

# Hyperosmotic stress-dependent NFκB activation is regulated by reactive oxygen species and IGF-1 in cultured cardiomyocytes

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**Abstract** We have recently shown that hyperosmotic stress activates p65/RelB NFκB in cultured cardiomyocytes with dichotomic actions on caspase activation and cell death. It remains unexplored how NFκB is regulated in cultured rat cardiomyocytes exposed to hyperosmotic stress. We study here: (a) if hyperosmotic stress triggers reactive oxygen species (ROS) generation and in turn whether they regulate NFκB and (b) if insulin-like growth factor-1 (IGF-1) modulates ROS production and NFκB activation in hyperosmotically-stressed cardiomyocytes. The results showed that hyperosmotic stress generated ROS in cultured cardiac myocytes, in particular the hydroxyl and superoxide species, which were inhibited by *N*-acetylcysteine (NAC). Hyperosmotic stress-induced NFκB activation as determined by IκBα degradation and NFκB DNA binding. NFκB activation and procaspase-3 and -9 fragmentation were prevented by NAC and IGF-1. However, this growth factor did not decrease ROS generation induced by hyperosmotic stress, suggesting that its actions over NFκB and caspase activation may be due to modulation of events downstream of ROS generation. We conclude that hyperosmotic stress induces ROS, which in turn activates NFκB and caspases. IGF-1 prevents NFκB activation by a ROS-independent mechanism.

**Keywords:** IGF-1; NFκB; Hyperosmotic stress; Reactive oxygen species; Caspase; Cardiomyocyte

phorylation of IKKα/β, degradation of IκBα, a parallel migration of p65 and RelB from the cytosol to the nucleus, binding of NFκB to the consensus DNA κB-site, activation of the NFκB-lux reporter gene and transient activation of caspases-9 and -3 [4]. Blockade of NFκB with an IκBα dominant negative overexpressing adenovirus prevented the activation of caspase but did not affect cell death in hyperosmotically-stressed cardiomyocytes. These observations led us to propose that hyperosmotic stress activated NFκB, which mediates caspase activation in cultured cardiomyocytes. We have also described that intracellular glutathione content decreases in cardiomyocytes exposed to hyperosmotic stress and its replenishment by *N*-acetylcysteine (NAC) protected cells from apoptosis induced by hyperosmotic stress [5]. These last evidences suggested that reactive oxygen species (ROS) may be involved in the apoptotic mechanism triggered by hyperosmotic stress.

Depending on the cell type, NFκB can be regulated by different stimulus such as oxidative stress, cytokines and growth factors [2,6–8]. There is little evidence about the regulation of NFκB by IGF-1 [9,10]. This growth factor plays an important role in the adaptation of cardiomyocyte to different kind of cell stress [11,12]. However, in cardiomyocytes, whether NFκB is regulated by ROS and IGF-1 remain unexplored. In this work, we investigated in cultured rat cardiomyocytes: (a) ROS generation by hyperosmotic stress, (b) NFκB activation by ROS and (c) ROS and NFκB regulation by IGF-1.

## 1. Introduction

NFκB is a transcription factor associated with cell adaptation to stress, immunity, inflammation, proliferation and apoptosis induced by a large number of different stimuli [1]. NFκB complexes can be found in all cell types and distinct reports suggest apparently opposing or contradictory functions for NFκB [1–3].

We have recently shown that the exposure of cultured rat cardiomyocytes to hyperosmotic conditions induced phos-

## 2. Materials and methods

### 2.1. Materials

Polyclonal antibodies against p65 and p50 were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against IκBα, procaspase-3/caspase-3 and procaspase-9/caspase-9 were purchased from Cell Signaling Technology Inc. (Beverly, MA). Dulbecco's modified Eagle's medium (DME), medium 199 (M199), sorbitol, NAC, 5,5-dimethylpyrroline 1-oxide (DMPO) were obtained from Sigma (St. Louis, MO, USA). PD98059 (PD, MEK-1 inhibitor) and LY294002 (LY, PI3-K inhibitor) were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). [<sup>32</sup>P] ATP was from NEN (Boston, MA). Human recombinant IGF-1 was a kind gift of Dr. C. George-Nascimento (Austral Biologicals, San Ramon, CA). The 2xNFκB-lux reporter gene was kindly donated by Dr. M. Karin (University of California, San Diego).

