Functional Cardiac Orexin Receptors: Role of Orexin-B/Orexin 2 Receptor in Myocardial Protection

Running Title: Functional Cardiac Orexin Receptors

Vanlata H Patel[1], *Emmanouil Karteris[2], Jing Chen[1], Ioannis Kyrou[1,3,4], Harman S Mattu[1,3], Georgios K Dimitriadis[1,3], Glenn Rodrigo[5], Charalambos Antoniades[6], Alexios Antonopoulos[6], Bee K Tan[1], Edward W Hillhouse[7,8], Andre Ng[5] & *Harpal S Randeva[1,3,4]

[1] Translational & Experimental Medicine, Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

[2] Biosciences, Brunel University, Uxbridge, UB8 3PH; UK

[3] Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism (WISDEM) and Human Metabolism Research Unit (HMRU) University Hospitals Coventry and Warwickshire NHS Trust, CV2 2DX, Coventry, UK

[4] Aston Medical Research Institute, Aston Medical School, Aston University, B4 7ET, Birmingham, UK

[5] Department of Cardiovascular Sciences, University of Leicester, Clinical Sciences Wing, Glenfield General Hospital, Leicester, LE3 9QP; UK

[6] Cardiovascular Medicine Division Radcliffe Department of Medicine, Oxford University Hospitals NHS Trust, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

[7] Weill Cornell University, New York, USA

[8] University of Leeds, Leeds, UK.

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Corresponding authors:
Prof Harpal S Randeva MBCHB, FRCP, MD, PhD
Translational & Experimental Medicine, Clinical Sciences Research Institute,
Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK
Tel: + 44 2476 572552
Fax: + 44 2476 523701
Email: Harpal.Randeva@warwick.ac.uk

Dr Emmanouil Karteris BSc, MSc, PhD
Brunel University London
College of Health and Life Sciences
Uxbridge, UB8 3PH, UK
Tel: 01895265892
Email: emmanouil.karteris@brunel.ac.uk

Abbreviations

AAR: area at risk

ANAR: area not at risk

BP: blood pressure

$[Ca^{2+}]i$: intracellular calcium

cAMP: Cyclic adenosine monophosphate

CNS: Central nervous system

cTnI: cardiac Troponin-I

CVD: cardiovascular disease

DAPI: 4,6-diamido-2-phenylindole

DORA: dual orexin (OX1R/OX2R) receptor antagonist

ERG: ETS-related gene

ERK: extracellular signal–regulated kinases

FITC: fluorescein isothiocyanate-conjugated

GPCR: G protein-coupled receptors

HCRTR2: hypocretin receptor 2

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HR: heart rate

ICV: intra-cerebroventricular

LA: left atrium

LCA: left coronary artery

LSVP mmHg: left ventricular systolic pressure

LV: left ventricle

LVDP: left ventricular developed pressure

LVEDP mmHg: left ventricular end diastolic pressure

MAP: mean arterial pressure

MAPK: mitogen-activated protein kinase

MTC: multiple tissue cDNA

Myosin Light Chain: MLC

NS: No supplement

NYHA: New York Heart Association

OR-A: Orexin A

OR-B: Orexin B

OX1R: orexin receptor 1

OX2R: orexin receptor-2

OXRs: orexin receptors

PGK1: Phosphoglycerate Kinase 1

PI3K: PI3 kinase

PVN: paraventricular nucleus

RA: right atrium

RISK: reperfusion injury signalling kinase

RPP: rate pressure product

RPP: rate pressure product

RSNA: renal sympathetic nerve activity

RT-PCR: reverse transcription-polymerase chain reaction

RV: right ventricle

SNS: sympathetic nervous system

Troponin: Tn

TTC: Triphenyl-tetrazolium chloride

Perspectives Section

(i) Several cardiovascular drugs exert their cardiac actions via GPCRs, and orexins acting via their GPCR receptors can affect the heart centrally. In this study we tested whether orexins and their receptors can exert a direct effect at cardiac level.

(ii) The human and rat heart constitutes a source of orexins and expresses functional OXRs. OR-B can exert direct cardioprotective effects in both *ex vivo* and *in vivo* rat heart models. In adults with heart failure there was a significant negative correlation between the severity of clinical symptoms (NYHA class) and OX2R expression in the human heart.

(iii) Based on the findings from our study, the use of orexin antagonists warrants further assessment and careful monitoring for CVD adverse effects, particularly in heart failure patients.

Abstract

Orexins/hypocretins exert cardiovascular effects which are centrally mediated. In this study we tested whether orexins and their receptors may also act in an autocrine/paracrine manner in the heart exerting direct effects. Quantitative RT-PCR, immunohistochemical and Western blot analyses revealed that the rat heart expresses orexins and orexin receptors. In isolated rat cardiomyocytes, only orexin-B (OR-B) caused an increase in contractile shortening, independent of diastolic or systolic calcium levels. A specific orexin receptor-2 (OX2R) agonist ([Ala^{11}, D-Leu^{15}]-Orexin B) exerted similar effects as OR-B, whereas a specific OX1R antagonist (SB-408124) did not alter the responsiveness of OR-B. Treatment of the same model with OR-B resulted in a dose-dependent increase of myosin light chain and troponin-I phosphorylation. Following ischaemia/reperfusion in the isolated Langendorff perfused rat heart model, OR-B, but not OR-A, exerts a cardioprotective effect; mirrored in an *in vivo* model as well. Unlike OR-A, OR-B was also able to induce ERK1/2 and Akt phosphorylation in rat myocardial tissue and ERK1/2 phosphorylation in human heart samples. These findings were further corroborated in an *in vivo* rat model. In human subjects with heart failure, there is a significant negative correlation between the expression of OX2R and the severity of the disease clinical symptoms, as assessed by the New York Heart Association (NYHA) functional classification. Collectively, we provide evidence of a distinct orexin system in the heart that exerts a cardioprotective role via an OR-B/OX2R pathway.

INTRODUCTION

Cardiovascular disease (CVD), including myocardial infarction, is the leading cause of morbidity and mortality in the Western world (1,2). Orexins, also referred to as hypocretins, exist in two functional forms, *i.e.* orexin A (OR-A) and orexin B (OR-B), which are derived from a common 130 amino acid precursor peptide (3,4) and exert their actions by activating the orexin-1 (OX1R) and orexin-2 (OX2R) receptors. OXRs belong to the GPCR superfamily (3) and are distributed throughout both central and peripheral sites where they regulate endocrine, metabolic and cardiovascular functions (5–7). Of note, several established cardiovascular drugs exert their cardiac actions via GPCRs, such as the muscarinic, angiotensin, adrenergic and endothelin receptors (8).

Growing evidence indicates that central orexin neurons are implicated in cardiovascular regulation (5).For example, in the central nervous system (CNS), both orexin and OX2R-containing nerve fibres have been identified in the paraventricular nucleus (PVN) (9), an area which constitutes a vital central site for integration of sympathetic outflow and cardiovascular function(10–12)(13–15) (16) (17). Thus, although orexins can influence the autonomic control of the cardiovascular system, little is known about any direct effects of orexins in the heart.

We and others have provided evidence for a wide distribution of orexins and their receptors peripherally (18–20). We have also demonstrated the presence of OXRs in human adipose tissue, where they modulate adipogenesis and adipose tissue metabolism (7). Herein, we hypothesised that orexins may also exert direct cardiac effects and we sought to investigate the expression and function of cardiac OXRs using *in vitro*, *ex vivo* and *in vivo* models, corroborating this with clinical data.

Materials and Methods

Drugs and experimental solutions

Study treatment agents were: OR-A, OR-B (Phoenix Peptides), OX2R agonist ([Ala11, D-Leu15]-Orexin B; Tocris), OX1R antagonist (SB-408124; Tocris), wortmannin (PI3Kinase inhibitor; Sigma-Aldrich) and MAPK inhibitor U0126 (Tocris). According to the manufacturer's specifications on biological activity, the used OX2R agonist ([Ala11, D-Leu15]-Orexin B; Tocris) is a "highly potent and selective OX2 receptor agonist; displays 400-fold selectivity over OX1 receptors. EC50 values are 0.13 and 52 nM for human OX2 and OX1 receptors respectively". Similarly, the used OX1R antagonist (SB-408124) is a "selective non-peptide orexin OX1 receptor antagonist (Kb values are 21.7 and 1405 nM for human OX1 and OX2 receptors respectively)". Triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich) was used to determine the infarct size following ischemia and reperfusion. All other reagents used were of the highest purity commercially available.

