Hyperosmotic stress activates p65/RelB NFκB in cultured cardiomyocytes with dichotomic actions on caspase activation and cell death

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Abstract **NFκB is a participant in the process whereby cells adapt to stress. We have evaluated the activation of NFκB pathway by hyperosmotic stress in cultured cardiomyocytes and its role in the activation of caspase and cell death. Exposure of cultured rat cardiomyocytes to hyperosmotic conditions induced phosphorylation of IKKα/β as well as degradation of IκBα. All five members of the NFκB family were identified in cardiomyocytes. Analysis of the subcellular distribution of NFκB isoforms in response to hyperosmotic stress showed parallel migration of p65 and RelB from the cytosol to the nucleus. Measurement of the binding of NFκB to the consensus DNA κB-site binding by EMSA revealed an oscillatory profile with maximum binding 1, 2 and 6 h after initiation of the hyperosmotic stress. Supershift analysis revealed that p65 and RelB (but not p50, p52 or cRel) were involved in the binding of NFκB to DNA. Hyperosmotic stress also resulted in activation of the NFκB-lux reporter gene, transient activation of caspases 9 and 3 and phosphatidylserine externalization. The effect on cell viability was not prevented by ZVAD (a general caspase inhibitor). Blockade of NFκB with AdIκBα, an IκBα dominant negative overexpressing adenovirus, prevented activation of caspase 9 (more than that caspase 3) but did not affect cell death in hyperosmotically stressed cardiomyocytes. We conclude that hyperosmotic stress activates p65 and RelB NFκB isoforms and NFκB mediates caspase 9 activation in cardiomyocytes. However cell death triggered by hyperosmotic stress was caspase- and NFκB-independent.**

Keywords: NFκB; Osmotic stress; Caspase; Cell death; Apoptosis

1. Introduction

Osmotic stress is one of the important mechanisms of tissue damage. Hypertonic stress may activate two opposing cellular signaling cascades that either lead to cell death or promote cell survival. The balance between these two pathways determines the fate of the cell. Hyperosmotic stress stimulates rapid and pronounced apoptosis in cultured cardiomyocytes [1,2].

There are five members of the NFκB family: Rel C(c-Rel), Rel A (p65), Rel B, NFκB1 (p105/p50) and NFκB2 (p100/p52) [3]. NFκB/Rel proteins exist as homo- or hetero-dimers and possess a conserved Rel homology domain that mediates dimerization as well as binding to DNA [4]. In most resting cells, NFκB is bound to its cytoplasmic inhibitory proteins, IκB (α,β, γ, ε or Bcl-3), and remains in the cytoplasm as a latent transcription factor [4]. Upon stimulation, the IκB kinase (IKK) complex, composed of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ, is activated and in turn phosphorylates IκBα and IκBβ proteins [4]. The phosphorylation triggers ubiquitin-dependent degradation of IκB proteins by the 26S proteosome, this process resulting in the release of NFκB [3,4]. Subsequently, NFκB translocates into the nucleus and activates transcription of specific target genes [3,4]. Additionally, there are other signaling factors that act more directly to activate NFκB via IκB or by direct phosphorylation of NFκB subunits [5,6]. Finally, there are combinatorial interactions at the level of the promoter between NFκB, its co-activators and other transcription factors, several of which are activated by MAPK and cytokine signaling pathways [7,8]. Thus, in addition to being a major mediator of cytokine effects in the heart, NFκB is positioned as a signaling integrator [9]. As such, NFκB functions as a key regulator of cardiac gene expression programs downstream from multiple signaling cascades in a variety of pathophysiological cardiac conditions [10–13]. It is not, however, known, whether hyperosmotic stress activates the NFκB system in cardiomyocytes and whether NFκB participates in the death and survival processes triggered by this insult.

