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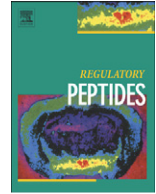

Activation of corticotropin releasing factor receptor type 2 in the heart by corticotropin releasing factor offers cytoprotection against ischemic injury via PKA and PKC dependent signaling

Anne K. Jonassen [a,*], Anita Wergeland [a], Erik Helgeland [a], Ole D. Mjøs [b], Bhawanjit K. Brar [c,d,**]

[a] Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen, Norway
[b] Department of Medical Physiology, Faculty of Medicine, University of Tromsø, Norway
[c] Department of Medicine, UCSD, San Diego, CA, USA
[d] VA San Diego Healthcare System, San Diego, CA, USA

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ABSTRACT

Corticotrophin-releasing factor receptor 2β (CRFR2β) is expressed in the myocardium. In the present study we explore whether acute treatment with the neuropeptide corticotrophin-releasing factor (CRF) could induce cytoprotection against a lethal ischemic insult in the heart (isolated murine neonatal cardiac myocytes and the isolated Langendorff perfused rat heart) by activating CRFR2. In vitro, CRF offered cytoprotection when added prior to lethal simulated ischemic stress by reducing apoptotic and necrotic cell death. Ex vivo, CRF significantly reduced infarct size from 52.1 ± 3.1% in control hearts to 35.3 ± 3.1% ($P<0.001$) when administered prior to a lethal ischemic insult. The CRF peptide did not confer cytoprotection when administered at the point of hypoxic reoxygenation or ischemic reperfusion. The acute effects of CRF treatment are mediated by CRF receptor type 2 (CRFR2) since the cardioprotection ex vivo was inhibited by the CRFR2 antagonist astressin-2B. Inhibition of the mitogen activated protein kinase-ERK1/2 by PD98059 failed to inhibit the effect of CRF. However, both protein kinase A and protein kinase C inhibitors abrogated CRF-mediated protection both ex vivo and in vitro. These data suggest that the CRF peptide reduces both apoptotic and necrotic cell death in cardiac myocytes subjected to lethal ischemic induced stress through activation of PKA and PKC dependent signaling pathways downstream of CRFR2.

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1. Introduction

The hypothalamic hypophysiotropic peptide corticotrophin releasing factor (CRF) is a 41-amino acid peptide produced in the hypothalamus as well as throughout the brain, where it plays a central role in behavioral and autonomic responses to stress. Peptides of the CRF family signal through the activation of two receptors, CRF receptor type 1 (CRFR1) and type 2 (CRFR2). The CRFR1 gene is expressed in human and rodents, while CRFR2 has three functional splice variants in human (α, β and γ) and two rodent variants (α and β). CRFR2 α is the major splice variant expressed in the rodent brain [1], whereas CRFR2β is expressed in peripheral tissue, with the highest levels in the skeletal muscle, heart and skin [2].

The main endocrine role for CRF is as the major stimulator of adrenocorticotropic hormone (ACTH) and b-endorphin release from the pituitary [3]. The peptide also has other direct functions in modulation of the immune and cardiovascular systems [4–7]. Although CRF is not found in abundance in murine heart tissue [8,9], the peptide has been shown to have a number of direct effects on mice [10], rat [11,12] and guinea pig hearts [13], presumably through activation of CRF receptor type 2 (CRFR2) that is highly expressed in myocytes and vessels within the heart [9]. It has been reported that a bolus injection of CRF into the left atrium of rat hearts, increases coronary flow, induces a positive inotropic effect and transiently increases atrial natriuretic peptide secretion [11]. Central administration of CRF has opposing effects to peripheral administration of the peptide, since central injection increases blood pressure and heart rate, whereas

Abbreviations: CRF, corticotrophin releasing factor; CRFR, corticotrophin releasing factor receptor; MEK½, mitogen activated protein kinase; PKC, protein kinase C; PKA, protein kinase A; ACTH, adrenocorticotropic hormone; UCN, urocortin; ERK½, extracellular signal-regulated kinases; Ast-2B, astressin 2B (CRFR2 antagonist); DMEM, Dulbecco's modified eagle's minimal essential medium; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; SI, simulated ischemia; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate buffered saline; LV, left ventricle; LVDP, left ventricle developed pressure; CF, coronary flow; RI, regional ischemia; EBD, Evans blue dye; RZ, risk zone; TTC, triphenyltetrazolium chloride; ANOVA, analysis of variance; H7, inhibitor of PKC; H-89, inhibitor of PKA; PD98059, inhibitor of ERK1/2; cAMP, 3′-5′-cyclic adenosine monophosphate; EC50, median effective concentration (required to induce a 50% effect); EPAC, exchange protein directly activated by cAMP; NMR, nuclear magnetic resonance.

