IGF-1 protects cardiac myocytes from hyperosmotic stress-induced apoptosis via CREB

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Abstract

Hyperosmotic stress stimulates a rapid and pronounced apoptosis in cardiac myocytes which is attenuated by insulin-like growth factor-1 (IGF-1). Because in these cells IGF-1 induces intracellular Ca^{2+} increase, we assessed whether the cyclic AMP response element-binding protein (CREB) is activated by IGF-1 through Ca^{2+}-dependent signalling pathways. In cultured cardiac myocytes, IGF-1 induced phosphorylation (6.5 ± 1.0-fold at 5 min), nuclear translocation (30 min post-stimulus) and DNA binding activity of CREB. IGF-1-induced CREB phosphorylation was mediated by MEK1/ERK, PI3-K, p38-MAPK, as well as Ca^{2+}/calmodulin kinase and calcineurin. Exposure of cardiac myocytes to hyperosmotic stress (sorbitol 600 mOsm) decreased IGF-1-induced CREB activation Moreover, overexpression of a dominant negative CREB abolished the anti-apoptotic effects of IGF-1. Our results suggest that IGF-1 activates CREB through a complex signalling pathway, and this transcription factor plays an important role in the anti-apoptotic action of IGF-1 in cultured cardiac myocytes.

Keywords: IGF-1; CREB; Apoptosis; Osmotic stress; Cardiac myocyte

Osmotic stress is one of the important mechanisms of tissue damage. We have previously shown that hyperosmotic stress stimulates a rapid and pronounced apoptosis in cultured cardiomyocytes [1] which can be attenuated by insulin-like growth factor-1 (IGF-1) [2]. This growth factor also has anti-apoptotic properties in different models of myocardial ischemia and infarction [3–5], and induces cardiac hypertrophy [6–8]. In cultured cardiac myocytes, IGF-1 activates multiple signalling pathways, including ERK, IP_3/Ca^{2+}, PKC, PI3-K/PKB, PLC-γ, and JAK-STAT [9,10].

Several transcription factors (such as NFAT, MEF, and NFκB) have been involved in cardiac hypertrophy [11] but their role in apoptosis has not been well studied. An emerging transcription factor that regulates apoptosis in several cell lines is the cyclic AMP response element-binding protein (CREB) [12,13] which is a 43 kDa protein that binds the CRE sequence [14]. CREB is activated by phosphorylation in serine 133 mediated by PKA, CaMK, ERK, PKC, p38-MAPK, and PI3-K/PKB [12,13,15]. CREB is activated by pro- and anti-apoptotic stimulus, and its function as a neuronal survival transcription factor is well described [13,15]. In adipocytes and pancreatic β cells, IGF-1 prevents apoptosis induced by serum deprivation and cytokines, respectively, through the activation of CREB [16]. In neurons, IGF-1 activates CREB through ERK, PI3-K/PKB, and p38-MAPK, regulating survival and differentiation [13]. CREB has been described in rat heart [17] and transgenic mice overexpressing a dominant negative CREB developed dilated cardiomyopathy [18]. Here we hypothesized that IGF-1 protects cultured cardiac myocytes from

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osmotic stress by the activation of CREB. We have previously shown that IGF-1 induces intracellular Ca^{2+} increase in cardiac myocytes through a PLC-IP_3 signalling pathway [10]. Because CREB is also a Ca^{2+}-activated transcription factor [12,15], we investigated the activation of CREB by IGF-1 through a Ca^{2+}-dependent signalling pathway. Our data suggest that IGF-1 activates CREB through MEK1/ERK, PI3-K, p38-MAPK, and also through CaMK and calcineurin, and mediates the anti-apoptotic effects of IGF-1 in cultured cardiac myocytes exposed to hyperosmotic stress.

Experimental

Animals. Rats were bred in the Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). This investigation conforms to the "Guide for the care and use of laboratory animals" published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

Culture of rat cardiac myocytes. Cardiac myocytes were prepared from hearts of 1- to 3-day-old Sprague–Dawley rats as described previously [9]. Cultured cardiomyocytes, assessed with an anti-β-myosin heavy chain antibody, were at least 95% pure.

