, as has been described previously (Baranowska-Kuczko *et al.*, 2016). Briefly, samples from the left ventricles were homogenized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). In addition, protein concentration was measured using the bicinchoninic acid method with BSA as a standard. Following this, homogenates were reconstituted in Laemmli buffer, separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with the corresponding primary antibodies in appropriate dilutions: CB_1 (1:200, cat no. Ab23703; Abcam, UK), CB_2 (1:500, cat no. Ab3561; Abcam, UK), FAAH1 (1:500; cat no. Ab54615; Abcam, UK) and GAPDH (1:500, cat no. sc-32 233; Santa Cruz Biotechnology, USA). Thereafter, nitrocellulose membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, USA). After adding a suitable substrate for horseradish peroxidase (Thermo Scientific, Rockford, IL, USA), protein bands were quantified densitometrically using a ChemiDoc visualization system EQ (Bio-Rad, Warsaw, Poland). Equal protein loading in each line was confirmed by Ponceau S staining. The level of protein expression was standardized to GAPDH.

Immunohistochemistry

For immunohistochemistry, the EnVision method was used (Baranowska-Kuczko *et al.*, 2016) using antibodies against: CB_1 (1:2000 rabbit; Anti-CB_1 cat no. Ab23703; Abcam, UK) and CB_2 (1:200 rabbit Anti-CB_2 cat no. Ab3561; Abcam, UK). To test the specificity of the antibodies, a negative control was included, in which the antibodies were replaced by normal rabbit serum (Vector Laboratories, CA, USA) at

the respective dilution (no staining), and a positive control, which was prepared from rat hippocampus for CB_1 and human tonsil for CB_2 (data not shown). The obtained results of immunohistochemical staining were viewed on an Olympus BX41 microscope with an Olympus DP12 camera under a magnification of 200× (20× lens and 10× eyepiece).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The results are given as the mean ± SEM (n = number of animals). The exact group size (n) for each experimental group/condition is provided in Tables 1–4 and 'n' refers to independent values, not replicates. Researchers were not blinded to the experimental conditions, but efforts were made to be close to the conditions of blinded assays, and our analysis did not include any subjective evaluation. Thus, (1) all the cardiac tissues were obtained *via* the same procedures; (2) they were treated in the same way; and (3) all data were obtained *via* direct recording of physiological parameters.

Maximal effects of agonists (E_{max}) and their potencies (as pEC_{50} values) were determined from the individual concentration–response curves. Values of E_{max} for isolated hearts and atria represent respective maximal changes from baseline and % of basal values, as their basal values in control normotensive SHAM and WKY were different in hearts (Table 2) and comparable in atria (see above). Due to dual cardiac effect of isoprenaline and the lack of the pronounced E_{max} for CP55940 in atria, E_{max} were determined for the following agonist concentrations: isoprenaline in the heart: 0.01 nM – 0.1 μM; and CP55940 in atria – 30 μM. Statistical analysis was performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA). Intergroup statistical comparisons were made by ANOVA followed by Bonferroni's multiple comparison test of the entire data set. *Post hoc* tests were only performed where the F-ratio of the ANOVA highlighted a significant difference ($P < 0.05$). The Student's t-test for paired and unpaired data was used as appropriate. Differences were considered significant at $P < 0.05$.

Materials

CP55940 (Tocris, Bristol, UK); heparin sodium (Polfa, Warsaw, Poland); 11-DOCA, N,N-dimethylformamide, (−)-isoprenaline (±)-bitartrate salt, Tween 80 (Sigma-Aldrich, Munich, Germany); URB597; Cayman Chemical Company, Ann Arbor, MI, USA); pentobarbital sodium (Biowet, Puławy, Poland). Stock solutions of isoprenaline were prepared in distilled water and further diluted with Krebs solution. CP55940 was dissolved in DMSO; further dilutions were made with Krebs solution such that the final concentration of the DMSO in the organ bath was less than 0.01%.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

General

Before application of the first dose of URB597, or its vehicle, SBP was about 70% higher in both hypertensive models. compared with normotensive controls (Table 1). URB597 reduced SBP in DOCA-salt by about 20% but did not change SBP in SHR. HR or body weight were also unchanged in DOCA-salt and SHR. Basal HR was reduced (by about 10%) in WKY and SHR both in the absence and the presence of URB597, 2 weeks after the experiment began.

Heart weight to body weight ratios were higher by about 50 and 20% in DOCA-salt and SHR compared with SHAM and WKY respectively (Table 1). The cardiac hypertrophy in DOCA-salt was connected with an increase in hypertrophy indices in all cardiac compartments. In contrast, in SHR, cardiac hypertrophy was associated with an increase in the ratio of left ventricular and septum weight to body weight only. URB597 reduced the elevated index of left ventricular and septum weight to body weight observed in DOCA-salt.

Diastolic stiffness was increased by about 40% in hearts of both hypertensive models (Table 1). URB597 diminished the enhanced diastolic stiffness observed in hypertension by about 20% in DOCA-salt, but not in SHR. Surprisingly, in normotensive animals, URB597 increased diastolic stiffness by about 15 and 50% in SHAM and WKY respectively.

Influence of hypertension and URB597 on basal parameters of isolated hearts

Values of all basal parameters of hearts isolated from DOCA-salt and SHAM were comparable (Table 2). In SHR, indices of cardiac contractility (LVP), work (RPP), maximal rate of LV contraction and relaxation were higher by about 50% than in WKY. These values were comparable with the respective values in DOCA-salt. URB597 did not modify any basal parameters of isolated hearts in normo- or hypertension.

Effects of hypertension and chronic URB597 administration on isoprenaline-induced cardiostimulatory effects in isolated hearts

Isoprenaline (0.01 nM – 1 μM) caused concentration-dependent increases of all cardiac parameters, which were comparable in hearts isolated from normotensive rats of both groups (for the respective pEC_{50} and E_{max} values determined for isoprenaline 0.01 nM – 0.1 μM, see Table 3; Figures 1 and 2). One exception was a lower increase in LVP (for E_{max} $P < 0.05$; but not its pEC_{50} value) in SHAM compared with WKY. Hypertension attenuated all cardiostimulatory effects of isoprenaline (mainly at the lower concentrations; Table 3; Figures 1 and 2). Maximal responses were reduced by about 50–60% for LVP (DOCA-salt and SHR) and by about 40% for $+(LVdP/dt)_{max}$ (DOCA-salt) and for $-(LVdP/dt)_{max}$ (SHR). Vasodilatory effects of isoprenaline on coronary arteries were completely prevented by hypertension. A weaker potency of isoprenaline was only observed for two parameters in SHR: $+(LVdP/dt)_{max}$ and RPP.