Animal Experiments

All animal experiments were performed in accordance with UK legal requirements and the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. This investigation also conforms to the guidelines for the welfare of animals in experimental neoplasia as they have been developed by the United Kingdom Coordinating Committee of Cancer Research (UKCCCR). Adult male Wistar rats (250-300 g) were housed in environmentally controlled conditions (22±2°C, humidity 40–60%) under a 12:12-h light-dark schedule (lights on 0600). After a week of habituation to these conditions, rats were sacrificed using cervical dislocation. Hearts were immediately removed for the following studies: (a) dissection and immediate snap-freezing for later tissue analysis; (b) isolation of ventricular cardiomyocytes by enzymatic digestion; (c) immersion in ice-cold oxygenated Tyrode's solution and then perfusion via the aortic cannula in the modified Langendorff model with Tyrode's solution at 37°C, as previously described (21). In brief, contractile parameters were measured by insertion of a fluid-filled latex balloon through

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the left atrium into the left ventricle, this was connected to a pressure transducer (MTL0380, ADInstruments Ltd, UK) and the balloon volume was adjusted to give end-diastolic pressure of <10 mmHg. Aortic perfusion pressure was monitored with a second pressure transducer in series with the aortic cannula. Data were continuously recorded using a Power Lab 8 preamplifier/digitiser (ADInstruments Ltd, UK). Hypothalamic dissection took place as previously described (22). All procedures were approved by the ethics committee and complied with the University's care and welfare guidelines, in strict accordance with the Home Office Guidance (PPL 70/7175) on Research and Testing using animals and the Animal (Scientific Procedures) Act, 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Human Cardiovascular Multiple Tissue cDNA (MTC) Panel

Commercially available human cardiovascular multiple tissue cDNA (MTC) panel (Clontech) were used for this study. All tissues were normal (*i.e.* non-diseased) and the amount of pooled patients varied from 11 up to 32. The sets of primers used for RT-PCR amplification were (i) OX1R sense 5'-ccttcctggctgaagtgaag-3' and antisense 5'-agtgggagaaggtgaagcag-3'; (ii) OX2R sense 5'-gtcgcaactggtcatctgct-3' and antisense 5'-cgtcctcatgtggtggttct-3'.

Harvesting of single-cell mRNA and cDNA synthesis

Under the microscope, single rat cardiomyocytes (n=6) were harvested via the patch clamp micro-electrode pipette, without losing the gigaseal to prevent contamination with extracellular fluid (23). Subsequently, the pipette contents were expelled into a sterile 0.5 ml tube.

Polymerase Chain Reaction (PCR)

Total RNA from different rat heart chambers (n=3) was extracted and cDNA synthesis was performed. PCR reactions were carried out as previously described (7).

Immunofluorescence for orexins and orexin receptors in cardiomyocytes

Adult rat cardiomyocytes, seeded on glass cover-slips were fixed with 4% paraformaldehyde in PBS. The primary rabbit antiserum OR-A and OR-B (Phoenix Europe GmbH, Karlsruhe, Germany) and the primary goat polyclonal OX1R (Santa Cruz Biotechnology Inc., USA) and a monoclonal OX2R antibody (Abcam, UK) were used at a 1:500 dilution. All dilutions were made in 3% BSA in PBS-0.01% Triton X-100. Specimens were incubated with primary antibody for 60 min, then washed three times with PBS (5 min each time) before incubation with anti-rabbit IgG-fluorescein isothiocyanate-conjugated (FITC) and Texas Red anti-goat secondary antibody (Vector laboratories, Peterborough, UK) for 45 min. The slides were then thoroughly rinsed with PBS, and the cell nuclei were visualized by applying the DNA-specific dye 4,6-diamido-2-phenylindole (DAPI) at a final concentration of 1.5 µg/ml.

Immunohistochemistry for orexin and orexin receptors in the rat heart

Whole heart tissue sections from adult rats (n=3) were cut at 3 µm. Endogenous peroxidase was blocked by incubating sections in a 1.5% H_2O_2 solution for 20 min. The detection system used was an immunoperoxidase-based system (Vector Universal Elite ABC kit) with a diaminobenzidine tetrahydrachloride visualisation agent. Sections were incubated in primary antibody (OX1R, OX2R, OR-A and OR-B) for 60 min at room temperature. Sections were then washed for 2x5 min in buffer. Secondary biotinylated antibody was applied for 30 min at room temperature. The avidin-biotin complex solution was then applied to the sections and incubated for 30 min at room temperature (Vector Elite ABC reagent). DAB solution was prepared according to the manufacturer's instructions and applied to the sections for 5 min. Sections were then dehydrated, cleared and mounted.

For controls, rat spleen, testes (positive controls) and thymus (negative control) were stained using paraffin-embedded tissues from US Biomax (#RA162, MD, USA). Furthermore, we have repeated the OR-A staining on rat heart array (#TR031; US Biomax MD, USA).

Western Blotting for PPO and Orexins

Protein lysates from rat brain, rat heart and human myometrium were separated using SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (15% polyacrylamide) under reducing conditions and then transferred onto immunoblot nitrocellulose membrane. Blocking of non-specific binding was achieved by placing the membrane in a blocking buffer (1% BSA in 1X TBST) at 4oC overnight. The following day, membrane was rinsed with TBST for 5-10 min. Membranes were then incubated with primary anti-rabbit OR-A and OR-B antibodies (Phoenix Peptides) diluted 1:500 and further incubated at 4oC overnight under gentle agitation. The next day, after rinsing the membrane three times in TBST for 10 min to remove unbound primary antibodies, the membranes were exposed to anti-rabbit IgG- HRP conjugate (Sigma Aldrich) (diluted 1:2000 in blocking solution) for 1 h at room temperature. Detection of immunocomplexes took place using the ECL method, exposing x-ray film (Fuji) at different time points (30 sec to 1 min) following by developing. Molecular weight approximations are taken by comparing the stained bands to that of the marker proteins.

Orexin mediated signalling in cardiomyocytes

Following the isolation of calcium tolerant cardiomyocytes (n=6), the cells were treated in a concentration (0.01-100 nM) and time (0-60 min) dependent manner with both OR-A and OR-B. Following these optimisation experiments, orexin-induced phosphorylation of various signalling cascades i.e. Akt, ERK1/2, Troponin-I and Myosin Light Chain were determined by western blot analyses, as described previously (24).

Measurement of cell contraction and intracellular calcium

Contraction and intracellular calcium were determined in isolated ventricular cardiomyocytes (n=6), as previously described (21). Cells were classified as either contractile or non-contractile in response to electrical stimulation. To measure $[Ca^{2+}]i$, myocytes were loaded with fura-2 and excited alternately at 340 and 380 nm with a monochromator and emitted light collected at > 520 nm and measured using a photomultiplier tube (Photon Technology International). Contraction strength is shown as percentage cell shortening and $[Ca^{2+}]_i$ as Fura2 ratio.

Determination of infarction following ischaemia/reperfusion in the isolated Langendorff perfused rat heart

All experiments lasted 195 min. Hearts were allowed to stabilize for 30 min with the standard Tyrode's solution perfusion. Contractile parameters from the data files were calculated by averaging a minimum of 6 cycles per data point to obtain the mean value for left ventricular systolic pressure, LVSP mmHg, and left ventricular end diastolic pressure, LVEDP mmHg (measured at t0, t30, t40, t75, t85, t100, t130, t160, and t195 min). The difference between these two pressure values represented the left ventricular developed pressure (LVDP, mmHg). Heart rate (HR, beats.min-1) was determined at the same time-points together with the rate pressure product (RPP, mmHg), which was calculated by multiplying the LVDP and HR (average value of LV Developed Pressure is obtained from averaging both systolic and diastolic values, then the mean is multiplied by HR to obtain RPP).

Global ischaemia was produced for 30 min by stopping flow of physiological solution to the heart. At the end of reperfusion, the heart was frozen at -20°C before being sliced into 2mm thick transverse sections and stained with 2% TTC, which reacts with intracellular dehydrogenases present in viable cells producing a red pigment and staining viable tissue red (whereas infarcted tissue remains pale) and then fixed in 10% formalin overnight to enhance the contrast. Each section was then photographed with a digital camera and then traced onto a clear acetate sheet to determine infarct size (% infarct area= infarct area on

each slice/total area of the heart slice X 100) (25); the area was calculated using computer-assisted planimetry [NIH image 1.57] (26–28).

For the inhibitor studies the rats were randomly assigned to one of the following groups: 1) control group, where they were subjected to 30 mins of global ischaemia followed by 120 mins of reperfusion, and the treated heart groups, which received inhibitor (wortmannin; 1umol/l or U0126; 10umol/l) together with OR-B perfusion which was added to Tyrode's buffer after stabilisation and perfused for 10 mins prior to 30 min global ischaemia and switched back to normal Tyrode's during 120 mins reperfusion. In addition, some hearts only received the inhibitors (wortmannin or U0126) to rule out any influence they may have directly upon infarct size.

Inducing ischemia in rats *in vivo*

Rats were anesthetized with 3% isoflurane inhalation and then intubated via a tracheostomy and ventilated with a mixture of O_2 and 1.5-3% isoflurane, using a rodent ventilator. A left thoracotomy was performed through the 4th intercostal space, pericardium opened and the left coronary artery (LCA) located and ligated with a 4-0 silk suture. The ligation was confirmed successfully when the anterior wall of the left ventricle (LV) turns pale around the area that was tied off using a snare. Following 30 minutes of regional ischaemia the snare was released and blood allowed to flow freely during 120 minutes of reperfusion. At the end of the experimental protocol the hearts removed and perfused onto the Langendorff system for staining. The ligation was tied off and hearts perfused with 1% Evans blue to demarcate the area at risk, followed TTC staining to identify the viable tissue staining deep red. The infarct area within the risk area was then calculated (I/R %). The same surgical procedure was followed for sham group without occlusion of the LCA (data not shown).