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## 2.2. Culture and treatment of cardiomyocytes

Cardiomyocytes were prepared from 3 day-old Sprague–Dawley rat hearts as described previously [4,5]. Rats were bred in the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile. All studies conformed with 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). Cardiomyocytes were plated at a final density of  $1\text{--}8 \times 10^3/\text{mm}^2$  on gelatin-coated 35-, 60-, or 100-mm Petri dishes or on gelatin-precoated 25-mm glass coverslips. Cultured cardiomyocytes were identified using an anti  $\beta$ -myosin heavy chain antibody as previously described [5]. Cell cultures were at least 95% pure.

## 2.3. ROS determination using dichlorofluorescein

Cardiomyocytes were pretreated with IGF-1 (10 nM) or NAC (1 mM) for 30 min before exposure to sorbitol-induced hyperosmotic stress (sorbitol, 600 mOsm). Dichlorofluorescein diacetate acetyl ester (DCF-DA, 10  $\mu\text{M}$ ) was added 10 min before cell lysis with 100  $\mu\text{L}$  NaOH (100 mM). Fluorescence was determined in cell extracts using a spectrofluorimeter (excitation: 488, emission: 525 nm) or registered by photography. Arbitrary units of fluorescence were corrected by protein content.

## 2.4. Electron spin resonance (ESR) spectroscopy

Cardiac myocytes were preincubated with IGF-1 (10 nM) for 30 min before sorbitol (600 mOsm) addition in the presence of DMPO (200 mM). Cells were incubated for 30 min, lysed with 0.5 ml triton X-100 1% (v/v), DMPO 200 mM in DME/M199 4:1 and incubated for 10 min at room temperature. In the presence of DMPO, hydroxyl radicals were trapped as the more stable spin adducts, DMPO-OH. ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G.

## 2.5. Western blot analysis

Western blots were performed as previously described [4]. Anti  $\beta$ -actin and anti I $\kappa$ B $\alpha$  were used at 1/1000 dilution. Anti I $\kappa$ B $\alpha$ , procaspase-3/caspase-3 and procaspase-9/caspase-9 antibody were diluted 1/1000 in TBST containing BSA 5% (w/v). Horseradish peroxidase-linked secondary antibody was used at 1/5000 dilution. Bands were detected using ECL. Films were digitalized and analysed by UN-SCAN-IT program software (Silk Scientific Corporation, Orem, UT, USA). Values were expressed as fold over time zero or control.

## 2.6. Electrophoretic mobility shift assay (EMSA) and supershift

NF $\kappa$ B binding activity was determined in nuclear fractions from cultured cardiomyocytes, prepared according to Schreiber et al. [13], using the double-stranded NF $\kappa$ B consensus oligonucleotide 5'-AGTTGAGGGGACTTCCCAGGC-3' [14]. Supershift assays were performed by incubating 5  $\mu\text{g}$  of nuclear extracts with 2  $\mu\text{g}$  of anti p65 or anti p50 antibodies for 2 h at room temperature [4]. As controls, 100-fold excess of a non-radioactive NF $\kappa$ B consensus and 1000-fold excess of mutated (5'-AGTTGAGGCGACTTCCCAGGC-3') oligonucleotides were used.

## 2.7. Transfections and gene reporter assays

Cardiomyocytes were transfected with 2 $\times$ NF $\kappa$ B lux and lacZ genes by the Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> method. Transfected cells were treated 24 h later with IGF-1 (10 nM) and/or sorbitol (600 mOsm). After 24 h incubation, cells were lysed and luciferase and  $\beta$ -galactosidase activities were assayed [15,16]. Luciferase activity was normalized against  $\beta$ -galactosidase activity.

## 2.8. Expression of results and statistical analysis

Data are given as means  $\pm$  S.E.M. of a number of independent experiments ( $n$ ) as indicated or as the mean of representative experiments performed on at least three separate occasions with similar outcome. 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.