URB597 tended to enhance, the reduced in hypertension increases in LVP, $+(LVdP/dt)_{max}$ and RPP (but not other parameters), which were induced by higher (0.01 nM –

Table 1

Influence of URB597 on physiological parameters of DOCA-salt and SHR and their respective normotensive SHAM and WKY

Parameters	SHAM	SHAM URB597	DOCA-salt	DOCA-salt URB597	WKY	WKY URB597	SHR	SHR URB597
n	10	10	10	11	10	9	10	10
SBP (mmHg)								
day 0	131 ± 7	131 ± 8	217 ± 16*	201 ± 13	104 ± 8	105 ± 6	183 ± 7*	199 ± 12
day 14	137 ± 5	118 ± 10	217 ± 17*	159 ± 10†,#	105 ± 6	92 ± 4	196 ± 10*	182 ± 7
HR (beats·min^{-1})								
day 0	362 ± 8	363 ± 10	360 ± 17	374 ± 11	343 ± 23	333 ± 9	383 ± 12	376 ± 10
day 14	357 ± 6	350 ± 14	370 ± 15	355 ± 11	280 ± 8#	317 ± 12†	357 ± 8*	349 ± 7#
Body weight (g)								
day 0	302 ± 7	291 ± 10	264 ± 2*	267 ± 4	333 ± 10	340 ± 11	288 ± 6*	295 ± 6
day 14	310 ± 8	304 ± 10	271 ± 9*	277 ± 6	334 ± 10	343 ± 10	287 ± 5*	291 ± 5
Heart weight/body weight (mg·g^{-1})	4.0 ± 0.2	4.7 ± 0.3	5.9 ± 0.3*	5.5 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	5.6 ± 0.1*	5.8 ± 0.1
LV + septum weight/body weight (mg·g^{-1})	2.26 ± 0.07	2.32 ± 0.11	3.60 ± 0.14*	3.15 ± 0.14†	2.53 ± 0.06	2.47 ± 0.04	3.33 ± 0.04*	3.54 ± 0.10
RV weight/body weight (mg·g^{-1})	0.61 ± 0.05	0.66 ± 0.04	0.87 ± 0.04*	0.74 ± 0.05	0.71 ± 0.03	0.67 ± 0.02	0.76 ± 0.02	0.78 ± 0.02
LA weight/body weight (mg·g^{-1})	0.073 ± 0.010	0.082 ± 0.004	0.155 ± 0.014*	0.129 ± 0.009	0.091 ± 0.005	0.088 ± 0.002	0.079 ± 0.002	0.076 ± 0.004
Diastolic stiffness constant (κ)	22.9 ± 0.7	26.3 ± 0.8†	32.4 ± 1.0*	26.2 ± 0.9†	21.6 ± 1.5	35.1 ± 1.1†	30.8 ± 0.9*	29.6 ± 2.8

Units are given in brackets. URB597 (1 mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. SBP and HR were recorded before (day 0) the first dose of URB597 or its vehicle and 14 days later. Heart and body weights were determined 12 h after the last dose of URB597 or its vehicle. Diastolic stiffness was measured at the beginning of Langendorff experiment. Weights of right and left ventricle (RV and LV), septum and left atrium (LA) were determined after the end of *in vitro* experiments. Data are given as the means ± SEM.

*$P < 0.05$ DOCA-salt and SHR versus SHAM and WKY respectively;

†$P < 0.05$, significant effect of URB597; ANOVA with Bonferroni *post hoc* test

#$P < 0.05$, significantly different from values before URB597 treatment (day 0 vs. day 14); Student's *t*-test for paired data.

Table 2

Influence of URB597 on basal parameters of hearts isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY

Parameters	SHAM	SHAM URB597	DOCA-salt	DOCA-salt URB597	WKY	WKY URB597	SHR	SHR URB597
n	10	10	10	11	10	9	10	10
HR (beats·min^{-1})	273 ± 7	293 ± 9	273 ± 9	269 ± 8	274 ± 14	254 ± 7	282 ± 10	260 ± 8
LVP (mmHg)	120 ± 8	131 ± 10	132 ± 10	131 ± 9	90 ± 5	90 ± 3	134 ± 10*	137 ± 8
+(LVdP/dt)$_{max}$ (mmHg·s^{-1})	3353 ± 217	3472 ± 274	3482 ± 240	3492 ± 268	2431 ± 222	2161 ± 631	3610 ± 204*	3307 ± 122
−(LVdP/dt)$_{max}$ (mmHg·s^{-1})	−2422 ± 174	−2846 ± 250	−2560 ± 240	−2798 ± 222	−1918 ± 125	−1924 ± 58	−2898 ± 234*	−3031 ± 165
RPP (mmHg·beats·min^{-1})	30 210 ± 2309	36 106 ± 3190	33 676 ± 2488	33 698 ± 2527	21 773 ± 1143	20 162 ± 620	34 243 ± 1587*	32 286 ± 1666
CPP (mmHg)	94 ± 8	92 ± 9	86 ± 8	85 ± 5	64 ± 4	66 ± 3	75 ± 3	76 ± 3

Units are given in brackets. URB597 (1 mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. Hearts were isolated 12 h after the final dose of URB597 or its vehicle. Data are given as the means ± SEM.

*$P < 0.05$, SHR significantly different from WKY; ANOVA with Bonferroni *post hoc* test; LVP, the maximum rate of positive +(LVdP/dt)$_{max}$ and negative −(LVdP/dt)$_{max}$ changes in LVP.

0.1 μM) isoprenaline concentrations in DOCA-salt hypertensive rats; in the case of maximal responses by about 35, 40 and 25% respectively (for the respective pEC_{50} and E_{max} values, see Table 3, Figures 1 and 2). In contrast, the only effect of URB597 in SHR was enhancement of the positive chronotropic effect of isoprenaline at higher (0.01 nM – 0.1 μM) concentrations (by approximately 40% for E_{max}). However, the potency of isoprenaline was not affected (Figure 2A′, Table 3).

In both normotensive groups, URB597 reduced the following cardiostimulatory effects of isoprenaline at lower concentrations: LVP, +(LVdP/dt)$_{max}$, −(LVdP/dt)$_{max}$ and RPP. Moreover, in SHAM, URB597 decreased the increases in LVP and HR induced by higher and lower concentrations of isoprenaline respectively (Figures 1 and 2). In WKY, URB597 diminished the potency of isoprenaline for +(LVdP/dt)$_{max}$ and RPP (Table 3). However, isoprenaline-induced coronary vasodilatation in hypertensive and normotensive rats (Figure 2C, C′) was not altered by FAAH inhibition.