Determination of infarction following ischaemia/reperfusion in the *in vivo* rat heart

At the end of reperfusion, the rat was overdosed with pentobarbital and the heart was rapidly removed and mounted onto the Langendorff apparatus as above. The left anterior

descending artery was ligated, 1 ml of 0.25% Evans Blue (in phosphate-buffered saline) was then injected into the heart and allowed to perfuse followed by normal Tyrode's for a further 2 minutes to delineate the area not at risk staining it blue (29). The heart was stored at -20°C before being sectioned into 2mm sections and stained with TTC to determine the infarct size as stated above. The percentage infarct tissue (I) within the area at risk (AAR) was calculated as I/AAR %. Furthermore, the ischaemic area (AAR) was distinguished from the area not at risk (ANAR).

Orexin in the human heart

The clinical study was approved by the Research Ethics Committee South-Central Oxford C (11/SC/0140). All participants provided written informed consent, in accordance with the Declaration of Helsinki.

Orexin gene expression in human myocardial tissue

To explore whether our findings from the cell cultures and animal model could be of relevance to cardiovascular disease in humans, we investigated OX2R gene expression levels in human myocardium samples. A cohort of 54 patients undergoing coronary artery bypass grafting with available myocardial cDNA library was used for this purpose (Table 1); relative expression was calculated using the Pfaffl method, with PGK1 as housekeeping gene (30).

Orexin signalling in the human myocardium

To examine the direct effects of OR-A and OR-B on intracellular signalling in the human heart, human myocardial tissue samples were collected from the right atrial appendage of 8 patients undergoing cardiac surgery. These myocardial tissue samples were washed in ice-cold Krebs HEPES buffer and then cut into thin strips containing all myocardial layers. The tissue was first equilibrated for 10 min in Krebs HEPES buffer at 37°C and then incubated for 20 min in the presence or absence of human recombinant OR-A (100 nM) or OR-B (100 nM)

(Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). At the end of the experiment, incubated tissue samples were collected and stored at -80°C until assayed. Western immunoblotting was used to examine the direct effects of OR-A and OR-B on phospho-Akt (Ser473), total-Akt, (Cell Signaling, Danvers, MA), and phospho/total ERK1/2 (Abcam, Cambridge, England, United Kingdom) in human myocardial tissue. Briefly, myocardial tissue samples were homogenized for 30 seconds using a pre-cooled electric Polytron homogenizer in 220 µl of lysis buffer (Invitrogen, UK) containing a protease plus phosphatase inhibitor cocktail (Roche Applied Science). Homogenates were spun at 13,000 rpm for 10 min at 4 °C. The protein concentration of the supernatants was then measured using the BCATM Protein Assay kit (Pierce, UK). Protein lysates were separated on 12% gradient SDS-NuPAGE gel (Invitrogen, UK), and proteins transferred to polyvinylidene difluoride membranes (Amersham, UK Ltd.), followed by blocking with 5% powdered skimmed milk. The membranes were incubated with the respective primary antibodies overnight and immunodetection of the primary antibodies was performed with horseradish-peroxidase-conjugated secondary antibodies (Promega), and enhanced chemifluorescence (Amersham Bioscience UK Ltd.), and quantified in relation to the house-keeping protein GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

All results are expressed as mean ± standard error (SEM), unless indicated otherwise. For western blotting experiments, the densities of the immunoreactive bands were measured using a scanning densitometer coupled to scanning software (ImageQuant; Molecular Dynamics, Amersham Pharmacia, UK). Data were evaluated as in the study by Maulik et al (31). Statistical analyses amongst the Langendorff model perfused groups were performed using one-way ANOVA followed by Bonferroni's post hoc test with significance determined at the level of $P<0.05$.

RESULTS

Detection of cardiac prepro-orexin and orexin receptors

Using qRT-PCR we demonstrate that both orexin receptors are expressed in the rat heart. Using rat hypothalamus as positive control, the expression of OX1R was significantly less in the heart ($P<0.001$), whereas OX2R expression levels were similar between the heart and the hypothalamus (Figure 1A). Both OXRs were uniformly expressed throughout the rat heart including the left ventricle (LV), left auricle (also known as left atrial appendage), interventricular septum, right ventricle (RV), and right auricle (Figure 1D). Single cell PCR from cardiomyocytes revealed expression of both OXRs in single cells investigated (Figure 1E).

Using Western blotting, we also confirmed the expression of OXRs at the protein level in total rat heart and cardiomyocyte cell lysates (data not shown). Immunohistochemical analysis of ventricular sections using specific antibodies for OX1R (Figure 1B-I) and OX2R (Figure 1B-II) revealed a widespread cytoplasmic and membrane distribution of both OXRs across the rat ventricle. Cellular distribution of OXRs was further investigated in rat cardiomyocytes *in vitro*. Immunofluorescence revealed a similar distribution of OX1R (Figure 1C-I) and OX2R (Figure 1C-II). This is consistent with the expression of GPCRs in the cytoplasm (due to trafficking or internalisation), as well as on the cell membrane (32). We then proceeded to study the expression of OXRs in the human heart using a cardiovascular multiple tissue cDNA panel demonstrating expression of both OXRs in multiple human cardiac compartments (Figure 1F).

We also demonstrate that prepro-orexin is expressed as a 302bp PCR product in the left ventricle (LV), left atrium (LA), interventricular septum, right ventricle (RV), and right atrium (RA) of the rat heart (Figure 1G). We have used Western blotting to demonstrate that the prepropeptide can be cleaved into full length OR-A (Figure 1H) and OR-B (Figure 1I), with molecular weights of approximately 3.5 kDa and 2.9 kDa, respectively. We then assessed the protein expression using immunohistochemistry. Antibodies were validated using rat thymus as negative control and rat spleen and rat testes as positive control (Figure 1J; Supplementary Figure 2). Subsequent immunohistochemical analysis of rat ventricular

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sections revealed protein expression of both cleaved peptides across the ventricle (Figure 1K; Supplementary Figure 2). Similar expression was evident in individual cardiomyocytes (Figure 1L).

Cardiomyocyte contractility and calcium measurements

Superfusion of cardiomyocytes with Tyrode's solution containing 10 nM OR-B increased the shortening of twitch contraction (Figure 2A), from 15.2±1.2% to 20.6±1.7% (n=6, *P<0.01*; Figure 2B). This was not associated with any change on either the amplitude (Figure 2A) or the rate of the Ca^{2+} transient decline (n=6, *P=NS*; Figure 2C). OR-A had no effect on the rat cardiomyocyte cell length even with concentrations as high as 100 µM (data not shown; P=0.07). The OR-B effect was replicated using the specific OX2R agonist ([Ala11, D-Leu15]-Orexin B) (Figure 2D), whilst it was not blocked by an OX1R specific antagonist (SB-408124) (Figure 2E), suggesting that these effects are predominantly mediated via an OR-B/OX2R pathway.

Furthermore, OR-B, but not OR-A (data not shown), significantly induced phosphorylation of troponin I at Ser^{23}/Ser^{24} in cardiomyocytes. This effect was dose-dependent, with a maximum activation at 100 nM of OR-B (*P<0.001*; Figure 2F). Similarly, OR-B, but not OR-A (data not shown), induced significant phosphorylation of the myosin light chain (MLC) at Ser^{19} with maximal effects at 100 nM and 15 min (*P<0.001*; Figure 2G).

OR-B exerts a cardioprotective effect

In both the isolated rat heart and the *in vivo* rat model, we measured left ventricular developed pressure (LVDP), LV pressure decay and HR. The rate pressure product (RPP) was then calculated; this was used as a clear and direct indication of the energy demand of the heart and, thus, a good measure of the energy consumption of the heart pre- and post-ischaemia.

Initially, pre-treatment with either OR-A, OR-B or OX2R agonist, prior to the ischaemic insult, did not alter the haemodynamic parameters or HR between any of the groups (data not

shown). Contractile parameters were recorded at various time points throughout the experimental protocol (Supplementary Figure 1). Following 30 min of global ischaemia in the isolated Langendorff perfused rat heart model, the expected decline in cardiac function during reperfusion, as observed in the control hearts, was not affected by pre-treatment with OR-A (Figure 3A). In contrast, the pre- and post- treatment group for both OR-B and the OX2R agonist resulted in significantly improved recovery compared to control during reperfusion (*P<0.05;* Figure 3A-B). This was further replicated in the *in vivo* rat model where the control group (saline treated) demonstrated poor cardiac functional recovery and cardiac function following 30 min of regional ischemia and 120 min reperfusion, whereas the OR-B pre-treated group [bolus injection (i.v.) of OR-B (100 nM) 10 min prior to regional ischaemia] showed significant improvement in functional recovery during reperfusion (*P<0.05;* Figure 3C). Concentration dependent effects were noted with OR-B (20–100nM), with a maximal response at 100 nM (data not shown).