2. Methods

2.1. Materials

Polyclonal antibodies against NFκB isoforms: p65, p50, p52, cRel and RelB used in Western blot, immunocytochemistry and supershift analysis were from Santa Cruz Biotechnology. Polyclonal antibodies against phospho-IKKα/β, IκBα, procaspase 3/caspase 3 and procaspase 9/caspase 9 were purchased from Cell Signaling Technology Inc. All other biochemicals were purchased from Sigma unless stated

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otherwise. [^{32}P]ATP was from NEN (Boston, MA) and ECL was from Perkin–Elmer Life Sciences (Boston, MA). Protein assay reagents were from Bio-Rad (Hercules, CA). The NFκB reporter gene, 2×NFκB-lux, was kindly donated by Dr. Michael Karin (University of California, San Diego). AdIκBα adenovirus was provided by Dr. X. Zhou (University of North Carolina, Chapel Hill). AdIκBα overexpresses a dominant negative form of IκBα (Ala32/Ala36) [14]. AdLacZ that express β-galactosidase, was used as infection control. Cardiomyocytes were transduced with adenoviral vectors using a multiplicity of infection (MOI) of 300, 24 h before hyperosmotic stress with sorbitol.

2.2. Culture and treatment of cardiomyocytes

Cardiomyocytes were prepared from neonatal hearts of Sprague-Dawley rats as described previously [2]. 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 $1–8 \times 10^3$/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 β-myosin heavy chain antibody as previously described [2]. Cell cultures were at least 95% pure.

2.3. Immunocytochemistry

Cardiomyocytes grown on coverslips were fixed with PBS containing 4% paraformaldehyde for 20 min and incubated in ice-cold 0.3% Triton X-100 for 10 min to permeabilize the cells. Nonspecific sites were blocked for 1 h at room temperature with 5% BSA in PBS. Cells were then incubated with p65, p50 or RelB antibodies at 1:100 dilutions at 4 °C overnight. Cardiomyocytes were washed with PBS and incubated with anti-rabbit IgG-FITC (1:1000). Nuclei were stained with propidium iodide (PI, 1 μg/ml). Secondary antibodies were anti-mouse IgG-Cy3 and anti-rabbit IgG-FITC. Fluorescence was evaluated in a scanning confocal microscope (Carl Zeiss Axiovert 135) and image analysis was made by LS Dummy software.

2.4. Preparation of cell extracts and Western blotting

Cardiomyocytes were scraped into cold lysis buffer: 20 mM HEPES, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM glycerol 1-phosphate, 0.06% (v/v) Triton X-100, 0.5 mM DTT, 0.1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Samples were centrifuged at $12000 \times g$ for 10 min at 4 °C and the protein contents of supernatants were determined by a Bio-Rad Bradford assay [2]. Soluble fractions were heated at 95 °C with 0.33 volumes of 4× SDS–PAGE sample buffer for Western blot analysis. Nuclear and cytosolic fractions from cultured cardiomyocytes were prepared as described by Courtois et al. [15]. Purity of cytosolic and nuclear fractions assessed by lactic dehydrogenase activity and c-fos levels were 90% and 66%, respectively. Phospho-IKKα/β, IκBα, procaspases 3 and 9 and caspase 3 and 9 levels were analyzed in total extracts. Cytosolic and nuclear proteins were evaluated with p65, RelB and p50 antibodies. Protein contents were checked using anti-β-actin and anti-TFIIB antibodies. Western blots were performed according to Galvez et al. [2]. The digitalized images were obtained by scanning the films. Then they were analysed by UN-SCAN-IT program software (Silk Scientific Corporation, Orem, UT, USA) and the values were expressed as fold over time zero or control.

2.5. Electrophoretic mobility shift assay (EMSA)

NFκB binding activity was performed as described previously [16] using the double-stranded NFκB consensus oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′, end-labeled with T4 kinase and 75 μCi of [γ-^{32}P]ATP. ^{32}P-labeled probe was incubated for 20 min at 4 °C in a 25 μl binding reaction mixture containing 2 μg of nuclear proteins and 1× binding buffer (500 ng poli (dG–dC), 10 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 12.5% glycerol, 1 mM DTT, 0.1% Triton X-100). The DNA–protein complexes were analyzed by electrophoresis on 6% non-denaturing polyacrylamide gels in Tris-borate–EDTA buffer. Supershift assays were performed incubating nuclear extracts with 4 μg of anti p65, anti RelB, anti p50, anti p52 or anti cRel antibodies for 2 h at room temperature followed by incubation with 30000 cpm of [^{32}P]-κB probe for 20 min at 4 °C and submitted to a 4% polyacrylamide electrophoresis in 0.5× Tris–borate–EDTA. As controls, 100-fold excess of non-radioactive NFκB consensus or 1000-fold excess of mutated (5′-AGTTGAGGCGAC-TTTCCCAGGC-3′) oligonucleotides were used.