Effects of hypertension and chronic URB597 administration on isoprenaline- and CP55940-induced changes in the contractile force of isolated left atria

Isoprenaline (0.01 nM – 3 μM) induced a concentration-dependent increase in the contractile force of atria isolated from normo- and hypertensive rats. Hypertension and URB597 did not alter the positive inotropic effect of isoprenaline in DOCA-salt and in SHAM (for the respective pEC_{50} and E_{max} values, see Table 4, Figure 3A).

The inotropic effect of the higher concentration of isoprenaline in atria isolated from WKY was higher than in SHAM (in the case of the E_{max} value by approximately 110%; $P < 0.05$). The E_{max} value in SHR was lower by about 60% than in WKY. URB597 almost completely restored the positive inotropic effect of isoprenaline in SHR. Interestingly, it also strongly enhanced isoprenaline-induced increase in contractile force observed in WKY. The maximal response was increased by about 50% (for the respective pEC_{50} and E_{max} values, see Table 4, Figure 3A′). There was no alteration in the potency of isoprenaline (Table 4) in either model of hypertension or after administration of URB597.

The vehicle alone for CP55940 reduced atrial contractile force by about 5–10% in all experimental groups (Figure 3), in hypertension and in the presence of URB597 (data not shown). CP55940 (1 nM – 30 μM) caused concentration-dependent decreases in the contractile force of left atria (a direct negative inotropic effect); stronger in WKY than in SHAM ($P < 0.05$ for pEC_{50} and E_{max} values; Figure 3B, B′; for the respective pEC_{50} and E_{max} values induced by CP55940 at 30 μM, see Table 4). The concentration–response curves for the negative inotropic effect of CP55940 in normotensive rats were shifted to the right, in atria isolated from DOCA-salt and SHR. URB597 completely restored the negative inotropic effect of CP55940 in the DOCA-salt and the potency of CP55940 in SHR. URB597 did not modify the negative inotropic effect of CP55940 observed in atria isolated from normotensive animals (Figure 3B, B′, Table 4).

Table 3

Influence of URB597 on the isoprenaline (0.01 nM – 1 μM)-induced changes in parameters of hearts isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY

		SHAM		SHAM URB597		DOCA-salt		DOCA-salt URB597	
	n	10		10		10		11	
Parameters		pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
HR (beats·min^{-1})		8.9 ± 0.2	107 ± 6	8.3 ± 0.2	98 ± 10	8.5 ± 0.2	89 ± 12	8.8 ± 0.3	94 ± 12
LVP (mmHg)		9.8 ± 0.7	55 ± 7	8.8 ± 0.4	30 ± 6*	8.7 ± 0.5	29 ± 6†	8.8 ± 0.3	39 ± 5
+(LVdP/dt)$_{max}$ (mmHg·s^{-1})		8.7 ± 0.4	4925 ± 522	8.5 ± 0.2	4124 ± 420	8.4 ± 0.2	2885 ± 397†	8.6 ± 0.2	3933 ± 512
−(LVdP/dt)$_{max}$ (mmHg·s^{-1})		9.3 ± 0.4	−2697 ± 303	8.8 ± 0.3	−2543 ± 335	8.5 ± 0.3	−2148 ± 325	8.7 ± 0.3	−2225 ± 350
RPP (mmHg·beats·min^{-1})		9.5 ± 0.5	30 487 ± 3894	8.7 ± 0.3	22 267 ± 2928	8.7 ± 0.4	20 097 ± 4442	8.7 ± 0.3	24 931 ± 4466
CPP (mmHg)		9.4 ± 0.6	−28 ± 6	9.3 ± 0.3	−32 ± 6	–	–	–	–
	n	WKY 10		WKY URB597 9		SHR 10		SHR URB597 10	
		pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
HR (beats·min^{-1})		8.5 ± 0.3	95 ± 7	9.0 ± 0.1	105 ± 7	8.4 ± 0.3	83 ± 11	8.8 ± 0.2	116 ± 11*
LVP (mmHg)		9.7 ± 0.3	81 ± 9	8.7 ± 0.1	85 ± 8	9.0 ± 0.6	32 ± 10†	8.6 ± 0.4	40 ± 8
+(LVdP/dt)$_{max}$ (mmHg·s^{-1})		9.4 ± 0.3	5526 ± 684	8.4 ± 0.2*	5279 ± 615	8.4 ± 0.2†	4413 ± 585	8.3 ± 0.2	4410 ± 454
−(LVdP/dt)$_{max}$ (mmHg·s^{-1})		9.2 ± 0.4	−2970 ± 290	8.6 ± 0.2	−3166 ± 451	8.3 ± 0.2	−1847 ± 177†	8.2 ± 0.2	−1878 ± 222
RPP (mmHg·beats·min^{-1})		9.5 ± 0.3	29 409 ± 3123	8.6 ± 0.1*	37 038 ± 1066	8.7 ± 0.2†	22 539 ± 2833	8.7 ± 0.2	24 680 ± 680
CPP (mmHg)		9.7 ± 0.5	−26 ± 4	9.1 ± 0.2	−28 ± 3	–	–	–	–

Values are based on the concentration–response curves shown in Figures 1 and 2. Maximal effects (E_{max}) determined for isoprenaline (0.01 nM – 0.1 μM) represent maximum changes from baseline. Units for E_{max} are given in brackets. Data are given as the means ± SEM.

*$P < 0.05$, significant effect of URB597.

†$P < 0.05$ DOCA-salt and SHR significantly different from SHAM and WKY respectively; ANOVA with Bonferroni *post hoc* test; LVP, the maximum rate of positive +(LVdP/dt)$_{max}$ and negative −(LVdP/dt)$_{max}$ changes in LVP.

Table 4

Influence of URB597 on the isoprenaline (0.01 nM – 3 μM)-induced positive inotropic and the CP55940 (1 nM – 30 μM)-induced negative inotropic effects in left atria isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY

	SHAM			SHAM URB597			DOCA-salt			DOCA-salt URB597		
Group	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*
Isoprenaline	8.1 ± 0.2	74 ± 9	9	7.9 ± 0.2	81 ± 12	9	8.3 ± 0.2	65 ± 13	10	8.2 ± 0.2	81 ± 15	11
CP55940	6.1 ± 0.3	−38 ± 4	10	6.8 ± 0.4	−36 ± 4	10	5.0 ± 0.2#	−25 ± 2	8	6.1 ± 0.2†	−48 ± 6†	10
	WKY			**WKY URB597**			**SHR**			**SHR URB597**		
	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*
Isoprenaline	8.3 ± 0.1	156 ± 12	9	8.3 ± 0.1	230 ± 15†	10	8.3 ± 0.1	67 ± 6#	10	8.6 ± 0.1	125 ± 11†	8
CP55940[a]	7.3 ± 0.2	−54 ± 4	9	7.3 ± 0.1	−48 ± 4	10	6.6 ± 0.2	−34 ± 6#	9	7.3 ± 0.2†	−36 ± 5	10

Values are based on the concentration–response curves shown in Figure 3. Maximal effects (E_{max}) are expressed in % of basal values.
[a]In the case of CP55940, E_{max} was determined for the concentration of 30 μM. Data are given as the means ± SEM.
†$P < 0.05$, significant effect of URB597;
#$P < 0.05$ DOCA-salt and SHR significantly different from SHAM and WKY respectively. ANOVA with Bonferroni *post hoc* test.