## 3. Results

### 3.1. Hyperosmotic stress stimulates ROS generation that is not prevented by IGF-1

We have previously shown that hyperosmotic stress decreased intracellular GSH levels in cultured cardiomyocytes [5]. In order to determine whether GSH reduction was due to ROS generation, cardiomyocytes were exposed to hyperosmotic stress with sorbitol (600 mOsm) and the fluoroprobe DCF-DA. Hyperosmotic stress caused a  $4.9 \pm 0.4$ -fold increase in ROS, as measured by DCF intensity (Fig. 1A). To confirm ROS formation by hyperosmotic stress, we used the ESR spin trap DMPO. Treatment of cardiomyocytes with 600 mOsm sorbitol in the presence of DMPO generated a four-line (1:2:2:1) ESR spectrum with AN = AH = 14.9 G (Fig. 1B). These ESR spectral characteristics were consistent with the formation of a relatively stable nitroxide-hydroxyl radical spin adduct (DMPO-OH) [17]. In order to evaluate whether superoxide was also a contributing radical, cells were also preincubated with superoxide dismutase (100 U/ml). The 1:2:2:1 ESR spectrum induced by sorbitol was prevented by the addition of this enzyme (Fig. 1B). When cells were pretreated with NAC, hyperosmotic stress-dependent ROS generation was significantly decreased to  $1.5 \pm 0.2$ -fold vs control ( $p < 0.05$ ). In contrast, IGF-1 did not prevent hyperosmotic stress-induced ROS formation ( $4.1 \pm 0.3$ -fold vs control). Basal level of ROS in cardiomyocytes in the presence of IGF-1 and NAC were  $2.1 \pm 0.3$  and  $1.0 \pm 0.1$ -fold vs control, respectively (Fig. 1C). In agreement with the results shown in Fig. 1C, IGF-1 did not decrease DMPO-OH generated in hyperosmotically-stressed cells, indicating that this growth factor did not prevent ROS formation induced by sorbitol-dependent hyperosmotic stress. IGF-1 alone induced barely detectable DMPO-OH generation (Fig. 1D).

### 3.2. ROS mediates NF $\kappa$ B activation by hyperosmotic stress

As we have recently shown that NF $\kappa$ B was activated in hyperosmotically-stressed cardiomyocytes [6] and since NF $\kappa$ B is a redox-sensitive transcription factor, we investigated whether ROS mediates NF $\kappa$ B activation by hyperosmotic stress, by treating cells with NAC. Fig. 2A depicts that hyperosmotic stress-dependent I $\kappa$ B $\alpha$  down-regulation was partially prevented by NAC ( $p < 0.05$ ). We next evaluated the effect of NAC on NF $\kappa$ B binding to a  $\kappa$ B site containing oligonucleotide. Fig. 2B shows that NAC prevents hyperosmotic stress-induced NF $\kappa$ B DNA binding. As specificity controls, addition of a 100-fold excess of non-radioactive oligonucleotide, but not a 1000-fold excess of a mutant form of the NF $\kappa$ B consensus sequence, specifically displaced the radioactive oligonucleotide bound to NF $\kappa$ B (Fig. 2B). Moreover, NAC prevented p65 DNA binding stimulated by hyperosmotic stress (Fig. 2C).

### 3.3. IGF-1 prevents NF $\kappa$ B activation by hyperosmotic stress

As shown in Fig. 3A, IGF-1 prevented I $\kappa$ B $\alpha$  degradation triggered by hyperosmotic stress and IGF-1 alone did not change I $\kappa$ B $\alpha$  basal levels. Moreover, cardiomyocytes were preincubated with PD98059 (PD) or LY294002 (LY) for 1 h, incubated with IGF-1 for 30 min, and then exposed to hyperosmotic stress for 2 h. PD and LY blocked the IGF-1-dependent protection of I $\kappa$ B $\alpha$  degradation induced by hyperosmotic stress (Fig. 3B). Both inhibitors did not change I $\kappa$ B $\alpha$