Effects of OR-B reducing the LV infarct size

In isolated rat heart model pre-treated with OR-B for 10 min prior to global ischaemia and reperfusion the LV infarct size was reduced from 61.5±3.0% in the control to 22.6±1.9% in the treated group (*P<0.001;* Figure 3D). In addition, the OR-B post-ischaemic treatment was just as effective at providing myocardial protection as pre-treatment, since it also significantly reduced the LV infarct size to 21.8±3.9% (*P<0.001;* Figure 3E). The OX2R specific agonist exhibited a similar protective effect to OR-B treatment, inducing a reduction in LV infarct size both during pre-treatment (25.7±3.6%) and at the start of reperfusion (34.7±3.1%, both *P values <0.001* compared to control*;* Figure 3D-E). However, pre-treatment with OR-A alone showed no significant reduction compared to control (58.3±3.0% *versus* 61.5±3.0%, respectively; P=NS) in the LV infarct size (Figure 3D). Moreover, in the *in vivo* model pre-treatment with a bolus injection (i.v.) of OR-B (and of ORX2Ra, but not of OR-A) 10 min prior to regional ischaemia and reperfusion significantly reduced the %LV infarct size compared to

the control (saline) group (66.7±3.0% *versus* 26.8±14.9% in the control and OR-B treated group, respectively; *P<0.001;* Figure 3F).

Involvement of distinct signalling pathways in myocardial protection

The isolated rat heart model was pre-treated either with an OX1R specific antagonist or a PI3 kinase (PI3K) inhibitor (wortmannin) in the presence or absence of OR-B prior to ischaemia reperfusion. Pre-treatment with either the OX1R specific antagonist or wortmannin alone showed no change in functional recovery compared to the control group (Figure 4A) and no effect on the %LV infarct size (61.5±3.0% in the control group *versus* 61.8±1.9% with OX1R antagonist alone, and 56.5±1.5% with wortmannin alone; P=NS, Figure 4B). Pre-treatment with OR-B together with wortmannin showed no myocardial protection on cardiac functional recovery (*P=NS;* Figure 4A); and, abolished the OR-B protective effect on LV infarct size (*P=NS*, Figure 4B), suggesting the importance of the PI3K/Akt pathway in OR-B induced cardioprotection. Improved functional recovery with OR-B pre-treatment was not inhibited by the simultaneous administration of an OX1R specific antagonist (*P<0.05;* Figure 4A). Similarly, the OR-B protective effect on %LV infarct size was not blocked by the OX1R specific antagonist (*P<0.001*; Figure 4B). Both these findings suggest that the noted effect is OX2R specific.

Given that ischaemic preconditioning protects the heart by phosphorylating the pro-survival kinases Akt and ERK1/2 at reperfusion, we investigated further whether orexins can affect their phosphorylation (33). Treatment with OR-B increased the phosphorylation of both Akt and ERK1/2 compared to basal levels (*P values <0.001*; Figure 4C-E); in contrast to OR-A which had no significant effect on ERK1/2 (Figure 4E) and Akt (data not shown). The OR-B effect was concentration-dependent (Figure 4D). The time course for the ERK1/2 activation was for 3, 5, 10, and 15 minutes. The involvement of a MAPK pathway was further corroborated using the selective MAPK inhibitor U0126, demonstrating that inhibition of the MAPK pathway using U0126 abrogates the protective effect of OR-B on LV infarct size (Figure 4F).

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OX2R expression in patients with heart failure, and involvement of the ERK1/2 pathway

To further evaluate the potential implication of orexins and their receptors in cardiac disease in humans, we studied OXR downstream signalling and expression in cardiac tissue samples from adult patients undergoing cardiac surgery and coronary artery bypass grafting. In the context of these studies, all human myocardial tissue samples were collected from the right atrial appendage during cardiac surgery.

Using Western blotting, we examined the direct effects of OR-A and OR-B on the phosphorylation of Akt and ERK1/2 in heart tissue samples from 8 patients. Treatment with OR-A or OR-B did not change Akt phosphorylation compared to basal levels (Figure 5A), whilst only OR-B significantly increased ERK1/2 phosphorylation compared to control (non-treated heart tissue; *P <0.05;* Figure 5B).

In addition, we measured OXR expression levels in heart tissue samples from heart failure patients undergoing coronary artery bypass grafting (n=54; Table 1). Our results demonstrated a negative correlation between the severity of heart failure symptoms and the expression of OX2R. As such, when patients were categorised using the New York Heart Association (NYHA) functional classification of heart failure (classes I to IV based on increasing severity of clinical symptoms) there was a significant decrease of OX2R in NYHA-II compared to NYHA-I and in NYHA-III/IV compared to NYHA-II and NYHA-I *(P values <0.05*; Figure 5C).

Discussion

In this study, we have shown that the heart constitutes a source of orexins and expresses functional OXRs. Moreover, our experiments revealed that OR-B can exert direct cardioprotective effects in both *ex vivo* and *in vivo* rat heart models.

Indeed, using the Langendorff perfused rat heart model, pre- and post- myocardial ischaemia treatments with both OR-B and a selective OX2R agonist resulted in significantly

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increased RPP which is a direct indication of the energy demand of the heart and, thus, a good measure of the energy consumption of the heart pre- and post-ischaemia.

In the case of the *ex vivo* studies that replicate the *in vivo* results, additional interactions between sympathetic activity, which is increased in ischaemia (34), and OXR signalling through cAMP (35) cannot be ruled out. Additionally, treatment of ventricular cardiomyocytes with OR-B, but not OR-A, lead to significantly increased Tn-I and MLC phosphorylation, followed by a concomitant increase in the strength of their twitch contraction. Of note, these findings agree with previous studies demonstrating that changes in the phosphorylation of key cardiac regulatory proteins can affect cardiac function (36,37).

This is the first observation of a differential specificity of OR-A and OR-B in the heart. OR-A activates both OX1R and OX2R receptors with similar potencies (~36 nM), whereas OR-B selectively activates OX2R compared to OX1R (~420 nM) (38). Both receptors belong to the GPCR superfamily and are highly promiscuous in terms of their G protein coupling characteristics (39). These tend to be cell- and tissue-specific and influence subsequent activation of downstream signalling pathways. For example, we have previously shown that expression of human OX2R is regulated by a complex involving a proximal PKA/PKC-regulated promoter and a distal promoter regulating tissue-specific expression of alternative transcripts which in turn post-transcriptionally regulate receptor levels (40). Therefore, future studies are required to provide further insight into the regulation of the promoter of OX2R in the myocardium.

Moreover, a dichotomous role of OXRs is evident in the rat brain, where OX1Rs mediate the neurobiological effects that drive drug seeking, whereas OX2Rs play a crucial role in sleep/wake cycle regulation and arousal (41). Interestingly, OR-A, but not OR-B, can activate electrophysiologically the subfornical organ of rat neurons (42). In a more recent study, OR-A (100 nM) and OR-B (100 nM) increased leptin gene expression in differentiated preadipocytes by 49.9% and 71.3%, respectively (43), indicative of differential modulation of transcriptional responses by these two peptides.

This phenomenon has been previously described for other GPCRs. In the case of smooth muscle cell CRH-receptors, urocortin, but not CRH, activated the MAPK signal transduction pathway despite similar binding affinities (44). Thus, it is possible that differences on OR-A and OR-B responses could also be influenced by the cardiac microenvironment, leading to alternative coupling of G proteins and activation of distinct signalling pathways. In previous studies, we have shown that OX2Rs can differentially couple to Gq, Gi/o and Gs proteins in different tissues (19,22,39). Emerging studies have also shown that OXRs can form functional dimers. It is possible that dimerization can also take place at the cardiac level and therefore influence pharmacological properties, such as the affinity of the receptors for orexin ligands, as well as cellular distribution and trafficking (45). Finally, polymorphisms of the OX2R gene have also been described previously (46). To this date, dimerization or polymorphisms have not been explored in the myocardium. Future studies should determine whether dimerization or polymorphisms of cardiac OXRs may affect ligand binding, or indirectly affect ligand binding by altering receptor structure.

In the present study, using an OX1R antagonist and an OX2R agonist, we conclude that the primary signalling at the rat heart is mediated via an OR-B/OX2R pathway. OXRs are able to activate certain key plasticity-regulating cascades, such as PI3K and ERK, and p38 MAPK. Here, pre-treatment with wortmannin abolished the OR-B protective effect on LV infarct size; suggesting the involvement of the PI3K/Akt pathway in OR-B induced cardioprotection. This signalling pathway has been implicated in myocardial protection; and is referred to as the reperfusion injury signalling kinase (RISK) pathway (33). Mediators that target the activation of the RISK (PI3K/Akt) pathway have been also shown to ameliorate recovery following ischaemic stress (25,47). In our study, OR-B, but not OR-A, was able to induce the phosphorylation status of both Akt and ERK1/2. Our data contradict previous studies showing that both orexin peptides can increase Akt phosphorylation. For example, in a study of neuronal cell cultures OR-B was more potent than OR-A, but the maximal effect of both peptides on the Akt activation was very similar (48). This apparent contradiction could be attributed to the fundamental differences between cardiomyocytes and neuronal cells

including morphological polarity, shape and function of action potentials, synaptic and homeostatic plasticity; not to mention the spatial expression of orexin receptors in the brain (49). As such, it is plausible that orexins exert their effect in an organ- and cell-specific manner.

The OR-B selectivity in post-translational modification of key mediators is corroborated by previous studies in other tissues. Indeed, in the human testis, when IP3 turnover was measured, OR-B exerted a more potent effect compared to OR-A (18). Similarly, in human H295R adrenocortical cells diverse roles have been noted for OR-A and OR-B in terms of activation of MAPKs and cortisol release; *i.e.* only OR-A (at 1nM) activated ERK1/2 and p38 (50).