Western blot analysis. Cardiomyocytes were treated with IGF-1 (10 nM) at indicated times or pretreated with IGF-1 (10 nM) for 30 min before exposure to hyperosmotic stress (sorbitol, 600 mOsm). At different times, total protein extracts [19] or nuclear and cytosolic protein extracts were prepared [20]. Western blots were performed as described [19]. Anti-β-actin monoclonal antibody (Sigma), anti-TFIIB (Santa Cruz Biotechnology), anti-phosphorylated $CREB^{Ser133}$ (p-CREB, Cell Signaling), and anti-CREB (Cell Signaling) polyclonal antibodies were diluted 1/1000 in 3% non-fat milk in Tris-buffered saline (pH 7.6) containing 0.1% (v/v) Tween 20.

Electrophoretic mobility shift assay (EMSA) and supershift. CREB binding activity was determined in nuclear fractions from cultured cardiomyocytes using the double-stranded oligonucleotide 5′-AGAG ATTGCCTGACGTCAGAGAGCTAG-3′ which contains the CRE consensus sequence [20]. Supershift assays were performed by incubating 5 μg of nuclear extracts with 2 μg of anti-CREB polyclonal antibody (10× Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. As controls, 100-fold excess of a non-radioactive CREB consensus and mutated (5′-AGAGATTGCCTGTGGTCAGAGAGCTAG-3′) oligonucleotides was used.

Recombinant CREB adenovirus. CREB adenovirus (Ad CREB) was provided by Dr. Charles Vinson (NIH, Bethesda, USA). Ad CREB overexpresses a dominant negative form of CREB (Ser133/Ala). As control an empty adenovirus (Ad Empty) was used. Cultured cardiac myocytes were transduced using a multiplicity of infection of 300, 24 h before IGF-1 or sorbitol treatment.

Apoptosis determination. Activations of caspase 9 and caspase 3 were determined by Western blot using anti-caspase 9 (Cell Signaling) or anti-caspase 3 (Cell Signaling) polyclonal antibodies. DNA laddering and cell viability were determined as previously described [19].

Expression of results and statistical analysis. Data are given as means ± SEM of a number of independent experiments (n) or are representative experiments performed on at least three separate occasions. Data were analysed by ANOVA and comparisons were performed using a protected Tukey's test. A value of $p < 0.05$ was set as the limit of statistical significance.

Results

CREB activation by IGF-1 in cultured cardiac myocytes

IGF-1 induced a rapid (6.5 ± 1.0-fold at 5 min) and transient (20 min) phosphorylation of CREB in cultured cardiac myocytes (Fig. 1A). Nuclear translocation of CREB was detected after 30 min of IGF-1 treatment. At that time, cytosolic levels of CREB decreased 0.3 ± 0.1-fold over control, while simultaneously nuclear levels of CREB increased 2.1 ± 0.4-fold over control (Fig. 1B). IGF-1 also induced CREB binding to DNA, which was detected after 15 min of exposure to IGF-1 (Fig. 1C). In order to verify the specificity of CREB binding to DNA, a supershift assay was performed. Preincubation of nuclear extracts with an anti-CREB antibody induced an electrophoretic mobility shift of the CREB–DNA complex (Fig. 1D). Moreover, 100-fold excess of non-labelled CRE but not a mutated CRE completely displaced [^{32}P]CRE oligonucleotide (Fig. 1E).

Taking together, these results show that IGF-1 induces phosphorylation and translocation of CREB to the nucleus and its binding to DNA in cultured rat cardiac myocytes.

Effect of hyperosmotic stress on IGF-1-induced CREB activation

Pretreatment of cultured cardiac myocytes with IGF-1 for 30 min induced a 2.3 ± 0.3-fold increase of CREB phosphorylation with respect to control. Hyperosmotic stress (600 mOsm) decreased IGF-1-induced CREB phosphorylation which reached basal values (0.8 ± 0.3-fold) after 10 min of treatment (Fig. 1B). Similar results were obtained using EMSA. Hyperosmotic stress (600 mOsm) decreased IGF-1-induced CREB binding to DNA after 2–4 h of incubation (Fig. 2B). These results indicate that hyperosmotic stress by sorbitol inhibited IGF-1-induced CREB activation.