2.6. Transfections and luciferase reporter assays

Cardiomyocytes in 60 mm dishes, were transfected with 2×NFκB lux and lacZ genes by the $Ca_3(PO_4)_2$ method. Transfected cells were treated by sorbitol (600 mOsm). After 8, 18 or 24 h incubation, cells were lysed and luciferase and β-galactosidase activities were assayed [17]. Luciferase activities were normalized against β-galactosidase activities.

2.7. Cardiomyocyte viability

The effect of osmotic stress on cardiomyocyte viability was measured using the CellTiter 96 proliferation assay (Promega, Madison, WI).

2.8. Annexin V/PI analysis

Annexin V/propidium iodide (PI) double staining was used to detect cell death. This assay does not discriminate between apoptosis and necrosis [18]. Cardiac myocytes were trypsinized, resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$) and labeled with annexin V-FITC and PI (1 μg/ml). Mixtures were incubated for 10 min in the dark at room temperature and analyzed immediately by flow cytometry in a Becton Dickinson FACSort. Results were acquired and analyzed by CELLQuest software.

2.9. Statistical analysis

Data shown are means ± S.E. of the number of independent experiments indicated (n) or representative experiments performed on at least three separate occasions with similar outcomes. Data were analyzed by analysis of variance and comparisons between groups 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 IKKα/β phosphorylation and IκBα degradation

IKKα/β activation was assessed in cardiomyocytes exposed to hyperosmotic stress (sorbitol, 600 mOsm). Results in Fig. 1A showed that phosphorylation of IKKα/β was early, reaching a peak at 30 min. Subsequently, there was a time-dependent degradation of IκBα (Fig. 1B). IκBα levels decreased after 30 min with a minimum 4–6 h post-stimulus. These results are consistent with a canonical activation of NFκB [4].

3.2. Hyperosmotic stress stimulates p65 and RelB translocation to the nucleus

Immunocytochemical analysis (Fig. 2A) showed that control cells have p65 and RelB localization both in cytoplasm and nucleus; p50 was mainly in the nucleus. When cardiomyocytes were rendered hyperosmotic with sorbitol up to 6 h, p65 and RelB increased their nuclear localization. However, p50 remained in the nucleus and its content was unchanged by hyperosmotic stress. Confocal imaging confirmed these results (Fig. 2B).

Fig. 2C shows that p65 levels increased significantly in the nuclear fraction after 30 min of hyperosmotic treatment. RelB levels were also increased in the nuclear fraction. The cytosolic content of p65 and RelB, decreased with similar kinetics, suggesting that both could be mobilized together. The cytosolic and nuclear contents of p50, the classical p65 heterodimer partner [4], did not change in cardiomyocytes exposed to hyperosmotic stress. p52 and cRel isoform distribution did