* Correspondence to: A.K. Jonassen, Department of Biomedicine, Faculty of Medicine, University of Bergen Jonas Lies vei 91, N-5009 Bergen, Norway. Tel.: +47 55 58 67 96, +47 55 58 67 33.
** Correspondence to: B.K. Brar, Department of Medicine, University of California & VA San Diego Healthcare System, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0838, USA. Tel.: +1 858 220 6273.
E-mail addresses: Anne.Jonassen@biomed.uib.no (A.K. Jonassen), bbrarzensun@gmail.com (B.K. Brar).

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peripheral administration produces a decrease in blood pressure and tachycardia [12].

Over a decade ago, we showed that a 24-hour treatment of neonatal rat cardiac myocytes with CRF prior to a 6-hour simulated ischemic insult reduced lactate dehydrogenase release from the cells, suggesting that the peptide was cardioprotective [14]. Moreover, it was demonstrated that the cardioprotective effect of pre-conditioned media was abolished by the CRF receptor antagonist alpha-helical CRF [14]. These data suggested that CRF is released from myocytes in response to mild ischemic stress to directly act on a CRF receptor 'pre-conditioning' the cells from a subsequent lethal ischemic stimulus. However, the intracellular signaling pathways that mediate the effects of CRF were not determined. The urocortin peptides (urocortin 1, UCN; urocortin 2 (UCN 2); urocortin 3 (UCN 3)) are members of the CRF family of peptides and protect murine myocardium from ischemic injury through signaling molecules including mitogen-activated protein kinase, extracellular kinase-1/2 (ERK1/2) [9,15] and protein kinase C (PKC) [16] downstream of CRFR2 [15].

The main aim of the present study was to determine whether acute treatment of murine cardiac myocytes or isolated ex vivo rat hearts with CRF peptide confers cardioprotection against a lethal ischemic insult. Secondly, the aim was to determine the signaling downstream of the CRFR2 receptor that mediates CRF peptide cardioprotection.

2. Materials and methods

2.1. Peptides and reagents

Synthetic rat/human (r/h) CRF peptide was obtained from Sigma-Aldrich (St Louis, MO). The peptide was dissolved in ddH_2O containing 0.1% BSA (pH 7.4), at a concentration of 1 mg/ml, aliquoted and stored at −70 °C until the day of use. The CRFR2 receptor antagonist [17], astressin 2B (Ast-2B) was obtained from Professor Jean Rivier at the Salk Institute, San Diego, CA. Again the peptide was dissolved at a concentration of 1 mg/ml in ddH_2O containing 0.1% BSA (pH 7.4), aliquoted and stored at −70 °C until the day of use. CRF was used at a concentration of 10 nM and the CRFR2 antagonist Ast-2B was used at a concentration of 100 nM. The PKC (H7) and PKA (H-89) inhibitors were purchased from Calbiochem and were used at concentrations of 6 nM [18] and 10 μM respectively [19]. The ERK1/2 inhibitor PD98059 (PD) was used at 5 μM (Cell Signaling Technology, Beverly, MA) [9,15–20]. Five percent CO_2, 0% O_2, and balance gas N_2 was obtained from Airgas West (Los Angeles, CA).

2.2. Preparation and treatment of neonatal mouse cardiac myocytes

Cardiomyocytes were isolated from hearts of C57BL/6 mice as before [15,21] using sequential digestion in collagenase type II (Worthington Biochem Inc., Lakewood, NJ). The cardiomyocyte cell suspension was transferred to 48-well, 2% (wt/v) gelatin-coated plates at a density of 10^4 cells/well for experiments involving assessment of cell death by trypan blue exclusion. After 24 h, cell medium was replaced with DMEM supplemented with 1% (v/v) fetal bovine serum for an additional 24 h before treatment. Within 2 days, a confluent monolayer of spontaneously beating myocytes was formed. For assessment of apoptosis, cells were plated in 96-well plates for analysis and quantification of single stranded DNA (ssDNA) apoptosis using a ELISA kit assay.