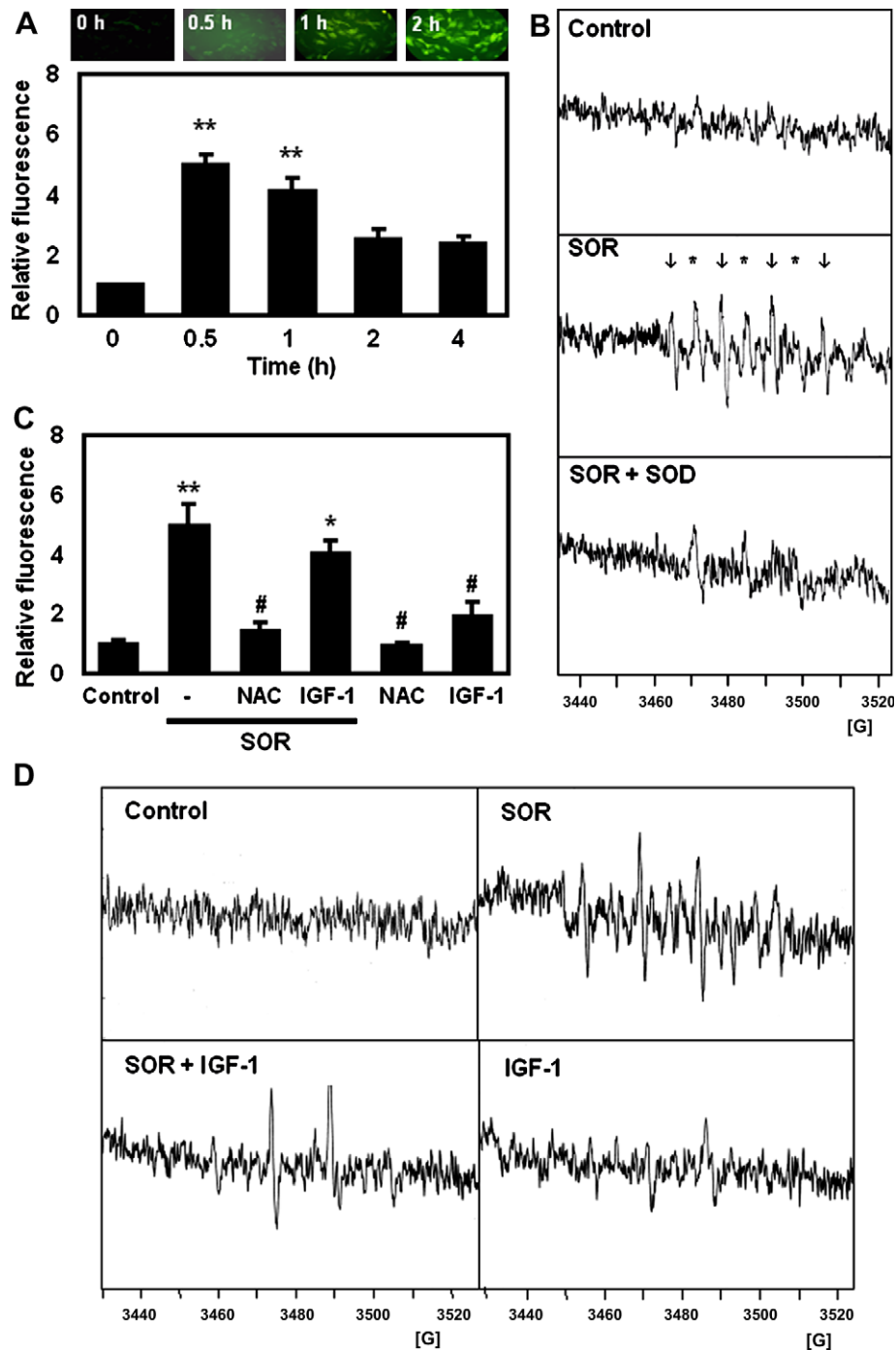


Fig. 1. Hyperosmotic stress induces ROS generation in cultured cardiomyocytes that is not prevented by IGF-1. (A) Cultured cardiomyocytes were preincubated with DCF-DA and then exposed to hyperosmotic stress with sorbitol (SOR, 600 mOsm) at indicated times. Fluorescence was determined as indicated in Section 2. \*\* $p < 0.01$  vs 0 h. (B) Cardiomyocytes were incubated with the spin trap DMPO (200 mM) and SOR in the presence or absence superoxide dismutase (SOD, 100 U/ml). Spectrometer conditions for the ROS detection were microwave frequency 9.83 GHz microwave power 20 mW, modulation amplitude 0.2 G/s, scan rate 1.25 G/s, time constant 0.5 s, number of scans: 20. ↓: spin adducts of hydroxyl and superoxide species; \*: radical unknown (probably DMPO decomposition). (C) Cells were preincubated with DCF-DA and exposed to hyperosmotic stress with sorbitol (SOR, 600 mOsm) in the presence or absence of *N*-acetylcysteine (NAC, 1 mM) or IGF-1 (10 nM). \*\* $p < 0.01$  and \* $p < 0.05$  vs control; # $p < 0.05$  vs SOR. (D) Cardiomyocytes were incubated with DMPO (200 mM) (control), DMPO and hyperosmotic stress (SOR, 600 mOsm), IGF-1 (10 nM) + SOR, or IGF-1. The ROS detection conditions were similar to those described above. Results are representative of three independent experiments.