IGF-1 signalling pathways involved in CREB activation

The two major routes for IGF-1 receptor signalling are PI3-K and ERK pathways [9,21]. To determine whether IGF-1-induced CREB phosphorylation involved PI3-K or MEK/ERK signalling pathways, we used chemical inhibitors of PI3-K (LY294002 or LY) and MEK1 (PD98059 or PD). Both PD and LY completely inhibited CREB phosphorylation induced by IGF-1 (Fig. 3A). Interestingly, a p38-MAPK inhibitor (SB203580 or SB) also blocked IGF-1-induced CREB phosphorylation (Fig. 3A).

Because IGF-1 induces Ca^{2+} transients in cultured cardiac myocytes, we tested whether IGF-1 activation of CREB requires Ca^{2+}-dependent signalling. BAPTA-AM (an intracellular calcium chelating agent), KN62 (Ca^{2+}/calmodulin kinase inhibitor), and CsA (calcineurin inhibitor) blocked IGF-1-induced CREB phosphorylation (Fig. 3B). Together, these results show that CREB phosphorylation induced by IGF-1 requires multiple signalling pathways in cardiac myocytes, including IGF-1-induced Ca^{2+} signalling.

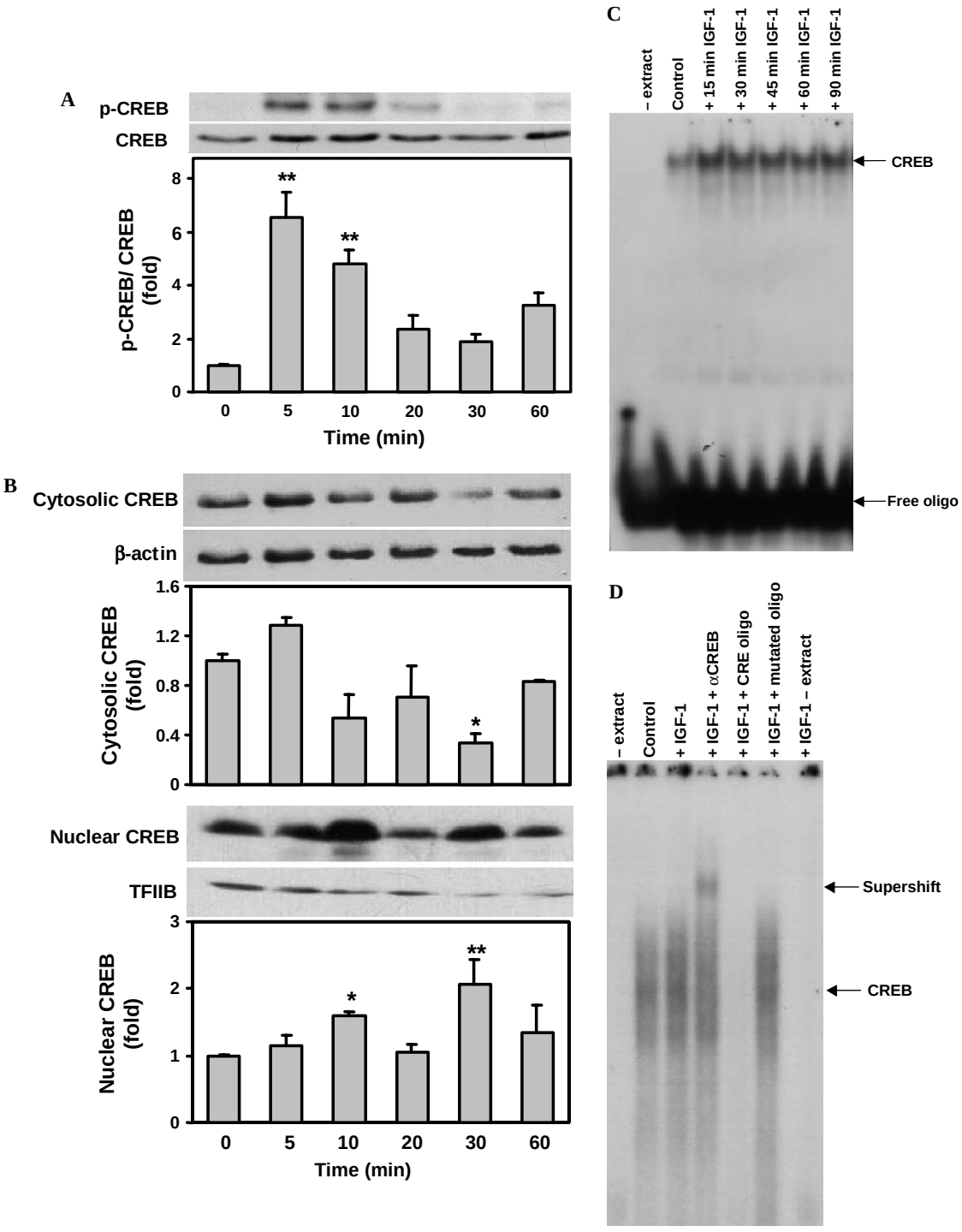

