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Characterization of the protective effects of cardiotrophin-1 against non-ischemic death stimuli in adult cardiomyocytes

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Abstract

The aim of this study was to investigate the cytoprotective effects of CT-1 against non-ischemic death stimuli in adult cardiomyocytes. Primary cultures of cardiomyocytes isolated from adult rats were stimulated with either angiotensin II (Ang II) or H_2O_2 in the presence or absence of CT-1. Cell death was determined by trypan blue exclusion, cell viability by MTT assay and apoptosis by TUNEL–Annexin-V staining. Intracellular pathways were analyzed by the employment of chemical inhibitors and by the assessment of signalling intermediates phosphorylation by Western blot analysis. CT-1 reduced ($p < 0.01$) total cell death and apoptosis induced by either Ang II or H_2O_2, and increased ($p < 0.01$) cell viability in cardiomyocytes exposed to these stimuli. These effects of CT-1 were abolished in the presence of antibodies specific for gp130 or LIFR and did not require RNA or protein synthesis. Both Wortmannin and PD98059 abolished protective effects of CT-1 against H_2O_2, whereas only Wortmannin inhibited protection against Ang II. In both cases, Akt kinase activation and Bad phosphorylation were observed. These findings suggest that CT-1 protects adult cardiomyocytes against Ang II- and oxidative stress-induced cell death, via gp130/LIFR and by means of the PI3K/Akt and the p42/44 MAPK intracellular cascades.

Keywords: Adult cardiomyocytes; Angiotensin II; Cardiotrophin-1; Oxidative stress; Survival

1. Introduction

The regulation of cell death/survival balance has emerged as an important topic in the maintenance of cardiac integrity not only during cardiac development but, most importantly, in the adult heart under pathological conditions. One of the mechanisms that terminally differentiated cells (i.e. cardiomyocytes) employ to protect themselves from deleterious stimuli is the release of survival cytokines capable of promoting cytoprotection in an autocrine/paracrine manner [1–3]. However, perpetuation of detrimental stimuli in chronic pathologies may turn this compensatory mechanism into a harmful pathological factor, because of the many deleterious effects that cytokines exert in myocardial structure and function [4,5].

Cardiotrophin-1 (CT-1) is a cytokine that belongs to the interleukin-6 (IL-6) family and exerts its cellular actions via the heterodimer constituted by the glycoprotein 130 (gp130) and the leukemia inhibitory factor receptor β (LIFR). CT-1 was first isolated from embryoid bodies as a 21.5 kDa protein that induced hypertrophy in neonatal cardiomyocytes [6], and its survival properties in the same cell type were described in early studies [7]. Cytoprotective effects of CT-1 have been demonstrated in neonatal cardiomyocytes

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subjected to serum deprivation [8,9], heat shock and lethal ischemia [10], and ischemia followed by reperfusion [11]. Most studies indicate that p42/p44 mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways are the two main intracellular signalling cascades that mediate CT-1 survival effects in neonatal cardiomyocytes [8,9,12,13] whereas its hypertrophic action is mediated by the Janus kinase/signal transducer and activator of transcription 3 pathway (Jak/STAT-3) [13].

Due to the multiple differences between neonatal and fully differentiated cells, the culture of adult cardiomyocytes is a preferred experimental model for molecular understanding of adult heart responses in vivo [14,15]. Adult cardiomyocytes have very little (if any) capability of undergoing mitosis, and this makes cell death an especially significant process in this cell type. In fact, these cells have developed sophisticated survival pathways to respond to stressful stimuli. The knowledge of CT-1 biological actions in adult cardiomyocytes is necessary to understand the precise role of this cytokine in cardiac pathologies. Protective effects of CT-1 in adult cardiomyocytes have been observed only in response to an acute death stimulus such as ischemia-reperfusion [16]. However, neither the protective capabilities of CT-1 to neutralize cell death induced by harmful stresses characteristic of chronic cardiac pathologies, nor the intracellular pathways activated by CT-1 in adult cardiomyocytes have been studied. We therefore investigated the survival effects of CT-1 in freshly isolated adult rat cardiomyocytes against two deleterious stimuli, angiotensin II (Ang II) and oxidative stress (i.e. H_2O_2), and analyzed the signalling pathways involved in these actions.

