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Moxonidine modulates cytokine signalling and effects on cardiac cell viability

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ABSTRACT

Regression of left ventricular hypertrophy and improved cardiac function in SHR by the centrally acting imidazoline I_1-receptor agonist, moxonidine, are associated with differential actions on circulating and cardiac cytokines. Herein, we investigated cell-type specific I_1-receptor (also known as nischarin) signalling and the mechanisms through which moxonidine may interfere with cytokines to affect cardiac cell viability. Studies were performed on neonatal rat cardiomyocytes and fibroblasts incubated with interleukin (IL)-1β (5 ng/ml), tumor necrosis factor (TNF)-α (10 ng/ml), and moxonidine (10^{-7} and 10^{-5} M), separately and in combination, for 15 min, and 24 and 48 h for the measurement of MAPKs (ERK1/2, JNK, and p38) and Akt activation and inducible NOS (iNOS) expression, by Western blotting, and cardiac cell viability/proliferation and apoptosis by flow cytometry, MTT assay, and Live/Dead assay. Participation of imidazoline I_1-receptors and the signalling proteins in the detected effects was identified using imidazoline I_1-receptor antagonist and signalling protein inhibitors. The results show that IL-1β, and to a lower extent, TNF-α, causes cell death and that moxonidine protects against starvation– as well as IL-1β –induced mortality, mainly by maintaining membrane integrity, and in part, by improving mitochondrial activity. The protection involves activation of Akt, ERK1/2, p38, JNK, and iNOS. In contrast, moxonidine stimulates basal and IL-1β-induced fibroblast mortality by mechanisms that include inhibition of JNK and iNOS. Thus, apart from their actions on the central nervous system, imidazoline I_1-receptors are directly involved in cardiac cell growth and death, and may play an important role in cardiovascular diseases associated with inflammation.

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1. Introduction

Left ventricular hypertrophy (LVH) and remodelling in hypertension and heart failure have been associated with increased circulating and cardiac expression of pro-inflammatory cytokines, tumor necrosis alpha (TNF-α) and interleukin (IL)-1β (Duerrschmid et al., 2013; Gullestad et al., 2012). These cytokines lead to cardiomyocyte hypertrophy and apoptosis in vivo and in vitro (Haudek et al., 2007; Hiraoka et al., 2001; Hu et al., 2009), and stimulation of cardiac fibroblast proliferation and differentiation into activated myofibroblasts, which produce large amounts of collagens. The ensuing structural changes, LVH and fibrosis, impede cardiac contractility and compromise cardiac performance. The intracellular signalling pathways include generation of reactive oxygen species (Khurana et al., 2014; Kohler et al., 2014), activation of phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B, PKB) (Chu et al., 2012; Tullio et al., 2013) and mitogen activated protein kinases (MAPKs): extracellular signal-regulated protein kinase (ERK), p38, and c-Jun NH_2-terminal protein kinase (JNK) (Chae et al., 2006; Haudek et al., 2007; Hiraoka et al., 2001; Sugden and Clerk, 1998; Yndestad et al., 2010).

Moxonidine is a centrally-acting sympatholytic imidazoline compound that shows higher affinity to non-adrenergic imidazoline I_1-receptors than α_2-adrenergic receptors. Moxonidine is used in hypertension treatment, including hypertension complicated with LVH (reviewed in (Mukaddam-Daher, 2012)). Our studies have shown that treatment of spontaneously hypertensive rats

Abbreviations: AGN192403, (2-endo-amino-3-exo-isopropylbicyclo[2.2.1]heptane); ANP, atrial natriuretic peptide; DMEM, Dulbecco's Modified Eagle Medium; ERK1/2, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; IL-1β, interleukin 1 beta; Imidazoline I_1-receptors, imidazoline receptors type 1; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH2-terminal protein kinase; LVH, left ventricular hypertrophy; MAPK, mitogen activated protein kinase; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; RVLM, rostral ventrolateral medulla; S1P, sphingosine 1 phosphate; TNF-α, tumor necrosis factor alpha

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(SHR) with moxonidine improves cardiac performance, attenuates LVH and collagen accumulation, in association with a transient apoptotic effect in SHR ventricles. The effects also include a substantial reduction in left ventricular IL-1β and circulating TNF-α and IL-6 levels, as well as attenuated ventricular Akt and p38 phosphorylation and inducible nitric oxide synthase (iNOS) expression (Aceros et al., 2011; Mukaddam-Daher et al., 2009; Paquette et al., 2008). On the other hand, moxonidine improves cardiac performance in cardiomyopathic hamsters in association with differential inflammatory/anti-inflammatory responses that culminate in reduced cardiac apoptosis (Stabile et al., 2011). The cardioprotective effects of in vivo moxonidine may be secondary to inhibition of sympathetic nerve activity, reduced noradrenaline release and cytokine levels, and subsequently, their downstream actions. Moxonidine may also directly act on cardiac cells to modulate the actions of cytokines. This hypothesis is supported by data showing that cardiac cells express cytokine receptors and imidazoline I_1-receptors (also known as nischarin), and in vitro moxonidine attenuates noradrenaline-induced cardiomyocyte apoptosis and fibroblast proliferation (Aceros et al., 2011). Accordingly, we sought to examine in primary cultures of neonatal rat cardiomyocytes and fibroblasts: i) the direct effect of moxonidine on cardiac cell viability and mechanisms involved, including the expression of imidazoline I_1-receptor/nischarin; and ii) to identify at what level moxonidine may regulate cytokine-induced cellular effects, focussing on their common signalling pathways, MAPKs, iNOS, and Akt (Aceros et al., 2011; Akira and Takeda, 2004; Edwards et al., 2001; Peng et al., 2009; Tesfai et al., 2011). The results demonstrate for the first time, the direct effect of moxonidine on cardiac cell viability. The mechanisms involve I_1-receptor activation and subsequent ERK and Akt activation in cardiomyocytes, and ERK, p38 and JNK, and Akt in fibroblasts. Furthermore, moxonidine modulates cytokine-induced effects on cardiomyocyte and fibroblast viability, through differential effects on cytokine-induced p38, JNK and iNOS.