Based on our findings, herein we propose the presence of a signalling pathway involving OX2R-ERK1/2-MLC in the heart with a net effect of increased Ca^{2+} sensitivity and cell length/contractility following OR-B superfusion in cardiomyocytes (21,51–54). Activation of ERK1/2 is involved in the activation of contractile responses through mechanisms involving direct phosphorylation of the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) (33,55–57).

In a seminal review by Spinazzi *et al*, a case is made for mediation of Ca^{2+} by orexin receptors (58). However, this effect of OX1R and OX2R on Ca^{2+}-release is through IP3 receptor activation, which, although evident in the hypothalamus, does not impact on the contraction of rat ventricular cardiac muscle under basal conditions, where the ryanodine receptors (RyR) dominate excitation-contraction (E-C) coupling (59). As such, this may have more of a role in pathophysiology of heart failure and arrhythmias (60). Interestingly, the dominant expression of IP3-receptors is on the nuclear membrane where they are thought to be involved in cardiac hypertrophy (61).

Conversely, inhibition of ERK attenuates force development by lowering MLC phosphorylation in cardiac tissue (62). Because of its central role in the regulation of contraction and relaxation of the heart, cTnI is also phosphorylated by OR-B to ensure

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appropriate function. Changes in the phosphorylation status of cTnI are well documented during acute cardiac events and in patients with heart failure (63).

We have expanded on these observations using human heart tissue samples from heart failure patients. So far, a number of studies indicated that there is a dysregulation of the orexin system in the CNS in models of heart failure (64–66). Here we further report that in adults with heart failure (NYHA classes I to IV) there was a significant negative correlation between the severity of clinical symptoms (NYHA class) and OX2R expression. The fact that OX2R expression was significantly lower in patients with NYHA classes III/IV compared to less symptomatic patients with NYHA class I/II, indicates that OX2R signalling is compromised in more severe heart failure. These observations are further supported by the findings of Perez *et al.*, where in a heart failure model, HCRTR2/OX2R-deficient mice exhibited poorer cardiac function, and greater myocardial scarring (67). Moreover, in the same study, differential OX2R expression data from microarray experiments was recorded in patients with dilated cardiomyopathy and ischemic cardiomyopathy (67).

Based on the established role that orexins have in maintaining the awake state, orexin antagonism has also been identified as a novel therapeutic approach for the treatment of insomnia (68). As such, suvorexant, a non-selective dual orexin (OX1R/OX2R) receptor antagonist (DORA), has been approved by the FDA, in 2014, for treating insomnia patients with difficulties in sleep onset and/or sleep maintenance (www.acc.org/latest-in-cardiology/articles/2015/06/16/08/40/new-insomnia-drugs-in-the-context-of-cardiovascular-disease - BELSOMRA® (suvorexant) package insert: www.merck.com/product/usa/pi_circulars/b/belsomra/belsomra_pi.pdf). To date, suvorexant use has not been associated with any reported adverse cardiovascular safety outcomes; however, it must be highlighted that it is unknown whether suvorexant has adverse effects in CVD patients, since patients with significant and/or recent CVD (*e.g.* with acute coronary syndrome or congestive heart failure) were excluded from the relevant trials (http://www.acc.org/latest-in-cardiology/articles/2015/06/16/08/40/new-insomnia-drugs-in-the-context-of-cardiovascular-disease). Therefore, and based on the findings from our study

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and from others (67), the use of orexin antagonists may warrant further assessment and careful monitoring for CVD adverse effects, particularly in heart failure patients (69).

Finally, our human *ex vivo* study results were also consistent with the *in vitro* rat data, since orexin treatment of human myocardial tissue demonstrated that OR-B, but not OR-A, was able to induce ERK1/2 phosphorylation. However, there was no change in Akt phosphorylation upon treatment with OR-A or OR-B. The latter could be due to the fact that chronic heart failure reduces Akt phosphorylation, so signalling is already compromised at basal levels. Indeed, compared to subjects with normal cardiac function, human skeletal muscle of heart failure patients demonstrates reduced Akt phosphorylation (70). It should be noted that the cardiovascular and musculoskeletal system are interrelated, whereby a functional deterioration in one is reflected similarly in the other (71). Differences in the phosphorylation of Akt can also be due to species differences, since certain proteins, like ERG, are differentially expressed in the rat and human heart (72).

We also acknowledge certain limitations with our experimental system using healthy young adult rats compared to patients used in this study that have certain comorbidities. To date, it has proven difficult to find the "perfect" translational *in vivo* model for CVD. This is primarily due to the high level of complexity, as well as heterogeneity of these diseases, in addition to potential impact/influence from environmental or genetic factors. Future studies can make use of different animal models for examples such as; heart failure, aged rats, or rats fed with a high fat diet to generate further data on the involvement of OR-B/OX2R at the cardiac level. However, to the best of our knowledge, there is no perfect *in vivo* model to represent the entire repertoire of defects/comorbidities at the cardiac level.

In summary, our present findings document that OR-B exhibits a protective role on cardiac function pre- and post- ischaemia, leading to reduction of the infarct size. We also provide novel conclusive evidence that the rat heart expresses functional OXRs and is a source of orexins. Cardiac OXRs can also influence contractile tone via mechanisms involving phosphorylation of TnI and MLC. Thus, in addition to the known CNS effects of orexins/OX2R (5,73), our data illustrate the importance of the OX2R as a potential

therapeutic target directly at the heart level, suggesting that targeted use of OX2R specific agonists may have a role in the prevention and treatment of CVD.

Disclosure

None

Author Contributions

HSR conceived and planned the experiments. VHP, JC, EK, CA, AA, BKT carried out the experiments. VHP, EK, IK, HSM, GKD, GR, CA, EWH, HSR, contributed to the interpretation of the results. HSR and EK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Figure Legends

Figure 1: **Expression of orexin-1 (OX1R) and orexin-2 (OX2R) receptors in the rat and human heart**.

Panel A: Quantitative RT-PCR analysis of OX1R and OX2R in the rat hypothalamus (hypo) and rat heart (***P<0.001 for heart *vs.* hypothalamus).

Panel B: Immunohistochemical analysis of rat heart ventricular sections (n=3) for OX1R (I) and OX2R (II). Magnification x400. Negative control (III) confirmed the specificity of the immunostaining.

Panel C: Immunohistochemical analysis of OX1R (I) and OXR2 (II) in rat cardiomyocytes and negative control (III). Identical results obtained from four independent experiments; measuring at least 50 cells.

Panel D: Expression of OX1R and OX2R in rat heart compartments (n=3). Lane 1 cDNA from left ventricle (LV); Lane 2 cDNA from left auricle; Lane 3 cDNA from septum; Lane 4 cDNA from right ventricle (RV); Lane 5 cDNA from right auricle; and Lane 6 is DNA marker.

Panel E: Single-Cell RT-PCR in cardiomyocytes (n=6): Lane-1: β-Actin; Lane-2: OX1R; Lane-3: negative control; Lane-4: OX2R.

Panel F: Expression of OX1R and OX2R in different human cardiac compartments: Lane-1: aorta; Lane-2: apex of the heart; Lane-3: left atrium (LA); Lane-4: right atrium (RA); Lane-5: right auricle; Lane-6: left auricle; Lane-7: left ventricle (LV); Lane-8: right ventricle (RV); Lane-9: interventricular septum; and Lane-10: atrioventricular (AV) node.

Panel G: Expression of prepro-orexin in rat heart chambers (n=3). Prepro-orexin is expressed as a 302bp PCR product in the left ventricle (LV) (lane 1), left atrium (LA) (lane 2), interventricular septum (lane 3), right ventricle (RV) (lane 4), and right atrium (RA) (lane 5). M=Marker; DNA ladder.

Panels H and I: Prepro-orexin (PPO) and the cleaved peptides Orexin-A (OR-A) and Orexin-B (OR-B) were detected using western blotting, with molecular weights of 15 kDa for prepro-orexin, 3.5 kDa and 2.9 kDa for OR-A (H) and OR-B (I), respectively; Lane 1: rat heart lysates; and Lane 2: rat hypothalamus lysates (n=3).

Panel J: Immunohistochemical analysis for OR-A on rat thymus section (I) and rat spleen (II).

Panel K: Immunohistochemical analysis of rat heart ventricular sections (n=3) for OR-A (I) and OR-B (II) and negative control (III). Magnification x400.

Panel L: Rat cardiomyocyte immunofluorescent analysis of OR-A (I), OR-B (II), and negative control (III). Identical results obtained from four independent experiments, measuring at least 50 cells.

Figure 2: Effects of orexin B (OR-B) on contractility of rat cardiomyocytes and on the phosphorylation of troponin I (TnI) and myosin light chain (MLC).

Using video-edge detection system, changes in cell length of individual cardiomyocytes (n=6) were recorded upon challenge with orexins.

Panel A & B: Superfusion of the myocyte with OR-B (10 nM) increased the strength of twitch contraction (**P<0.01).

Panel A & C: No effect on the amplitude of the Ca^{2+} transient was observed.

Panel D: Similar effects on the strength of twitch contraction was observed following treatment with a specific orexin-2 receptor (OX2R) agonist (OX2Ra: [Ala11, D-Leu15]-Orexin B) (*P<0.05).