2.3. SsDNA apoptosis using ELISA

Apoptotic cell death was assessed using a commercial single-stranded DNA (ssDNA) apoptosis ELISA (Enzyme-Linked ImmunoSorbent Assay detection) kit (Chemicon) according to the manufacturer's instructions. This ELISA technique is sensitive and relies on selective denaturation of DNA in apoptotic cells by formamide, and detection of denatured DNA with monoclonal antibody to single-stranded DNA as described previously [22].

2.4. Exposure of cardiomyocytes to lethal simulated ischemia

For lethal simulated ischemia (SI), cells were incubated in the hypoxic chamber at 37 °C for 6 h in a humidified atmosphere of 5% CO_2, 0% O_2, balance gas N_2 at a pressure of 4 lb/in^3 using Esumi ischemic buffer as before [15] and reoxygenated for 2 h. Esumi ischemic buffer contains 137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl.2H_20$, 4 mM HEPES, 10 mM deoxyglucose, and 20 mM sodium lactate (pH 6.2). Untreated cells were cultured in a normoxic environment in Esumi control buffer containing 3.8 mM KCl, 10 mM glucose, and no sodium lactate (pH 7.4). CRF was added to the cells 10 min prior to the SI insult. The cell signaling inhibitors were added 5 min prior to the CRF peptide (Fig. 1A). Cell death was measured using trypan blue exclusion or the apoptosis ELISA kit. For trypan blue exclusion, the cells were harvested as described previously [9,15]. After the addition of an equal volume of 0.4% trypan blue (Sigma) in PBS, the percentage of blue cells (dead cells)/total cells was counted by scoring 250 cells, a minimum of three times per well, using a hemocytometer. For neonatal cardiomyocytes, 6 h of SI and 2 h of reoxygenation were used in order to achieve approximately 40–60% cell death.

2.5. Isolated Langendorff rat heart preparation

The Langendorff perfusion model was used to investigate the cardioprotective actions of CRF on infarct size [9,15]. All experiments were approved by the Norwegian State Commission for Laboratory Animals, and carried out in accordance with the European Communities Council Directive of 1986 (2010/63/EU). Male Wistar rats (250–350 g) fed with a standard diet were heparinized (200 IU) and anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The hearts were excised and rapidly mounted onto a Langendorff perfusion system as described before [23]. A water-filled latex balloon, connected to a hydrostatic pressure transducer (SP844, Memscap, Norway) and coupled to a high performance data acquisition system (Power Lab 8/30, Chart Pro software-MLS250), was inserted into the left ventricle (LV) through an incision in the left atrium and inflated to set an initial end diastolic pressure of 5–10 mm Hg. Coronary flow (CF) was measured by timed collection of effluent over 1 min at each sampling point. A 3–0 silk suture was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischemia was achieved by pulling the snare and was confirmed by a substantial fall in both left ventricular developed pressure (LVDP) and CF, while ischemic reperfusion was achieved by releasing the snare. All hearts underwent 20 min of stabilization [9,15,23], 30 min of regional ischemia (RI), and then 120 min of reperfusion. CRF (10 nM) was present for 10 min before the lethal ischemic insult (Fig. 1B). To explore the signaling pathways that mediate the protective effect of CRF, the following inhibitors were added 5 min prior to the 10 min CRF treatment [9,15,23]; ERK1/2 inhibitor PD 98059(5 μM), PKC inhibitor H7 (6 nM), PKA inhibitor H-89 (10 mM) and CRFR2 inhibitor Ast-2B (100 nM). At the end of the experiment the ligation was re-tied and Evans blue dye (EBD) 0.2% (w/v) was infused to demarcate the risk zone (RZ). The hearts were frozen at −20 °C and thereafter cut into 2-mm thick slices from the apex to the atrioventricular groove. Then the slices were stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37 °C for 20 min, before fixation in 4% (v/v) formalin solution to enhance the contrast of the stain, and then compressed to a uniform thickness by placing them between two glass plates separated by a 2-mm spacer. The area of the left ventricle, the infarcted area (TTC negative) and the risk zone (none blue) were traced on an acetate transparency, and the infarct size was determined using a computerized planimetry program where the infarct size/risk ratio (%) was determined by expressing the infarcted (TTC negative) area in percent of the risk zone (RZ; EBD negative). Determination

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