Influence of hypertension and chronic administration of URB597 on cardiac expression of CB_1, CB_2 receptors and FAAH

Analysis of CB receptor and FAAH expression in left cardiac ventricles by Western blotting showed a single immunoreactive band of the molecular size expected for CB_1 receptors (60 kDa), CB_2 (40 kDa) and FAAH (67 kDa). The expression of CB_1, CB_2 receptors and FAAH decreased in tissues from hypertensive DOCA-salt rats, whereas in SHR, only FAAH was down-regulated. URB597 did not modify expression levels in normotensive animals. It just prevented the previously observed decrease in FAAH expression and tended to do so with respect to CB_1 and CB_2 receptor expression, in DOCA-salt but not in SHR (Figure 4).

Immunolocalization of CB_1 and CB_2 receptors (Figure 5) revealed their appearance in cardiomyocytes of the left cardiac ventricle, although the immunoreactivity for CB_1 receptors appeared more intense. Both CB_1 and CB_2 immunoreactivities were less intense in both models of hypertension (especially in DOCA-salt) compared with their respective normotensive controls. URB597 increased labelling for CB_1 receptors in both hypertensive models and for CB_2 receptors in DOCA-salt only. Interestingly, in both models of hypertension treated with URB597, a clearly visible signal for CB_1 receptors was noticed (stronger in SHR rather than DOCA-salt rats), in the region of the intercalated discs of the heart.

Discussion

Chronic URB597 administration produces age-dependent hypotension in DOCA-salt hypertensive rats (Toczek *et al.*, 2016), which is partly dependent on vascular changes (Baranowska-Kuczko *et al.*, 2016). Here, we examined whether the potential hypotensive effect of chronic FAAH inhibition in hypertension was dependent on cardiac performance. Cardiac function was assessed in two *in vitro* models using tissue taken from the same animal: Langendorff hearts and paced left atria in which contractility is dependent or independent of contraction frequency respectively.

Changes related to hypertension

Hypertensive animals had higher SBP, cardiac hypertrophy and diastolic stiffness. Isoprenaline-induced vasodilatation and its ino- (with the exception of atria from DOCA-salt animals), chrono- and lusitropic effects in hearts and/or atria were attenuated in hypertension. This may result from a reduction of myocardial β-adrenoceptor density in SHR (Böhm *et al.*, 1988). In DOCA-salt hypertension, no change or a decrease in the number of β-adrenoceptors has been reported (Böhm *et al.*, 1992).

The cannabinoid receptor agonist CP55940 induced a concentration-dependent decrease in atrial contractile force, with a stronger effect in WKY compared with SHAM. The maximum negative inotropic effect of CP55940 was comparable with that induced by AEA in atrial muscle of humans (Bonz *et al.*, 2003) and rats (Sterin-Borda *et al.*, 2005). This is the first study to demonstrate that the negative

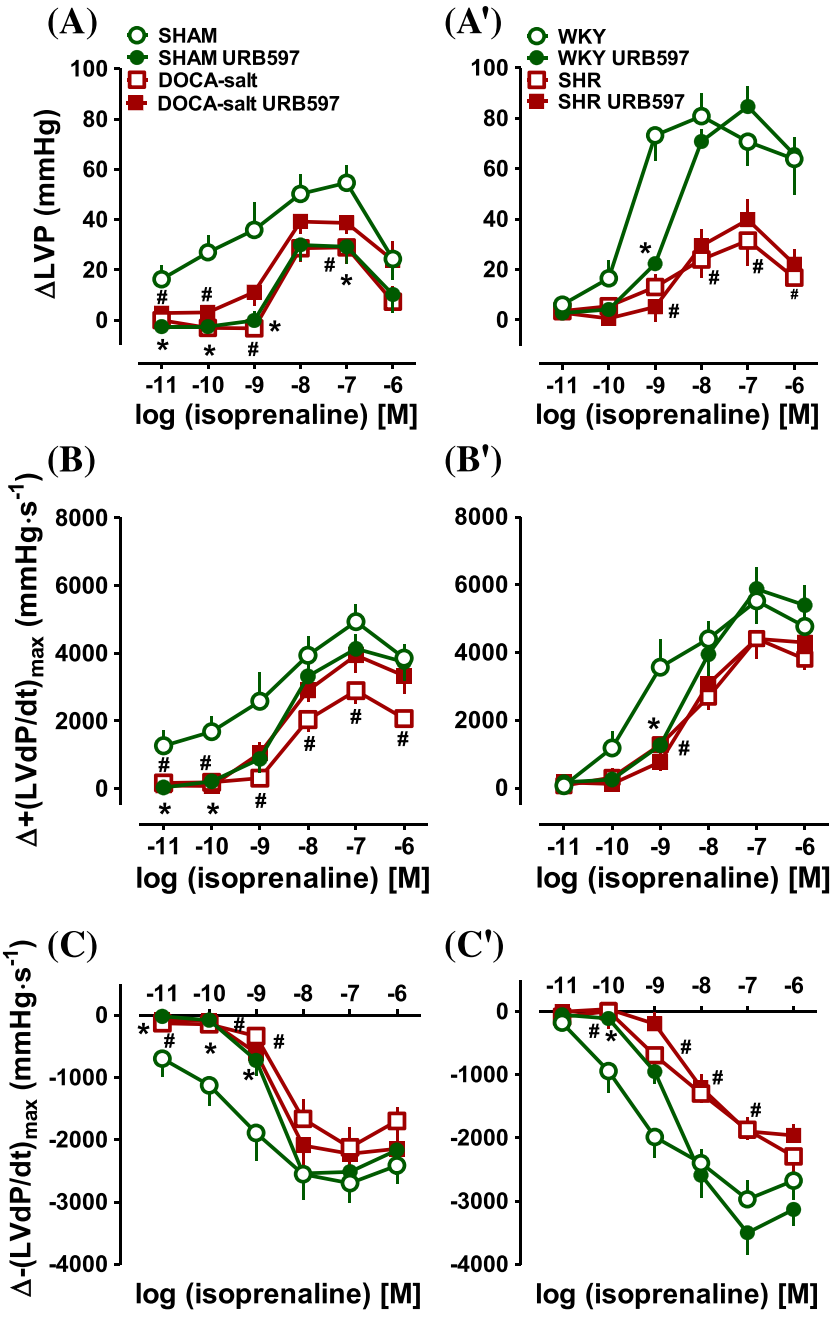

Figure 1

Influence of URB597 on the isoprenaline-induced changes in LVP (A, A′), the maximum rate of positive $+(LVdP/dt)_{max}$ (B, B′) and negative $-(LVdP/dt)_{max}$ (C, C′) changes in LVP (ΔLVP) of hearts isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY. URB597 (1-mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. Values shown are changes from baseline (Table 2). Data are given as the means ± SEM of 9–11 rats. *$P < 0.05$, significant effect of URB597, in normotensive rats only: $^{\#}P < 0.05$, DOCA-salt and SHR significantly different from SHAM and WKY respectively; ANOVA with Bonferroni *post hoc* test. In few cases, SEM is smaller than or equal to the size of symbols.