basal levels (data not shown). These experiments suggest that ERK and PI3-K are involved in the protection of I $\kappa$ B $\alpha$  degradation by IGF-1 in response to hyperosmotic stress. EMSA assays depicted that hyperosmotic stress also stimulated

NF $\kappa$ B DNA binding activity which was prevented in the presence of IGF-1 (Fig. 3C). IGF-1 alone did not stimulate NF $\kappa$ B DNA binding (Fig. 3C). Supershift analysis showed that p65, but not p50, bound to DNA (Fig. 3D) in

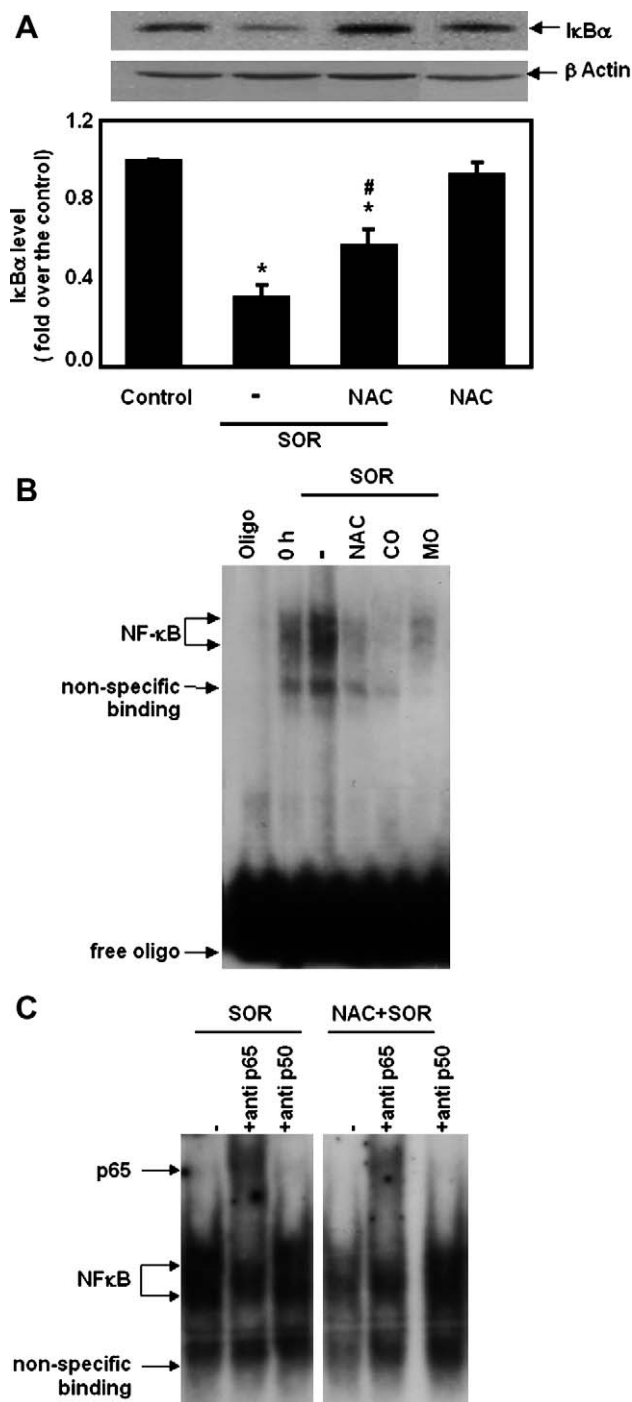


Fig. 2. NAC prevents I $\kappa$ B $\alpha$  degradation and NF $\kappa$ B DNA binding induced by hyperosmotic stress in cultured cardiac myocytes. (A) Cardiomyocytes were exposed for 2 h to hyperosmotic stress with sorbitol (SOR, 600 mOsm), SOR + NAC (1 mM) or NAC alone. Membranes were revealed with anti-I $\kappa$ B $\alpha$  antibody or anti- $\beta$ -actin. Results are representative of at least three independent experiments. \* $p < 0.05$  vs control, # $p < 0.05$  vs sorbitol. (B) Nuclear extracts were obtained from non-treated cardiomyocytes (0 h), or after 2 h treatment with SOR or SOR + NAC (1 mM). Oligo corresponds to controls without nuclear extracts. 100-fold excess of non-radioactive NF $\kappa$ B oligonucleotide (CO) or 1000-fold excess of a mutant NF $\kappa$ B oligonucleotide (MO) were used as controls using nuclear extract from cardiomyocytes treated with SOR. EMSA was performed as indicated in Section 2. (C) Nuclear extracts were incubated with anti p65 or anti p50 antibodies as described in Section 2. Results are representative of at least three independent experiments.

hyperosmotically-stressed cardiomyocytes and this NF $\kappa$ B subunit composition was not changed by the preincubation with IGF-1 (Fig. 3D). To assess whether NF $\kappa$ B-dependent transcription was stimulated by IGF-1, cardiomyocytes were cotransfected with 2 $\times$ NF $\kappa$ B lux reporter and lacZ genes. IGF-1 did not alter the ratio luciferase/ $\beta$ -galactosidase activity respect to controls (Fig. 3E). However, IGF-1 completely prevented the increase in luciferase/ $\beta$ -galactosidase activity ratio stimulated by hyperosmotic stress ( $p < 0.05$  vs sorbitol) (Fig. 3F).