Fig. 1. IGF-1 activates CREB in cultured cardiac myocytes. Cells were treated with IGF-1 (10 nM) and total protein extracts or nuclear and cytosolic protein extracts were prepared at indicated times. (A) Phosphorylated CREB (p-CREB) and total CREB levels were determined in total cell protein extracts by Western blot using anti-p-CREB and anti-CREB polyclonal antibodies. (B) Total CREB levels were determined in nuclear and cytosolic protein extracts by Western blot using anti-CREB polyclonal antibody. Cytosolic and nuclear CREB levels were normalized using β-actin and TFIIB, respectively. (C) EMSA. Nuclear extracts were obtained from non-treated cardiac myocytes (control) or after 15, 30, 45, 60, and 90 min treatment with IGF-1 (10 nM). Lane 1: control without nuclear extract. EMSA was performed as indicated in Experimental. (D) Supershift. Nuclear extracts were obtained from non-treated cardiomyocytes (control) or after 30 min treatment with IGF-1 (10 nM). Nuclear extracts were incubated with anti-CREB antibody (αCREB) as described in Experimental. 100-fold excess of non-radioactive CRE oligonucleotide (CRE oligo) or 100-fold excess of a mutant CRE oligonucleotide (mutated oligo) were used as controls. Results are the average ± SEM ($n = 3$). Gels are representative of at least three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control.