Panel E: Addition of an OX1R specific antagonist (OX1R Ant.: SB-408124) did not alter the OR-B cardiomyocyte contractile response (*P<0.05).

Panel F: OR-B was able to induce phosphorylation of troponin I in a concentration dependent manner (**P<0.01, ***P<0.001).

Panel G: OR-B was able to induce phosphorylation of MLC in a concentration dependent manner (**P<0.01, ***P<0.001). For the above experiments, equal protein loading was confirmed, using *β*-actin as an internal control. B= basal (no Orexin B).

Figure 3: Cardiac functional parameters and infarct size determination following ischaemia (n=8).

Panels3A-C. Panels depict the effects on left rate pressure product (RPP) = heart rate (HR) x left ventricular developed pressure (LVDP), following 30 min of ischaemia (45 min to 75 min) for:

Panel 3A: Control (Con); pre-treatment: orexin A (OR-A), orexin B (OR-B) or orexin-2 receptor (OX2R) agonist (OX2Ra) in the *in situ* rat model (*ex vivo;* isolated Langendorff model) (*P<0.05, compared to control).

Panel 3B: Control, post-treatment OR-B or OX2Ra in the *in situ* rat model (*P<0.05, compared to control).

Panel C: Control, bolus pre-treatment OR-A, OR-B, OX2Ra in the *in vivo* rat model (*P<0.05, compared to control).

Panels 3D-F. Depict the effects on the left ventricle (LV) infarct size (% LV; transverse sections of the rat heart were obtained at the end of the experimental protocol and the sections were stained using triphenyl-tetrazolium chloride so that the infarcted tissue appears pale, whilst viable tissue stains red). More specifically:

Panel D: Control; pre-treatment with OR-A, OR-B and OX2Ra in the *ex-vivo* model (***P<0.001, compared to control).

Panel E: Control; post-treatment with OR-B or OX2Ra in the *ex-vivo* model (***P<0.001, compared to control).

Panel F: *In vivo* model with bolus pre-treatment with OR-A, OR-B and OX2Ra prior to regional ischaemia and reperfusion (***P<0.001, compared to control)**.**

Figure 4: Orexin B (OR-B)/orexin-2 receptor (OX2R) effects on key signalling pathways involved in myocardial protection using the isolated Langendorff perfused rat heart model (n=8).

Panel A: Cardiac functional performance measured and represented as left rate pressure product (RPP) in rat hearts pre-treated with control (saline treated), OR-B plus OX1R antagonist, OX1R antagonist alone, OR-B plus wortmannin (wort; a PI3Kinase inhibitor), and wortmannin alone (*P<0.05 compared to control).

Panel B: Left ventricle infarct size determination (%LV) in pre-treated hearts with OR-B plus OX1R antagonist, OX1R antagonist alone, OR-B plus wortmannin, and wortmannin alone (***P<0.001 compared to control). Dotted line across denotes control levels.

Panel C: OR-B time-dependent treatment and Akt phosphorylation: exposure of rat cardiomyocytes to OR-B (100 nM) induced maximal phosphorylation at 5 min. Change in the phosphorylation was evident as early as 2 min post-treatment (*P<0.05, ***P<0.001 compared to basal).

Panel D: OR-B induced a dose-dependent induction in the phosphorylation status of Akt, becoming significant at 1 nM and reaching a plateau at 100 nM (**P<0.01, ***P<0.001 compared to basal).

Panel E: OR-B treatment increased the phosphorylation of ERK1/2 compared to basal levels (***P<0.001). Isoproterenol (ISO) and Angiotensin-II (Angio-II) were employed as positive controls.

Panel F: inhibition of the MAPK pathway using a selective MAPK inhibitor (U0126) abrogates the protective effect of OR-B on LV infarct size (*P<0.05, **P<0.01, ***P<0.001 compared to control; a: P<0.05 compared to OR-B).

Figure 5: Orexin-induced signalling and orexin receptor expression in heart samples from cardiac surgery patients.

Panel A-B: In human heart tissue obtained from patients undergoing coronary artery bypass (n=8), both orexin A (OR-A) and orexin B (OR-B) showed no change in Akt phosphorylation compared to the non-treated heart tissue (100 nM). Change in the phosphorylation state was evident for ERK1/2 signaling in the OR-B treatment group (*$P<0.05$ compared to control).

Panel C: Expression of orexin-2 receptor (OX2R) in heart samples from heart failure patients (n=54) demonstrated a significant decrease of OX2R in NYHA-II compared to NYHA-I patients ([‡]$P<0.05$) and in NYHA-III/IV compared to NYHA-II (*$P<0.05$) and to NYHA-I patients ([†]$P<0.05$). NYHA: New York Heart Association Functional Classification for heart failure stages.

Supplementary Figure 1: Experimental protocol for the isolated Langendorff perfused rat heart and the *in vivo* model of ischaemia reperfusion (n=8).

Protocol (1): presents the control pre-treatment study, which consisted of 30 minutes (min) stabilisation followed by 30 min global ischaemia and 120 min reperfusion. For the treatment groups, hearts were perfused with Tyrode's+treatment agent for 10 min prior to 30 min global ischaemia followed by 120 min reperfusion.
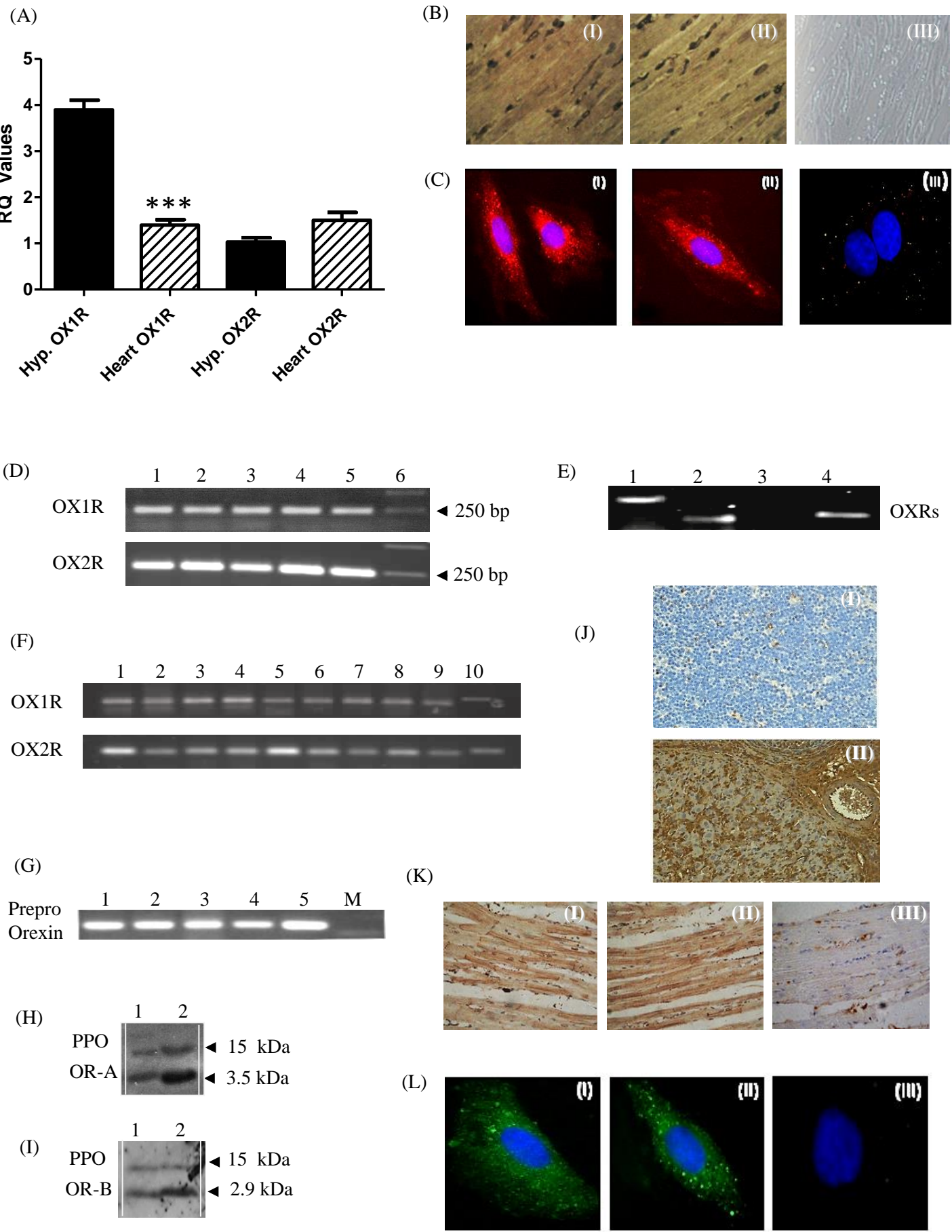
Protocol (2): presents the post-treatment study group, hearts were perfused with Tyrode's+treatment for 30 min into the start of reperfusion after which the hearts were perfused with Tyrode's only.
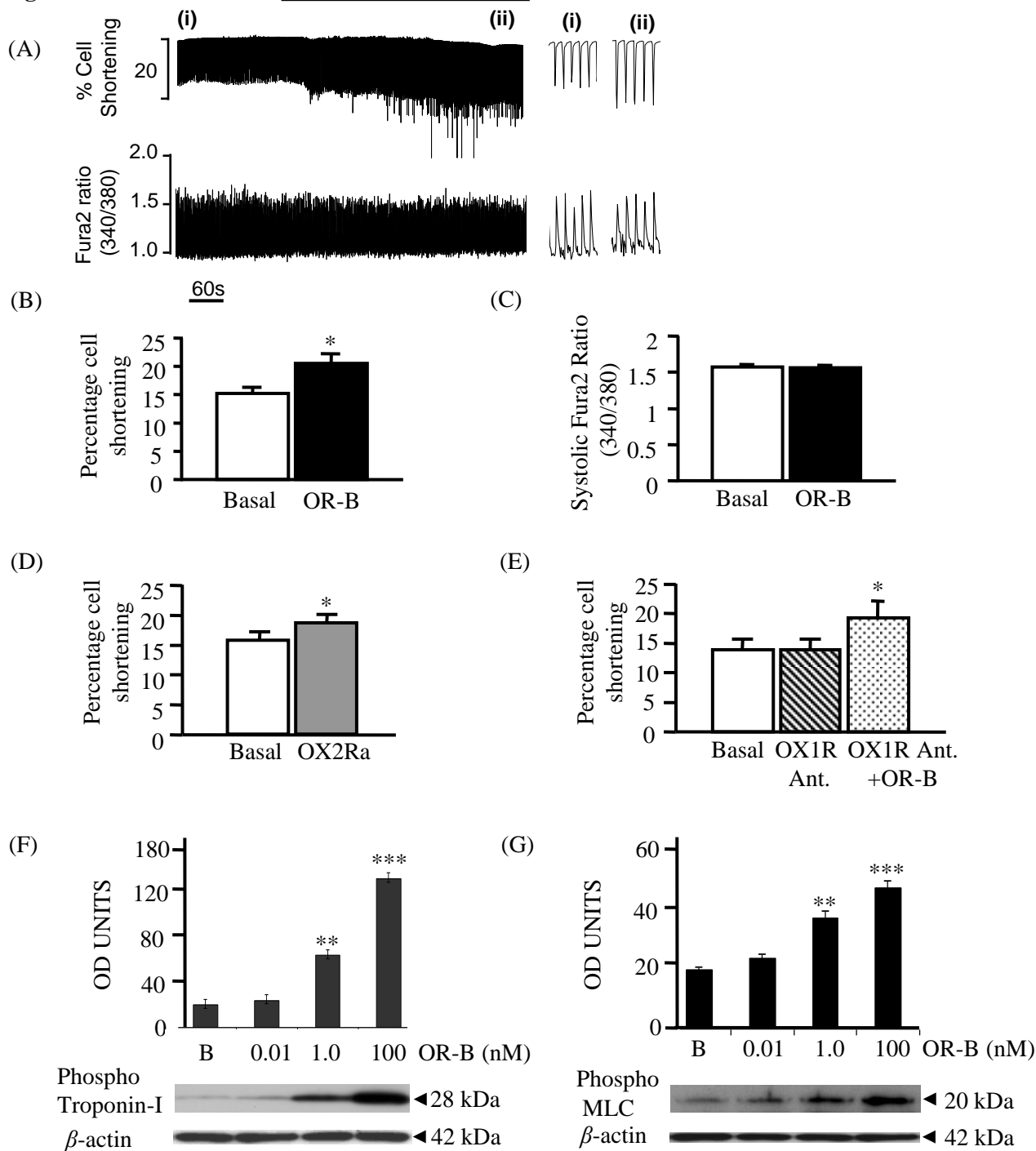
Protocol (3): presents the *in vivo* rat model of regional ischaemia and reperfusion; the pre-treatment was with a bolus intravenous (i.v.) injection (*e.g.* of orexin B 10 nmol) 10 min prior to 30 min regional ischaemia and 120 min reperfusion.