inotropic effects of CP55940 are diminished in hypertension. This was accompanied by decreased ventricular immunoreactivity and/or expression of FAAH and CB receptors (especially in DOCA-salt), which can induce negative (CB_1) and positive (CB_2) inotropic responses in rat left atria (Sterin-Borda *et al.*, 2005). By contrast, Bátkai *et al.* (2004) reported an up-regulation of cardiac CB_1 receptors and FAAH, associated with an AEA-induced sustained decrease in cardiac contractility, in anaesthetized SHR. However, Bátkai *et al.* (2004) used older rats for their *in vivo* experiments (8–10 months vs. 8–10 weeks in our study), in which the negative inotropic effect of AEA was modified by a decreased HR. We examined atrial but not ventricular contractility. However, hypertension-induced

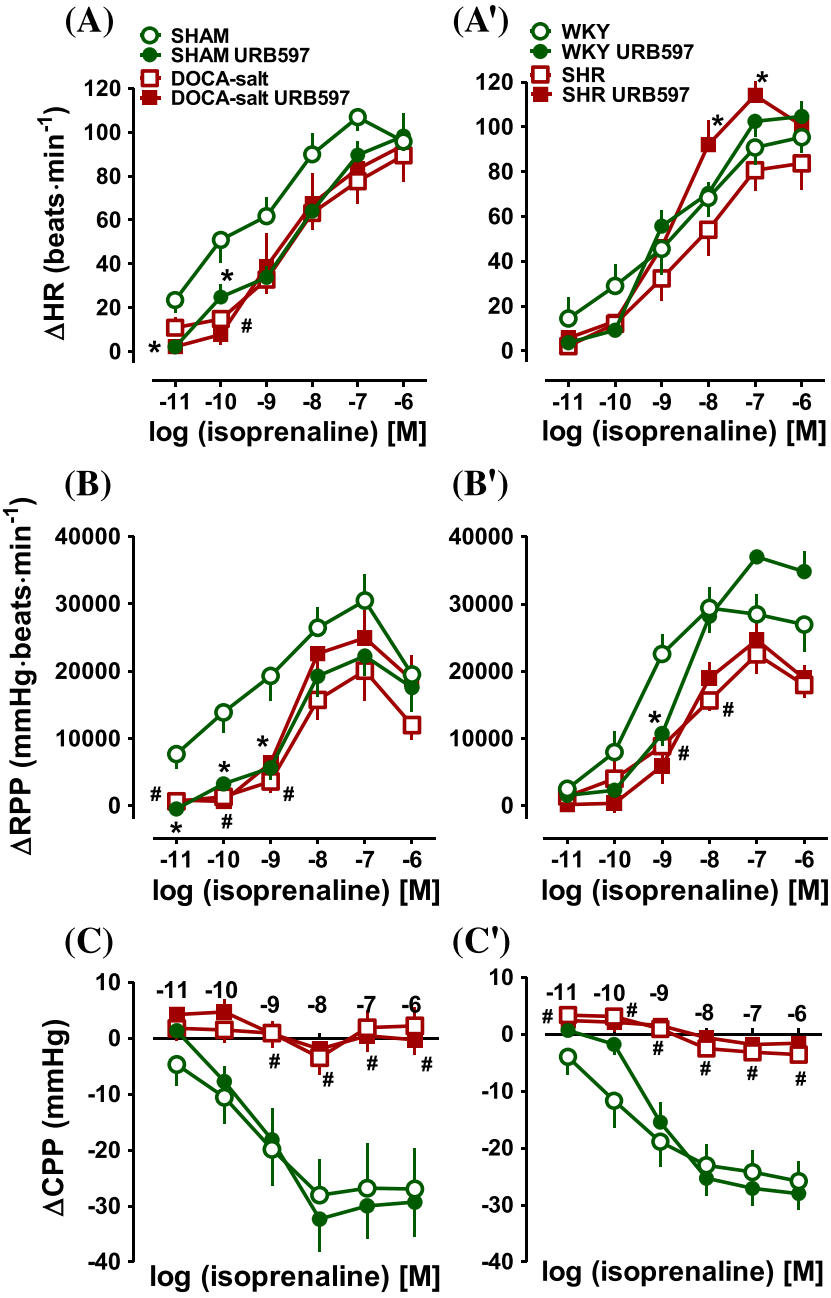

Figure 2

Influence of URB597 on the isoprenaline-induced changes in HR (A, A′), RPP (B, B′) and CPP (C, C′) of hearts isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY. URB597 (1 mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. Values shown (ΔHR etc) are changes from baseline (Table 2). Data are given as the means ± SEM of 9–11 rats. *$P < 0.05$, significant effect of URB597, in normotensive rats only with the exception of HR in SHR; $^{\#}P < 0.05$, DOCA-salt and SHR significantly different from SHAM and WKY respectively; ANOVA with Bonferroni *post hoc* test. In few cases, SEM is smaller than or equal to the size of symbols.

ventricular changes are accompanied by modifications of atrial function (Pluteanu *et al.*, 2015). In contrast to the reduced effects and expression of cardiac CB_1 receptors in DOCA-salt reported here, we have found in the same model up-regulation of vascular CB_1 receptors and CB_1 receptor-mediated vasodilatation (Baranowska-Kuczko *et al.*, 2016) and enhanced inhibition of sympathetic noradrenaline release (Toczek *et al.*, 2015) in resistance vessels. This suggests that these receptors may play a protective role in hypertension.