2. Materials and methods

2.1. Cell cultures

Ventricular cardiomyocytes and fibroblasts were isolated, enzymatically digested, and purified from 1 to 2 day old Sprague Dawley rat pups, using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp, Lakewood, NJ, USA) following manufacturer instructions, as we previously described (Aceros et al., 2011). Recovered cells were pre-plated on plastic flasks twice, for 30 min each, to reduce non-myocyte cell numbers. The adherent cells are mostly fibroblasts while cardiomyocytes are in the suspension.

Isolated Cardiomyocytes seeded at a density of 2.5×10^5 per ml were cultured in Dulbecco's Modified Eagle Medium (DMEM), low glucose, 4 mM L-Glutamine, 1 mM Sodium Pyruvate (Invitrogen, Burlington, ON), supplemented with 10% FBS (Fetal Bovine Serum, Qualified, Canada Origin, Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (P/S) (Invitrogen, Burlington, ON, Canada). The medium was changed every 2 days.

Cytosine arabinoside (cyt Ara, 10 μM) was added throughout the cardiomyocyte culture period to inhibit non-cardiomyocyte outgrowth (Condorelli et al., 2002). Because of its possible toxicity (Gonzalez-Juanatey et al., 2004), and to rule out possible influence on cytokine-induced cell death, cyt Ara was omitted in some cultures. However, cytotoxicity assays, performed simultaneously did not show differences between the cells cultured with or without cyt Ara, at the concentration used, so results were pooled. Furthermore, microscopic evaluation of α-sarcomeric actin (mouse, Sigma-Aldrich A2172, Oakville, ON, Canada) confirmed purity (> 95%) of cardiomyocyte cultures.

Fibroblasts reaching 70% confluence after 3 days of culture were sub-cultured to reduce the possible contamination by cardiomyocytes and endothelial cells, then seeded at a density of 1.25×10^5 per ml in six-well plates, in triplicate.

The cultured cardiomyocytes and fibroblasts were a pooled cell population from 11–15 neonatal rat ventricles in each experiment. Four to five independent experiments done in triplicate were performed for each condition. All experimental procedures were approved by the Ethical Committee of CRCHUM following the Canadian Guidelines.

2.2. PCR analysis

Nischarin mRNA (accession number NM_022656.2) was detected in cardiomyocytes collected after 3 days of isolation and in fibroblasts on first and second passages. Normal C57BL/6 mouse (Charles-river, St-Constant, QC) brain and heart were used as positive control for nischarin mRNA.

Cardiomyocytes, seeded at a density of 2.5×10^5 per ml and fibroblasts at a density of 1.25×10^5 per ml in six-well plates, in triplicate, as well as tissues were collected and snap frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent; equal amounts of RNA were reverse transcribed to cDNA using Maloney murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase), then amplified with Taq polymerase (all from Invitrogen, Burlington, ON, Canada). cDNA was then amplified by PCR using the following primers for nischarin:

Fw: AGAGGCTCCAGCTCCAAAC; Rv: TTCTTGTCCCCAAATTCTGA, with an expected product of 706 bp. After 40 cycles with an annealing temperature of 60 °C, the product was migrated using 1% agarose gel with SYBR safe DNA gel stain (Invitrogen, Burlington, ON, Canada). S18 amplification was used as loading control. Images were taken with Typhoon trio imager (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada).

2.3. Cell treatment

Cardiomyocytes on day 3 after isolation and fibroblasts at the second passage were treated and harvested simultaneously under identical experimental conditions, using the same concentrations and duration of treatments. All cells were grown in DMEM containing 10% FBS, then rendered quiescent by incubation overnight in DMEM supplemented with 0.1% FBS. All procedures were performed using DMEM+0.1%FBS, hereafter termed DMEM, as control vehicle, unless otherwise specified.

Synchronized quiescent cells were then incubated for different time points, either in freshly prepared DMEM alone or containing 10^{-7} or 10^{-5} M moxonidine (Solvay Pharmaceuticals, Hannover, Germany), TNF-α (10 ng/ml; Recombinant rat, catalog number CLR08, Cedarlane Canada, Burlington, ON, Canada), or IL-1β (5 ng/ml; Recombinant rat, catalog number EN-RR202420, Thermo Scientific, Rockford, IL, USA), each alone or in combination with moxonidine. The concentrations of TNF-α and IL-1β and incubation times were chosen following published reports showing increased reactive oxygen species production and iNOS expression in neonatal rat cardiomyocytes, respectively (Higuchi et al., 2002), (LaPointe and Sitkins, 1996). Incubation in DMEM supplemented with 10%FBS (DMEM+10%FBS, replete condition) or H_2O_2 (10^{-4} M), which induces neonatal cardiomyocyte apoptosis (Long et al., 2004), were used as positive and negative controls. Incubation with lipopolysaccharides from Escherichia coli 0111:B4 (LPS, 100 ng/ml) (Sigma-Aldrich, Oakville, ON, Canada) was used as positive control for iNOS expression (Li et al., 2009b). The participation of imidazoline I_1-receptor/nischarin was confirmed

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