Supplementary Figure 2: A: Rat testes (positive control), B and C: Different sections of rat heart stained positive for OR-A, D: Rat Heart; a region that is not positive for OR-A; demonstrating antibody specificity. Magnification x40
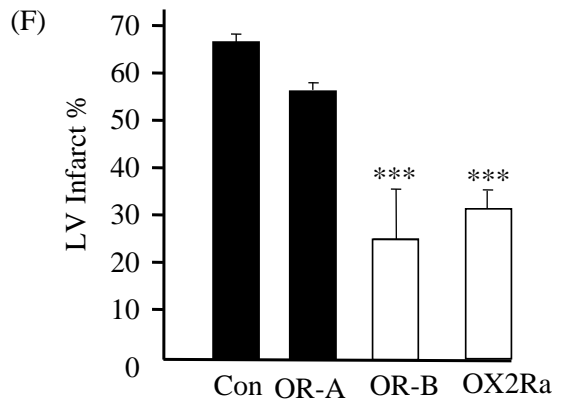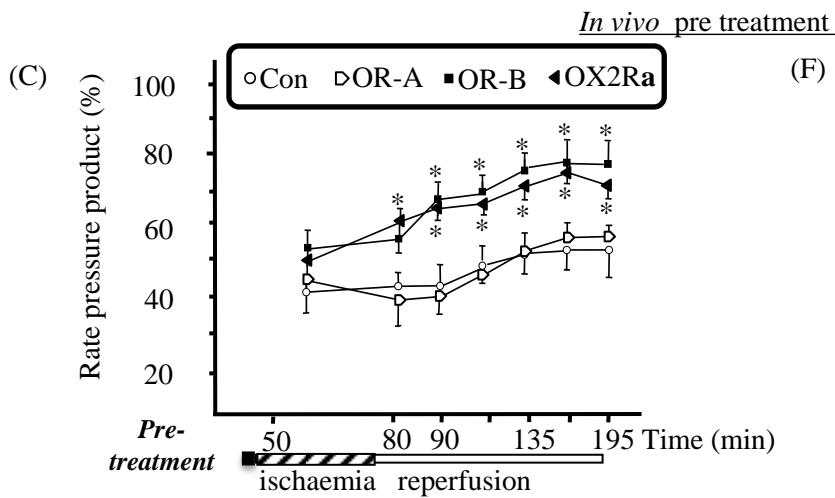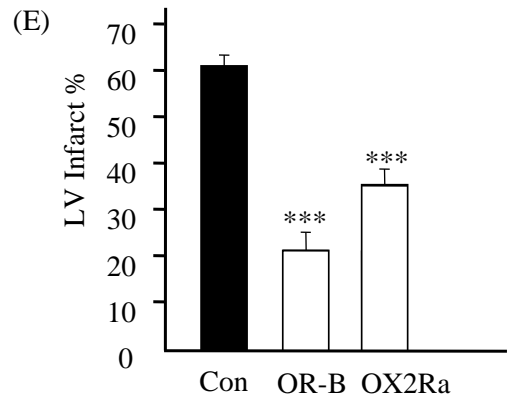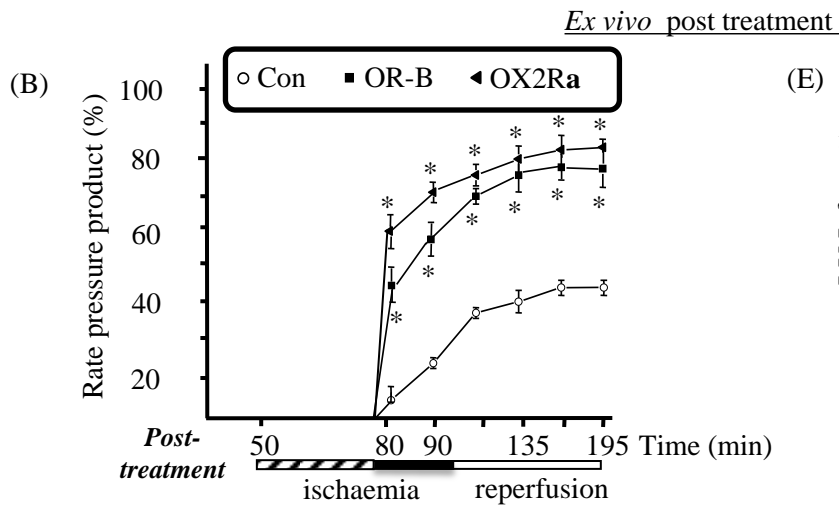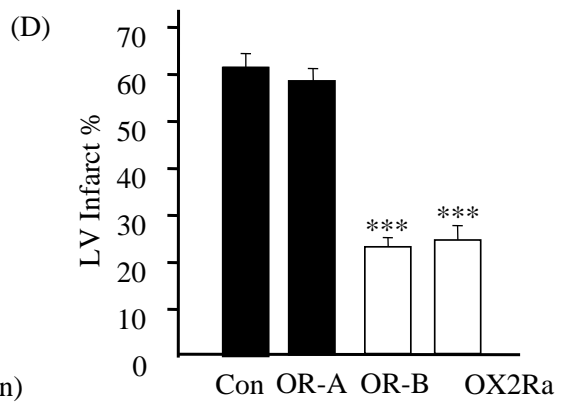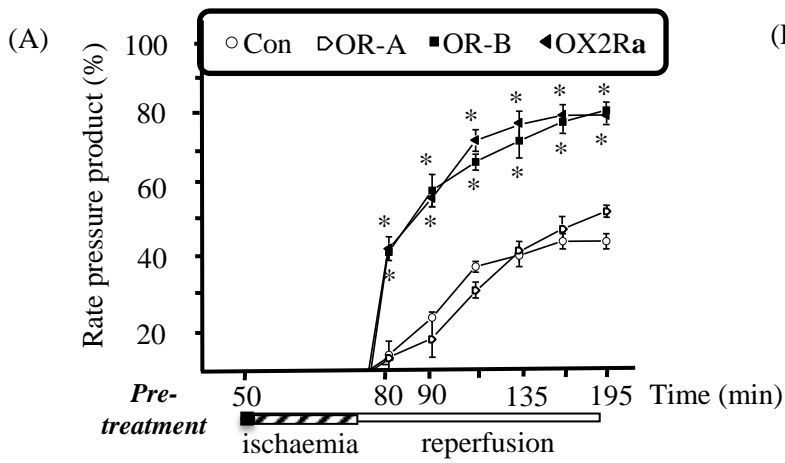
Table 1. Selected key patient characteristics prior to cardiac surgery and tissue collection for the heart failure study patients, including age, gender, hypertension, hyperlipidaemia, type 2 diabetes mellitus, smoking status and body mass index (BMI). Data are presented as mean±SEM, or as frequencies.
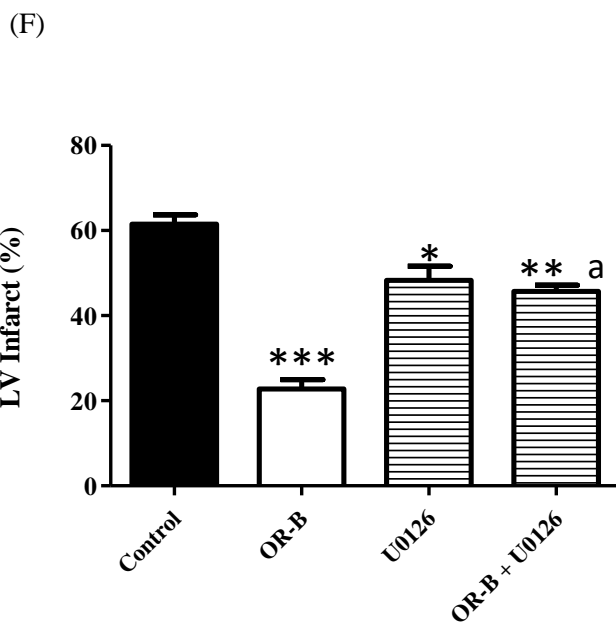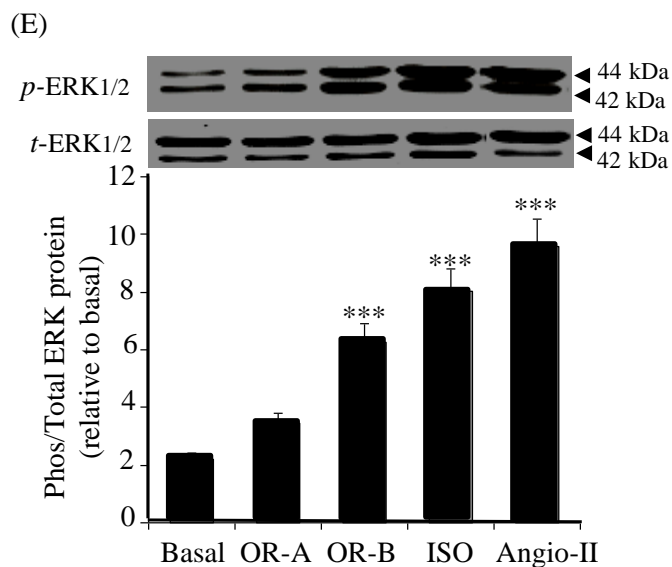
Figure 1