Influence of chronic URB597 administration on hearts of hypertensive rats

FAAH inhibitors are postulated to possess anti-hypertensive potential, because their acute systemic injection decreased

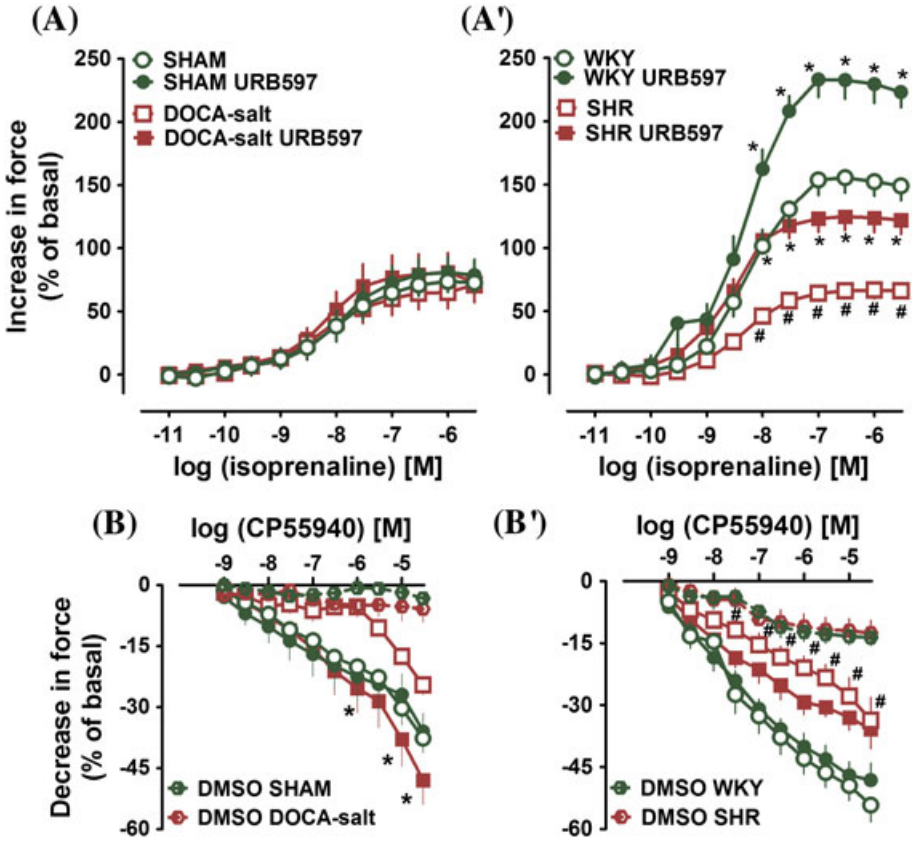

Figure 3

Influence of URB597 on the isoprenaline-induced (A, A′) increases and on the CP55940-induced (B, B′) decreases in contractile force of left atria isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY. URB597 (1 mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. Data are expressed in % of basal values (see Results). Data are given as the means ± SEM of 8–11 rats; *$P < 0.05$, significant effect of URB597; $^{\#}P < 0.05$, DOCA-salt and SHR significantly different from SHAM and WKY respectively; ANOVA with Bonferroni *post hoc* test. In few cases, SEM is smaller than or equal to the size of symbols.

BP in SHR partially through normalization of cardiac contractile performance, associated with up-regulation of cardiac CB_1 receptors and FAAH (Bátkai *et al.*, 2004; Pacher *et al.*, 2008; Godlewski *et al.*, 2010; O'Sullivan, 2015). We found that the effect of chronic FAAH inhibition was dependent on the hypertension model. More marked alterations were observed in secondary hypertension. This was probably due to the more dynamic progress of hypertension in DOCA-salt compared with SHR (the comparable hypertension of about 200 mmHg before the first dose of URB597 was obtained after 4 weeks of DOCA-salt procedure vs. 8–10 weeks of hypertension development in SHR) and the potential development of adaptive changes in SHR (Zicha and Kunes, 1999).

Chronic URB597 administration decreased BP only in DOCA-salt (consistent with our previous studies; Baranowska-Kuczko *et al.*, 2016; Toczek *et al.*, 2016) but not in SHR. We measured BP 12 h after the final dose of URB597 (1 mg·kg^{-1}) in conscious rats. Higher doses were not used due to potential side effects and neurotoxicity (1.5 and 3-mg·kg^{-1}; Su *et al.*, 2015). In contrast, the strong hypotensive effects of acute administration of the FAAH inhibitors URB597 (used at the maximally effective dose of 10 mg·kg^{-1}; Bátkai *et al.*, 2004) and AM3506 (Godlewski *et al.*, 2010) were determined immediately after *i.v.* injection in older anaesthetized SHR.

The present results demonstrate that URB597 induced a fall in BP in DOCA-salt hypertension, which was partly mediated by vascular (Baranowska-Kuczko *et al.*, 2016) and cardiac mechanisms. URB597 reduced hypertrophy of the left ventricle and septum and cardiac diastolic stiffness (observed in hypertension) and completely restored the negative inotropic effect of CP55940 in isolated atria. These changes were accompanied by enhanced ventricular expression of CB_1 and CB_2 receptors (immunohistochemistry) and FAAH (Western blot) that was reduced in hypertension. It is the first report demonstrating the up-regulation of FAAH expression following chronic FAAH inhibition. So far, a decrease (Baranowska-Kuczko *et al.*, 2016; Su *et al.*, 2016) or no change (Okine *et al.*, 2012; Baranowska-Kuczko *et al.*, 2016) in FAAH expression has been demonstrated in other tissues, after chronic URB597 administration. We can only speculate that in our hands, inhibition of enzyme activity might cause a compensatory increase in enzyme expression (King *et al.*, 1991). The possible involvement of cardiac mechanisms in the hypotensive effect of URB597 is partly confirmed by the