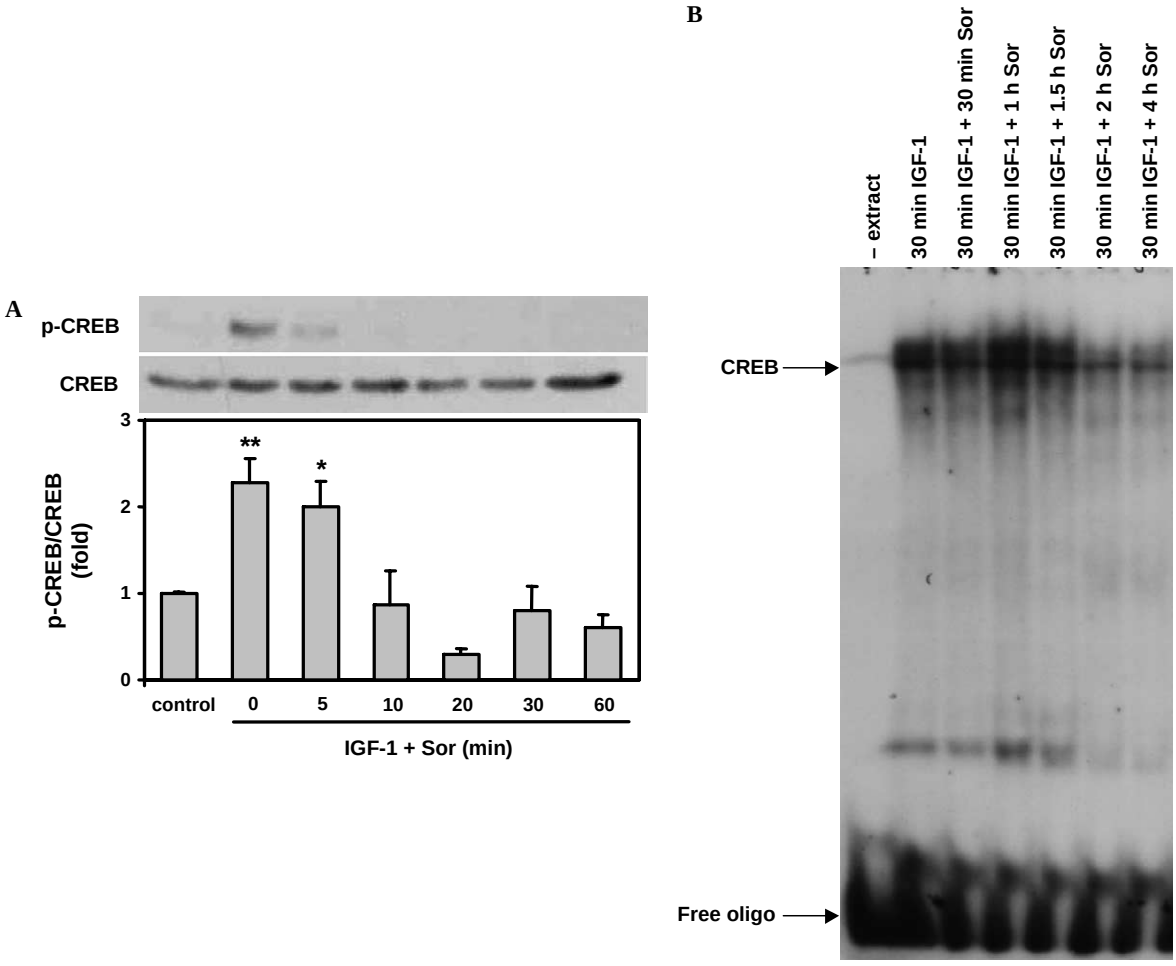

Fig. 2. Hyperosmotic stress inhibits IGF-1-stimulated CREB activation in cultured cardiac myocytes. Cells were preincubated with IGF-1 (10 nM) for 30 min and then exposed to hyperosmotic stress with sorbitol (Sor, 600 mOsm). At indicated times, total protein extracts or nuclear protein extracts were prepared. (A) Phosphorylated CREB (p-CREB) and total CREB levels were determined in total cell protein extracts by Western blot using anti-p-CREB and anti CREB polyclonal antibodies. (B) EMSA. Nuclear extracts were obtained from cardiomyocytes treated 30 min with IGF-1 or pretreated 30 min with IGF-1 followed by exposure to hyperosmotic stress with sorbitol (Sor) at indicated times. Lane 1: control without nuclear extract. EMSA was performed as indicated in Experimental. Results are the average ± SEM ($n = 3$). Gels are representative of at least three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control.

Participation of CREB in the anti-apoptotic effects of IGF-1 in cultured cardiac myocytes