#### 3.4. NAC and IGF-1 attenuate procaspase fragmentation stimulated by hyperosmotic stress

In cardiac myocytes exposed for 2 h to hyperosmotic stress, the cleavages of procaspase-9 to caspase-9 and procaspase-3 to caspase-3 were recently observed [4]. We have also shown that caspase activation by hyperosmotic stress was mediated by NF $\kappa$ B [4]. We assessed whether NF $\kappa$ B-dependent caspase activation was regulated by ROS and IGF-1. As shown in Fig. 4A, NAC only prevented fragmentation of procaspase-9 to caspase-9. In contrast, caspase-3 degradation triggered by hyperosmotic stress was prevented by IGF-1 (Fig. 4B).

## 4. Discussion

The main findings of this work were (a) hyperosmotic stress generated ROS in cultured cardiac myocytes, in particular the hydroxyl and superoxide species, which were inhibited by NAC; (b) NF $\kappa$ B activation by hyperosmotic stress was mediated by ROS; (c) IGF-1 did not regulate hyperosmotic stress-dependent ROS production; and (d) this growth factor inhibited NF $\kappa$ B activation.

Hyperosmotic stress stimulated ROS production. The sources for these ROS may have different subcellular origins such as mitochondria and NADPH oxidase. Armstrong and Jones demonstrated that respiratory chain complex III increased ROS production when glutathione levels were depleted [18] and mitochondrial aconitase iron cluster also generate hydroxyl radical by Fenton reaction [19]. However, superoxide radical and H<sub>2</sub>O<sub>2</sub> generated by NADPH oxidase have also shown to participate in NF $\kappa$ B activation during muscle differentiation [20] and interleukin-1 receptor 1 signaling [21].

NF $\kappa$ B has been implicated as an important osmosignaling molecule that is activated in response to hyperosmolarity in both renal medullary interstitial, endothelial cells and cardiomyocytes [4,22,23]. Our results indicate that hyperosmotic stress generates ROS that may be responsible for NF $\kappa$ B activation. Therefore, we propose that in cardiomyocytes, NF $\kappa$ B is an osmosignaling factor that detects ROS formation upon hyperosmotic stress. However, the involved mechanism on NF $\kappa$ B redox regulation remains to be elucidated.