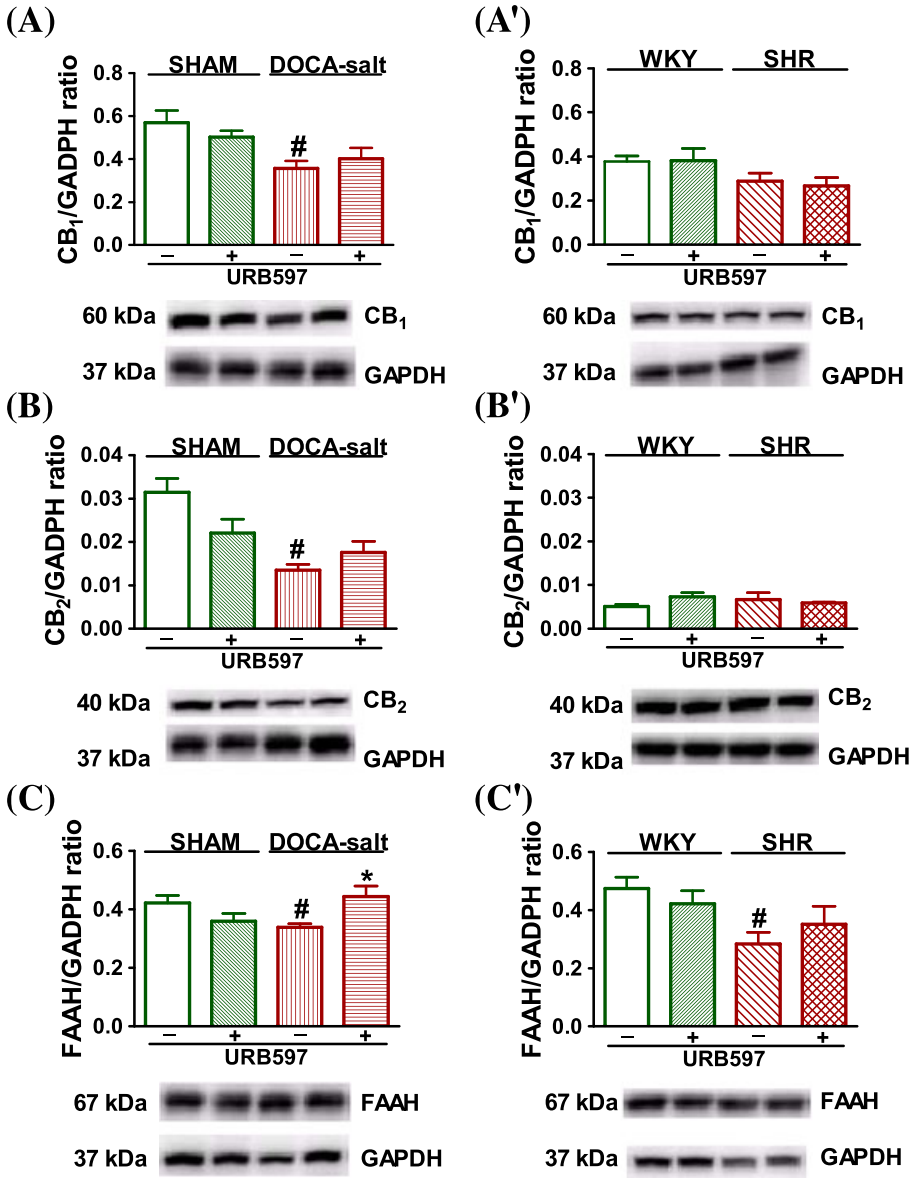

Figure 4

Western blots of CB_1 (A, A′), CB_2 (B, B′) and FAAH (C, C′) protein in left ventricle isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY. URB597 (1 mg·kg^{-1}) or its vehicle was injected i.p. every 12 h for 14 days. On the bottom of the panel, representative Western blots for CB_1, CB_2, FAAH and GADPH (which served as loading control) are given. Mean ± SEM of six rats; *$P < 0.05$, significant effect of URB597; $^{\#}P < 0.05$, DOCA-salt and SHR significantly different from SHAM and WKY respectively; ANOVA with Bonferroni *post hoc* test.

observation that, in SHR, the same procedure did not decrease BP or normalize cardiac hypertrophy, diastolic stiffness, the negative inotropic effect of CP55940 or expression of CB receptors and FAAH. Our results on isolated atria from DOCA-salt validated the findings from *in vivo* experiments (Bátkai *et al.*, 2004) that changes in cannabinoid-induced inotropic effects are related to a direct influence on the myocardium but not through inhibition of sympathetic tone. Moreover, the significance of CB_1 receptor mediated cardiac alternations underlined observations that chronic administration of the CB_1 receptor antagonist **rimonabant** improved cardiac function and remodelling after myocardial infarction, in obese SHR (Slavic *et al.*, 2013).

The most visible effects of URB597 in SHR were the normalization (atria) and enhancement (hearts) of the positive ino- and chronotropic effects of isoprenaline respectively. It remains to be established whether these changes are related to redistribution of CB_1 receptors to the region of intercalated discs, clearly observed in SHR. We are the first to demonstrate this intriguing relationship between cardiac intercalated discs and CB_1 receptors. Intercalated