(A)

(B) (I) (II) (III)

(C) (I) (II) (III)

(D)
	1	2	3	4	5	6	
OX1R							◄ 250 bp
OX2R							◄ 250 bp

E)
	1	2	3	4	
					OXRs

(F)
	1	2	3	4	5	6	7	8	9	10
OX1R										
OX2R										

(G)
	1	2	3	4	5	M
Prepro Orexin						

(H)
	1	2	
PPO			◄ 15 kDa
OR-A			◄ 3.5 kDa

(I)
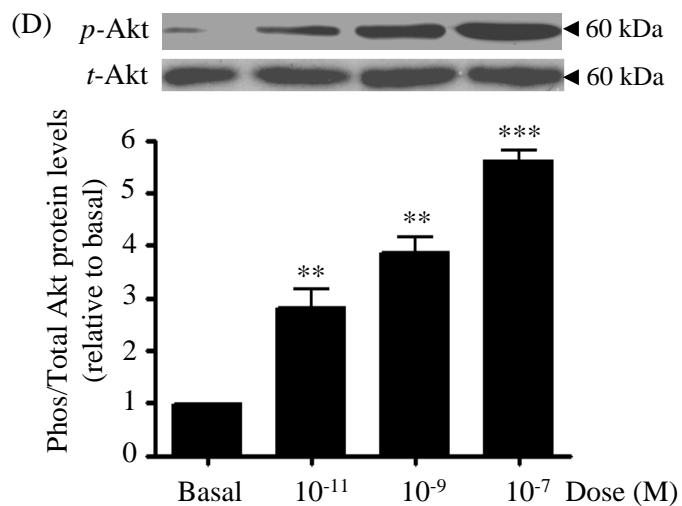|	1	2	
PPO			◄ 15 kDa
OR-B			◄ 2.9 kDa

(J) (I) (II)

(K) (I) (II) (III)

(L) (I) (II) (III)

Figure 2

(A)

10 nM Orexin-B

(i)   (ii)   (i)   (ii)

% Cell Shortening

20

Fura2 ratio (340/380)

2.0

1.5

1.0

60s

(B)

Percentage cell shortening

25
20
15
10
5
0

          Basal   OR-B

*

(C)

Systolic Fura2 Ratio (340/380)

2

1.5

1

0.5

0

          Basal   OR-B

(D)

Percentage cell shortening

25
20
15
10
5
0

          Basal   OX2Ra

*

(E)

Percentage cell shortening

25
20
15
10
5
0

     Basal   OX1R   OX1R Ant.
             Ant.   +OR-B

*

(F)

OD UNITS

180

120

80

40

0

      B   0.01   1.0   100   OR-B (nM)

***

**

Phospho
Troponin-I   ◄28 kDa

β-actin   ◄42 kDa

(G)

OD UNITS

60

40

20

0

      B   0.01   1.0   100   OR-B (nM)

***

**

Phospho
MLC   ◄ 20 kDa

β-actin   ◄ 42 kDa

Figure 3

Figure 4

Figure 5

Table 1

Participants (n)	54
Age (years)	65.2 +/- 1.3
Male gender (%)	50 (92.6)
Hypertension (%)	30 (55.6)
Hyperlipidaemia (%)	31 (57.4)
Type 2 diabetes mellitus (%)	16 (29.6)
Smoking - active (%)	26 (48.1)
- ex (%)	13 (24.1)
BMI (Kg/m^2)	27.29 +/- 0.50

Protocol 1
(pre-treatment protocol *in situ*)

| 0 | 30 | 40 | 45 | 75 | 80 | 90 | 105 | 135 | 165 | 195 |

baseline   Pre-
treatment   ← **GLOBAL ISCHAEMIA** →   **REPERFUSION** →
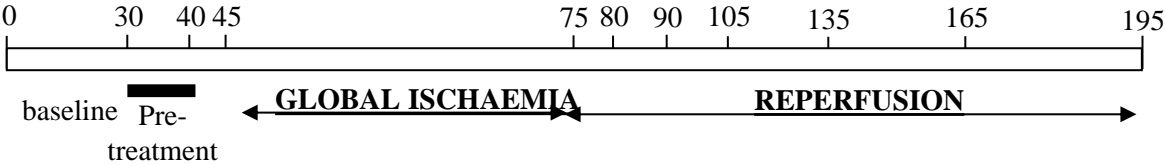
Protocol 2
(post-treatment protocol *in situ*)

| 0 | 30 | 40 | 45 | 75 | 80 | 90 | 105 | 135 | 165 | 195 |

baseline   ← **GLOBAL ISCHAEMIA** →   **REPERFUSION** →
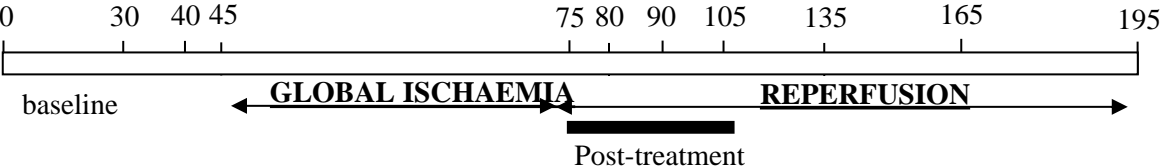
Post-treatment

Protocol 3
(pre-treatment *in vivo*)

| 0 | 30 | 40 | 45 | 75 | 80 | 90 | 105 | 135 | 165 | 195 |

baseline   ← **REGIONAL ISCHAEMIA** →   **REPERFUSION** →

Bolus
injection

A.

B.

C.

D.