IGF-1 inhibited the activation of NF $\kappa$ B induced by hyperosmotic stress, as assessed by I $\kappa$ B $\alpha$  degradation, EMSA and NF $\kappa$ B reporter gene expression. The mechanisms involved in this effect were through ERK1/2 and PI3K, which are the main signaling pathways activated by IGF-1 in cultured cardiomyocytes [24]. There are only a few reports suggesting NF $\kappa$ B regulation by IGF-1. In astrocytes, IGF-1 inhibits NF $\kappa$ B activation by preventing I $\kappa$ B $\alpha$  degradation [9]. In HT29-D4 cells, the action of IGF-1 depended on the enhancement of sur-

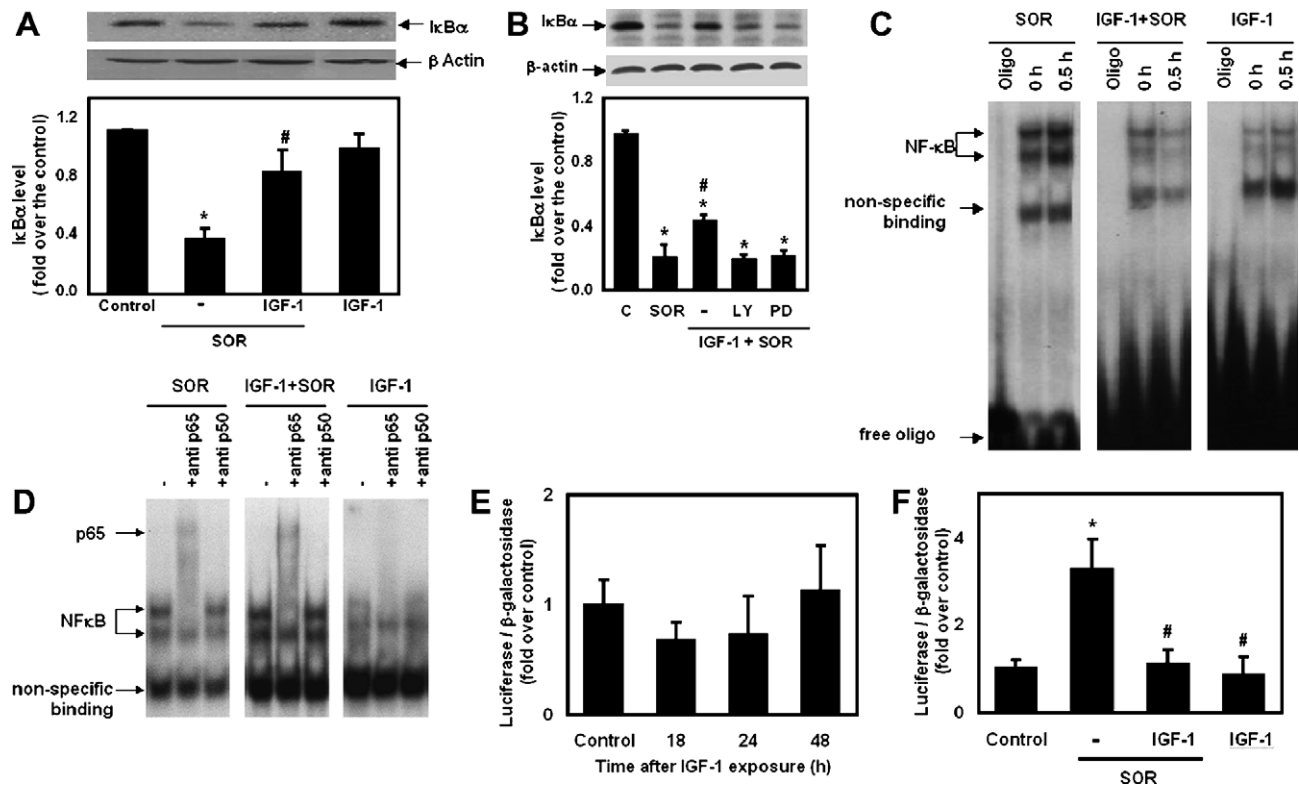


Fig. 3. IGF-1 prevents IκBα degradation, NFκB DNA binding and NFκB functional activation in hyperosmotically-stressed cardiac myocytes. (A) Cardiomyocytes were exposed to hyperosmotic stress with sorbitol (SOR, 600 mOsm), SOR + IGF-1 (10 nM) or IGF-1 alone, and (B) cells were preincubated with PD98059 (PD, 100 μM) or LY294002 (LY, 50 μM) and then treated with SOR and IGF-1. Membranes were revealed with anti-IκBα antibody or anti-β-actin as indicated in Section 2. Results are representative of at least three independent experiments. \**p* < 0.05 vs control, #*p* < 0.05 vs sorbitol. (C) Nuclear extracts were obtained from non-treated cardiomyocytes (0 h), or after 0.5 h treatment with Sorbitol (SOR, 600 mOsm), SOR + IGF-1 (10 nM) or IGF-1 alone. Oligo corresponds to controls without nuclear extracts. 1000-fold excess of non-radioactive NFκB oligonucleotide (CO) or 100-fold excess of a mutant NFκB oligonucleotide (MO) were used as controls using nuclear extract from cardiomyocytes treated 0.5 h with SOR. (D) Nuclear extracts were incubated with anti p65 or anti p50 antibodies as described in Section 2. Results are representative of at least three independent experiments. (E) Temporal study of IGF-1 activation of NFκB lux reporter gene. Cardiomyocytes were cotransfected with 2xNFκB lux reporter and β-galactosidase genes and treated with IGF-1 (10 nM) at indicated times. Luciferase and β-galactosidase activities