To evaluate the participation of CREB in the anti-apoptotic effects of IGF-1, cultured cardiac myocytes were transduced with either an empty adenovirus (Ad Empty) or an adenovirus containing a dominant negative CREB (Ad CREB). In cultured cardiac myocytes transduced with Ad Empty, caspase 3 and caspase 9 were activated by hyperosmotic stress (600 mOsm). This activation was attenuated by preincubation with IGF-1 (10 nM) for 30 min (Figs. 4A and B). In contrast, cells transduced with Ad CREB did not modify hyperosmotic stress induced caspase 3 and caspase 9 activation, but completely abolish IGF-1 dependent attenuation of caspase activation (Figs. 4A and B). The same effect was observed when DNA laddering and cell viability were assessed. In cardiac myocytes transduced with Ad Empty, hyperosmotic stress (600 mOsm) increased DNA laddering (1.45 ± 0.05-fold) and reduced cell viability (0.38 ± 0.02-fold) was observed. However, preincubation with IGF-1 (10 nM) for 30 min prevented hyperosmotic stress-induced increase of DNA laddering (1.05 ± 0.10-fold) and reduction on cell viability (0.67 ± 0.02-fold) (Figs. 4C and D). Transduction with Ad CREB did not modify hyperosmotic stress-induced DNA laddering (1.52 ± 0.20-fold) and reduction on cell viability (0.31 ± 0.02-fold). However, preincubation of Ad CREB transduced cardiomyocytes with IGF-1 (10 nM) for 30 min did not prevent sorbitol induced DNA laddering (1.45 ± 0.15-fold) and cell viability (0.49 ± 0.02-fold) (Figs. 4C and D).

These results show that Ad CREB prevents anti-apoptotic effects of IGF-1, indicating that IGF-1 signals through

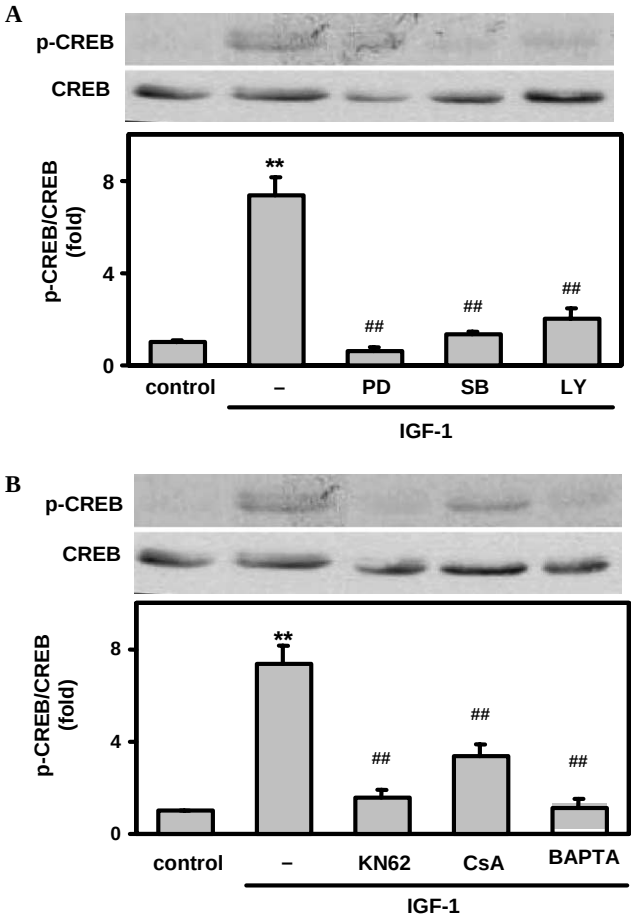

Fig. 3. Different signalling pathways are involved in CREB phosphorylation by IGF-1 in cultured cardiac myocytes. Cells were preincubated for 30 min in panel A with PD98059 (PD, 50 μM, MEK-1 inhibitor), SB203580 (SB, 10 μM, p38-MAPK inhibitor), LY294002 (LY, 50 μM, PI3-K inhibitor), or in panel B with KN62 (1 μM, CaMK inhibitor), cyclosporine A (CsA, 0.5 μM, Cn inhibitor) or BAPTA-AM (BAPTA, 100 mM, intracellular Ca^{2+} chelating agent) and then treated with IGF-1 (10 nM) for 5 min. Total protein extracts were prepared, and phosphorylated CREB (p-CREB) and total CREB levels were determined by Western blot using anti p-CREB and anti CREB polyclonal antibodies. Results are the average ± SEM ($n = 3$). $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs IGF-1 alone.