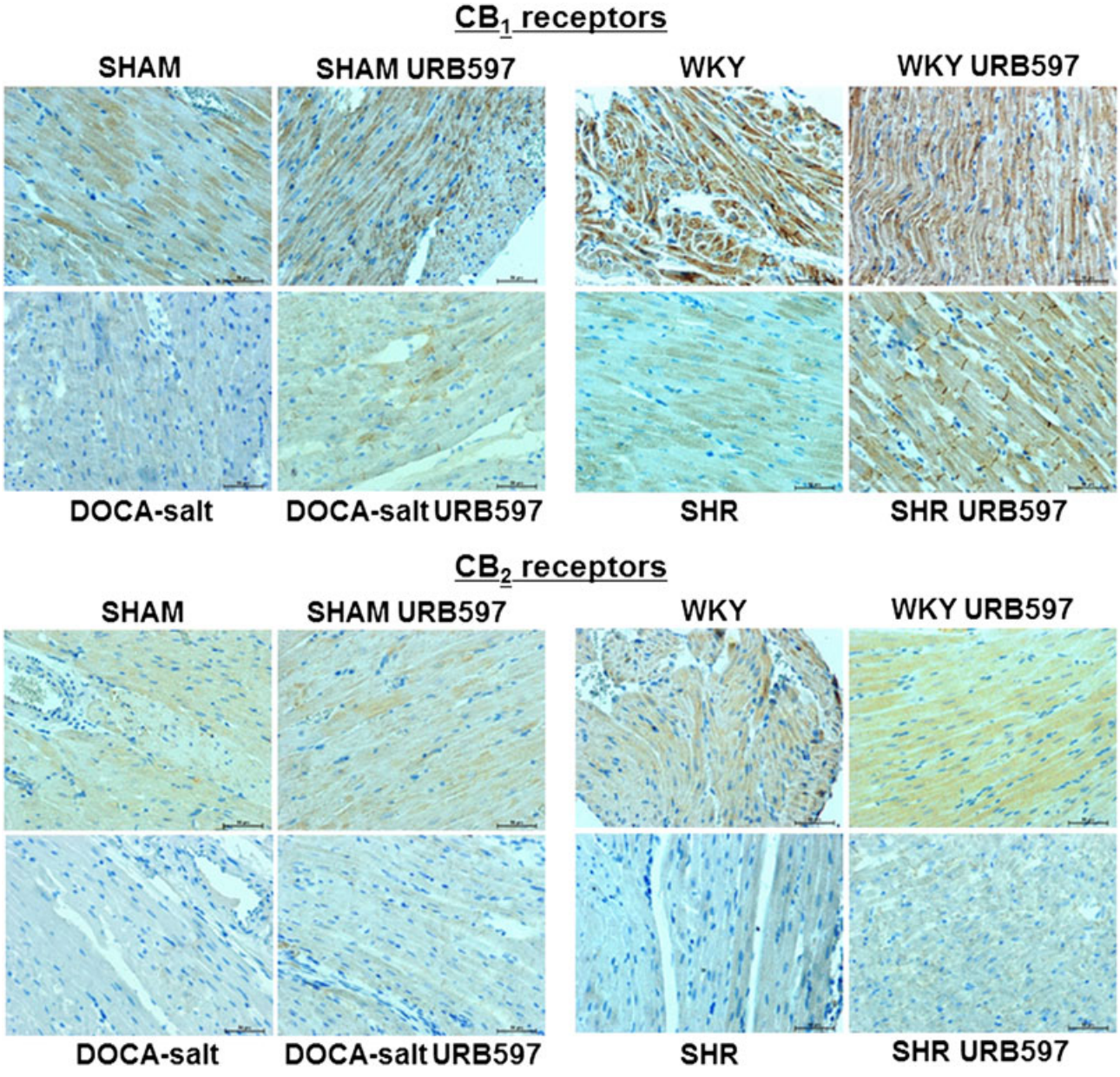

Figure 5

Representative micrographs of immunohistochemical staining of CB_1 and CB_2 receptors in cross sections of left ventricle isolated from DOCA-salt and SHR and their respective normotensive SHAM and WKY treated i.p. every 12 h for 14 days with URB597 (1 mg·kg^{-1}) or its vehicle. Scale bar: 50 μm.

discs are responsible for mechanical (desmosomes and adheren junctions) and electrical (gap junctions) communication between adjacent cardiomyocytes (Campbell *et al.,* 2014). AEA can inhibit gap junctions in certain cell types, such as endothelial cells, but not myocytes (Oz, 2006). Improving cardiac gap junction communication, which is also modified by β-adrenoceptor stimulation (Campbell *et al.,* 2014), has been proposed as a new antiarrhythmic mechanism (Dhein *et al.,* 2010). Thus, normalization of basal HR and prevention of arrhythmia by chronic URB694 administration to stressed animals (Carnevali *et al.,* 2015b) could be associated with gap junction modification.

Pre-incubation of ventricular papillary muscle (isolated from rats with cirrhotic cardiomyopathy) with the CB_1 receptor antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-piperidin-1-ylpyrazole-3-carboxamide restored its blunted contractile response to isoprenaline (Gaskari *et al.,* 2005). In our hands, chronic URB597 administration restored the positive inotropic effect of isoprenaline in atria, which was reduced in SHR and tended towards the same in isolated hearts from DOCA-salt.

Influence of chronic URB597 administration on hearts of normotensive rats

To date, it is generally accepted that endocannabinoids are not involved in the regulation of cardiovascular parameters under physiological conditions, as genetic ablation or acute pharmacological blockade of CB receptors or FAAH does not modify basal haemodynamic parameters including left ventricle stiffness (Pacher *et al.,* 2005; Malinowska *et al.,* 2012; O'Sullivan, 2015) and parameters of isolated rat hearts (Wagner *et al.,* 2005). Surprisingly, we found that chronic URB597 administration produced two detrimental effects in normotensive rats, namely, increased cardiac diastolic stiffness and modified cardiostimulatory effects of isoprenaline. Thus, it diminished the following effects of lower concentrations of isoprenaline on the heart: ino- and lusitropic actions, increases in RPP and the chronotropic effect (the latter in SHAM only). Moreover, the positive inotropic effect of higher concentrations of isoprenaline was enhanced in atria from WKY. These data indicate that the modification of β-adrenoceptor function by cannabinoids, found in acute experiments (Maslov *et al.,* 2004; Gaskari *et al.,* 2005; Liao *et al.,* 2013; Carnevali *et al.,* 2015b; see Introduction), may also extend to chronic studies.

There is no clear explanation of the negative effect of URB597 in normotensive rats. In the light of pro-fibrotic effects of CB_1 receptor activation in diabetic mice (Rajesh *et al.,* 2012) or anti-fibrotic influence of the CB_1 receptor antagonist rimonabant in experimental metabolic syndrome (Slavic *et al.,* 2013), it could be not so surprising to see increased cardiac stiffness in response to URB597 increasing level of endocannabinoids acting *via* CB receptors. URB597 is generally regarded as a selective FAAH inhibitor *(*Piomelli *et al.,* 2006). However, proteome-wide studies identified the off-target effects of URB597 (Ahn *et al.,* 2007). Thus, side-effects of URB597 might be connected with its off-target response and with direct or indirect activation of alternative pathways of AEA metabolism. Importantly, other unexpected effects of chronic URB597 administration in SHAM animals have been observed previously in small resistance arteries: impaired acetylcholine-induced vasodilatation, potentiated phenylephrine-induced vasoconstriction (Baranowska-Kuczko *et al.,* 2016) and enhanced oxidative stress in liver (Biernacki *et al.,* 2016). Similarly, cardiac injury and enhanced oxidative/nitrative stress were greater in $FAAH^{-/-}$ compared with $FAAH^{+/+}$ mice, and AEA was reported to enhance cell death in human cardiomyocytes pretreated with an FAAH inhibitor (Mukhopadhyay *et al.,* 2011).

In summary, the present findings show that hypotensive effect of chronic FAAH inhibition depends on the model of hypertension used and partly correlates with improved cardiac performance. The fall in BP in response to chronic URB597 administration was observed only in DOCA-salt rats. This was associated with decreased left ventricular hypertrophy and cardiac diastolic stiffness and an enhanced negative inotropic atrial response to CP55940. The most visible influence of URB597 in SHR was an enhanced positive inotropic effect to isoprenaline in isolated hearts, accompanied by a redistribution of CB_1 receptors to the intercalated disc regions. However, chronic URB597 administration induced side-effects in normotensive rats such as enhanced cardiac diastolic stiffness and modified cardiostimulatory effects to isoprenaline, stressing the importance of examination of bidirectional interactions of endocannabinoids with the cardiac effects of β-adrenoceptors. Thus, the use of FAAH inhibitors as potential pharmacotherapy in normotensive patients, for example, for control of pain and inflammation (Fowler, 2015) or psychological-cardiac comorbidity (Carnevali *et al.,* 2016), should be approached cautiously, especially considering the side effects of FAAH inhibitors that have been reported in clinical studies (Mallet *et al.,* 2016).

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Author contributions

A.P.-B. designed and conducted isolated heart experiments, analysed data and co-wrote the manuscript; J.W. performed isolated atria experiments and analysed the data; M.T. performed the *in vivo* studies and helped in interpretation of data; M.B.K. contributed to the revised manuscript; I.K. designed and analysed immunohistochemical experiments; E.H.-S. designed and analysed Western blot experiments; B. M. conceived, designed and supervised experiments and wrote the manuscript. All authors approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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