were determined as assessed in Section 2. Results are the average ± SEM of three independent experiments. (F) IGF-1 attenuates NFκB lux reporter gene activation by hyperosmotic stress. Cardiomyocytes were cotransfected with 2xNFκB lux reporter and β-galactosidase genes and treated with sorbitol (SOR, 600 mOsm), SOR + IGF-1 (10 nM) or IGF-1 alone (10 nM). \**p* < 0.05 vs control, #*p* < 0.05 vs SOR.

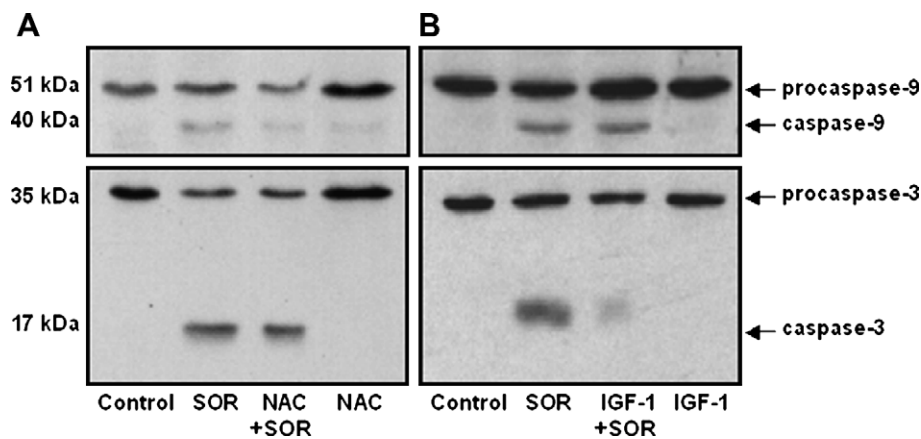


Fig. 4. Effects of NAC and IGF-1 on procaspase-9 and -3 fragmentation triggered by hyperosmotic stress in cultured cardiomyocytes. (A) Cells were exposed for 2 h to hyperosmotic stress with sorbitol (SOR, 600 mOsm), SOR + NAC (1 mM) or NAC; (B) sorbitol (SOR, 600 mOsm), SOR + IGF-1 (10 nM) or IGF-1 and Western blot analysis of procaspase and caspase-9 and -3 were performed. Gels are representative of at least three independent experiments.

vival initiated by TNF $\alpha$  and mediated by NF $\kappa$ B, which acted in concert to suppress the proapoptotic signals [10].

The relationship between hyperosmotic stress and ROS formation opens a new mechanism for caspase induction by hyperosmolarity. Previous work in mouse Balb/c 3T3 fibroblasts and human Hep 3B and A431 cells, has shown that hyperosmotic shock stimulates caspase activation, being blocked by antioxidants [25].

Caspase regulation by IGF-1 has been described in different systems [26,27], however, little is known about a concomitant effect of IGF-1 over both caspases and NF $\kappa$ B activation. In burn-induced inflammatory responses, IGF-1 prevented caspase-3 and activated NF $\kappa$ B [28]. In our model, IGF-1 did not decrease ROS generation induced by hyperosmotic stress, suggesting that its actions over NF $\kappa$ B and caspase activation may be due to modulation of events downstream of ROS generation.

We conclude that hyperosmotic stress induces ROS, which in turn activates NF $\kappa$ B and caspases. However, IGF-1 prevents NF $\kappa$ B activation by a ROS-independent mechanism.

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