CREB to protect cardiac myocytes from apoptosis triggered by hyperosmotic stress.

Discussion

We showed here that in cultured rat cardiac myocytes, IGF-1 activates CREB through several signalling pathways, including ERK, PI3-K, p38-MAPK, CaMK, and calcineurin. It has been previously shown that IGF-1 induces phosphorylation of CREB in pituitary cells through Ras-MAPK pathway [22], in embryonic dorsal root ganglia neurons by PI3-K-Akt [23], in skeletal muscle cells by CaMK [24], in pancreatic β-cell through PI3-K [16], in breast epithelial cells [25], in cerebral cortical neurons [26], and in pheochromocytoma cells through p38-MAPK [27]. However, this is not a general action of IGF-1 because this growth factor did not induce CREB phosphorylation in cerebellar granule neurons [28]. Our results showed that IGF-1 also activates CREB through the Ca^{2+}-dependent signalling pathways, CaMK and calcineurin. These results collectively described for the first time that these signalling pathways as well as Ca^{2+} transients [10] could be involved in the activation of transcription factors such us CREB.

In cultured rat cardiac myocytes, Mehrhof et al. [29] showed that IGF-1 induces CREB phosphorylation in a PI3-K- and MEK1-dependent manner. In our work, CREB phosphorylation in serine 133 was induced through several signalling pathways activated by IGF-1, stimulating both CREB translocation to the nucleus and binding to DNA. Our results, together with those of Mehrhof et al. [29], unequivocally demonstrated that IGF-1 induces phosphorylation and activation of CREB in cardiac myocytes, mediated by a complex signalling pathway.

The role of CREB in cell survival has been largely described in neurons [13,23,26,28] and cancer cells [25,30]. CREB mediates survival by enhancing transcription of anti-apoptotic bcl-2 family members [31,32]. CREB-dependent Bcl-2 pathway participates in the resveratrol-induced preconditioning of the heart [33] and in the IGF-1 reduction of cardiomyocyte death induced by hypoxia [29]. Here, we demonstrated that CREB mediates anti-apoptotic effects of IGF-1 in cultured rat cardiac myocytes, determined by activation of both caspases 3 and 9, DNA laddering, and cell viability. On the other hand, hyperosmotic stress by sorbitol, which induces a strong and rapid apoptosis in cultured cardiomyocytes [1], decreased IGF-1-dependent phosphorylation of CREB. These results suggest that the pro-apoptotic stimulus (hyperosmotic stress) antagonized IGF-1 action on CREB activation, indicating that CREB plays a crucial role in the survival/death balance.

In neuroblastoma cells, staurosporine triggered apoptosis by caspase-dependent cleaving of CREB [34]. In hippocampus, the dephosphorylation of CREB played a major role in the lethal toxicity induced by kainic acid [35]. In cerebellar granule neurons, oxidative stress activated a calpain-dependent decline and dephosphorylation of CREB, inducing cell death [36]. However, we observed that the overexpression of a CREB inactive form did not modify the hyperosmotic stress-induced apoptosis in cultured cardiac myocytes. These last results also suggested that CREB was not involved in the apoptosis induced by hyperosmotic stress in cardiac myocytes.

We conclude that IGF-1 induces activation of CREB through several signalling pathways, including the Ca^{2+}-dependent CaMK and calcineurin, and mediates the anti-apoptotic effects of IGF-1 observed in cultured cardiac myocytes exposed to hyperosmotic stress. Pro-apoptotic stimulus antagonizes IGF-1-induced CREB activation suggesting that CREB is an important factor involved in the survival/death regulation.