2. Results

2.1. CT-1 protects adult cardiomyocytes from angiotensin II- and H_2O_2-induced cell death

Three different methodological approaches were employed to investigate whether CT-1 was capable of decreasing cell death induced by Ang II and H_2O_2. First, total cell death was quantified by trypan blue exclusion assay, based on the incapacity of death cells to exclude trypan blue and thus quantifying both oncotic and apoptotic cells. Second, cell viability was estimated by MTT cell respiration assay, which measures the mitochondrial capability to reduce MTT substrate on live cells. Finally, to investigate whether CT-1 was able to decrease specifically programmed cell death, the apoptotic index was quantified by means of TUNEL–Annexin-V double staining method. Preliminary dose–response experiments, performed by means of trypan blue exclusion, revealed that CT-1 was completely ineffective at doses lower than 10^{-10} mol/L, it decreased Ang II- and H_2O_2-induced cell death by 35% ($p < 0.05$) and by 32% ($p < 0.05$), respectively, at 10^{-10} mol/L, and by 50% ($p < 0.01$) and by 47% ($p < 0.01$), respectively, at 10^{-9} mol/L. Hence, 10^{-9} mol/L was employed in all subsequent survival experiments. Incubation with CT-1 alone during 24, 48 and 72 h did not modify cell death (data not shown).

Fig. 1 (Panel A) shows that CT-1 decreased total cell death induced by either Ang II (from $30.7 \pm 0.9\%$ to $15.1 \pm 0.4\%$, $p < 0.01$) or H_2O_2 (from $23.9 \pm 0.5\%$ to $12.7 \pm 0.4\%$, $p < 0.01$). Similarly, the presence of CT-1 increased cell viability from 51% to 74% ($p < 0.01$) in cells stimulated with Ang II, and from 59% to 79% ($p < 0.01$) in cells treated with H_2O_2 (Panel B). Finally, we found that the protective effects of CT-1 involve not only total cell death but also specifically apoptotic cell death. Thus, CT-1 decreased the apoptotic index from $6.14 \pm 0.26\%$ to $2.50 \pm 0.09\%$ ($p < 0.01$) in Ang II-treated cells, and from $4.66 \pm 0.19\%$ to $2.76 \pm 0.14\%$ ($p < 0.01$) in H_2O_2-treated cells (Panel C).

To establish whether the heterodimer gp130/LIFR mediated the protective effects of CT-1, cells were incubated with antibodies specific for the two sub-units prior to the addition of the cytokine. Fig. 2 shows that survival effects of CT-1 were completely abolished ($p < 0.01$) when gp130 or LIFR was blocked with a specific antibody, indicating that the interaction of CT-1 with the heterodimer is necessary to exert survival actions in adult cardiomyocytes.

We next analyzed whether mRNA and/or protein synthesis was necessary to obtain protective actions of CT-1, by incubating the cells with the transcription inhibitor actinomycin D or with the protein synthesis inhibitor cycloheximide prior to the addition of CT-1 and death stimuli. As illustrated in Fig. 2, the survival effect of CT-1 was not affected by either of these compounds indicating that, in these experimental conditions, mRNA or protein synthesis was not required to observe cytoprotective effects of CT-1. The inhibitors did not modify cell death when incubated alone (data not shown).

2.2. CT-1 activates PI3K/Akt, p42/44 MAPK and STAT-3 in adult cardiomyocytes

To investigate the intracellular signalling cascades activated by CT-1, we examined by Western blot the degree of phosphorylation exhibited by the intracellular intermediates. Incubation of adult freshly isolated cardiomyocytes with CT-1 increased phosphorylation of Akt, p42/44 MAPK and STAT-3 in 15 min and for up to 60 min. No changes in p38 MAPK and c-Jun-N-terminal kinase (JNK) phosphorylation were observed in cells incubated with CT-1 for the same time periods. (Fig. 3, Panel A). The blockade of the CT-1 receptor