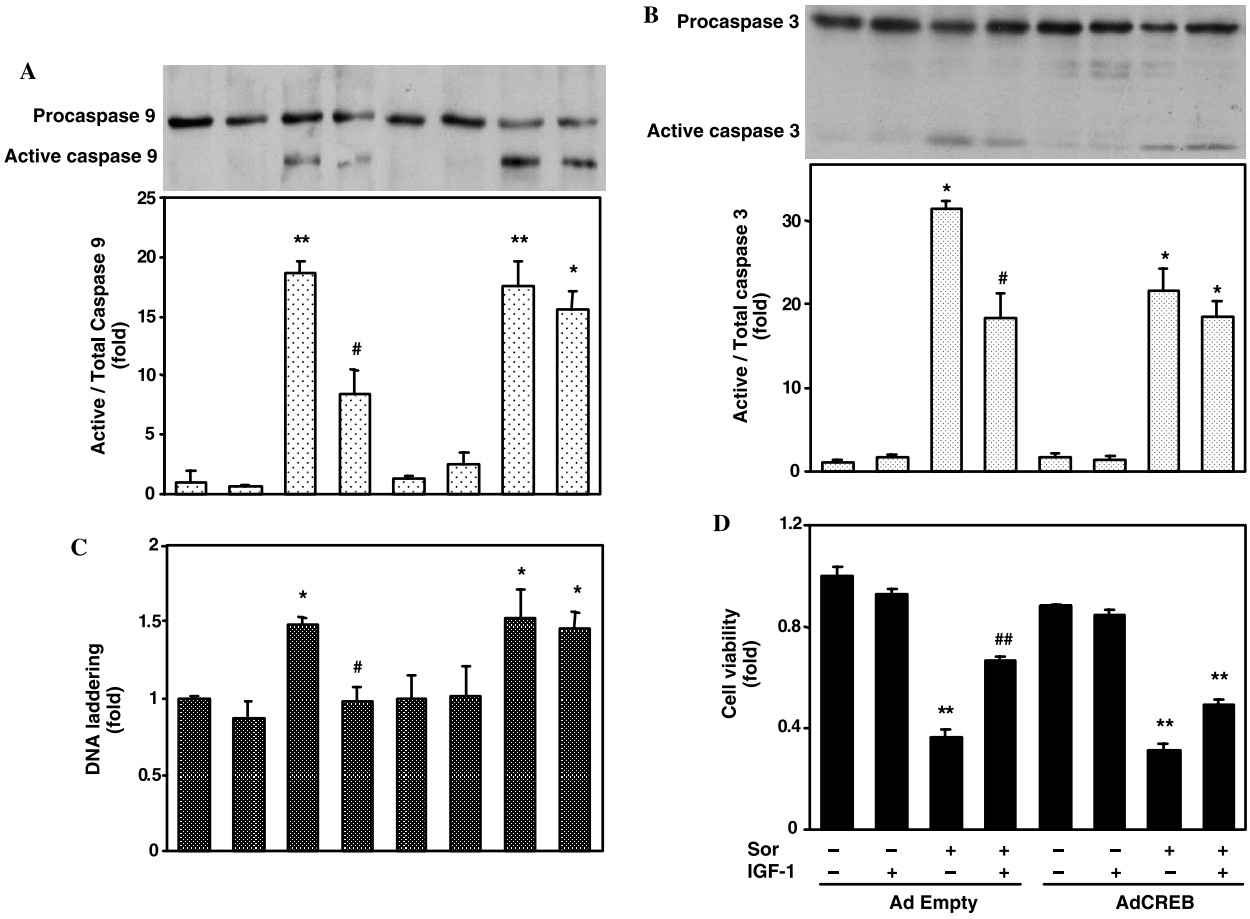

Fig. 4. CREB mediates anti-apoptotic effects of IGF-1 in cultured cardiac myocytes. Cells were transduced with either an empty adenovirus (Ad Empty) or an adenovirus containing a dominant negative CREB (Ad CREB). Transduced cells were treated with IGF-1 (10 nM), sorbitol (Sor, 600 mOsm) or IGF-1 plus Sor. (A) Caspase 9 activation, (B) caspase 3 activation, (C) DNA laddering, and (D) cell viability were determined as described in Experimental. Results are the average ± SEM ($n = 3$–4). $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#}p < 0.05$ vs sorbitol, and $^{\#\#}p < 0.01$ vs sorbitol.

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