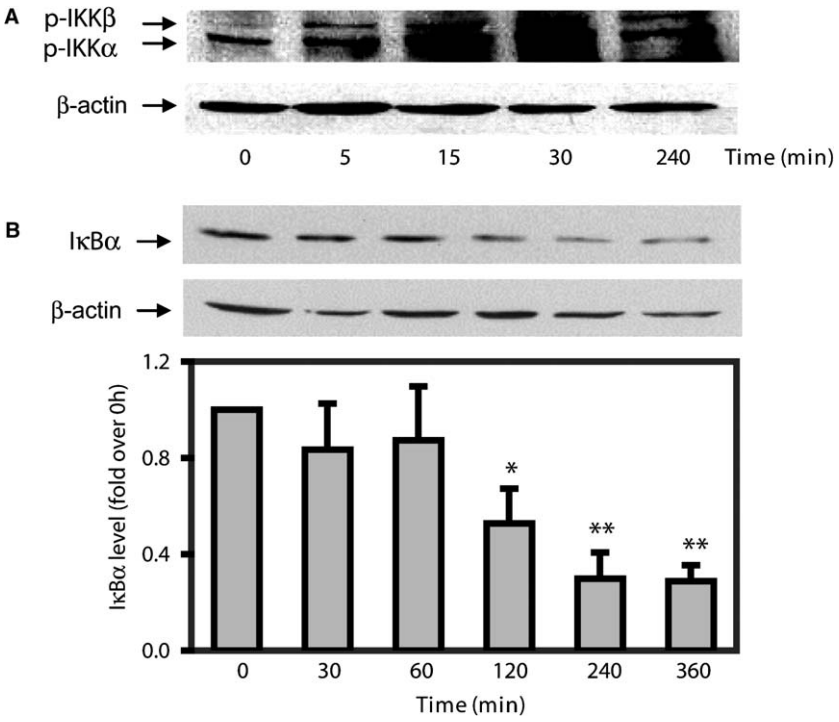

Fig. 1. Hyperosmotic stress by sorbitol induced IKK phosphorylation and IκBα degradation. Cardiomyocytes were incubated with hyperosmotic sorbitol (Sor) and protein extracts were prepared at the times indicated. (A) Phosphorylated IKKα/β (p-IKKα/β) and (B) IκBα levels were determined by Western blot ($n = 5$); $^{*}P < 0.05$ and $^{**}P < 0.01$ versus time 0 min.

not change after hyperosmotic stress stimulation (data not shown).

3.3. NFκB functional activation in hyperosmotic-stressed cardiomyocytes

When triggered by various stimuli NFκB induces changes in the rates of transcription of target genes [19]. To address whether hyperosmotic stress results in binding of NFκB to DNA, we performed EMSA assays with a ^{32}P-labeled κB consensus sequence. Results showed an oscillatory time dependent NFκB DNA binding activity with maximum at 1, 2 and 6 h (Fig. 3A and B, $P < 0.05$). These results are in agreement with those showing degradation of IκBα and translocation of p65 and RelB. NFκB transcriptional activity in cardiomyocytes co-transfected with the 2×NFκB-Lux and β-galactosidase genes was determined from the ratio of the luciferase/β-galactosidase activities. The accumulation of luciferase expression and further activity was detectable after 18 h in hyperosmotically stressed cardiomyocyte (Fig. 3C, $P < 0.05$).

We also carried out supershift assays to identify which of the NFκB isoforms are involved in the DNA binding induced by hyperosmotic stressed cardiomyocytes. A NFκB supershift was observed only with p65 and RelB antibodies (Fig. 3D). The p65 supershift was much stronger than that of RelB suggesting that there is either little affinity between the κB probe and RelB or that the formed RelB-containing dimer is scarce within the cardiomyocyte.

3.4. NFκB-dependent activation of caspases by hyperosmotic stress is not associated to cardiomyocyte death

The exposure of cardiomyocytes for 1–2 h to hyperosmotic stress induced fragmentation of procaspases 9 and 3 into caspases 9 and 3, respectively (Fig. 4A). Increase of caspase 9 proteolytic activity was also detected after 2 h of sorbitol treatment (data not shown). Hyperosmotic stress decreased the viability of cardiomyocytes but was not prevented by ZVAD (Fig. 4B). To evaluate the role of NFκB in the activation of caspases stimulated by hyperosmotic stress, cardiomyocytes were transduced with AdIκBα, an adenovirus that overexpresses a dominant negative protein that contains Ser^{32}Ala and Ser^{36}Ala mutations. After hyperosmotic stress, AdIκBα prevented the translocation of p65 to the nucleus (data not shown). These changes inhibit phosphorylation, prevent proteasomal degradation of IκBα and hence NFκB dissociation, thereby preventing translocation to the nucleus [14]. In cardiomyocytes transduced with AdIκBα, the activation caspase 9 induced by hyperosmotic stress, more than the activation of caspase 3, was significantly attenuated (Fig. 5). These results showed that NFκB mainly mediated caspase 9 activation in cultured cardiomyocytes.

To study the role of NFκB in cell death, we evaluated phosphatidylserine externalization and PI incorporation by FACS analysis in transduced cardiomyocytes with AdIκBα. Hyperosmotic stress started to increase annexin V binding after 4 h (data not shown), reaching a maximum after 6 h (almost 3-fold respect to control, Table 1, $P < 0.01$). PI staining revealed cardiomyocyte exposed to hyperosmoric stress had no a significant compromised membrane integrity (Table 1), suggesting that necrosis was not stimulated by hyperosmotic stress. The adenoviral transduction with AdIκBα did not significantly change the % annexin V positive cells or % PI positive cells, indicating that NFκB does not participate in cardiomyocyte death. Controls using AdLacZ showed no differences with non-transduced cells.

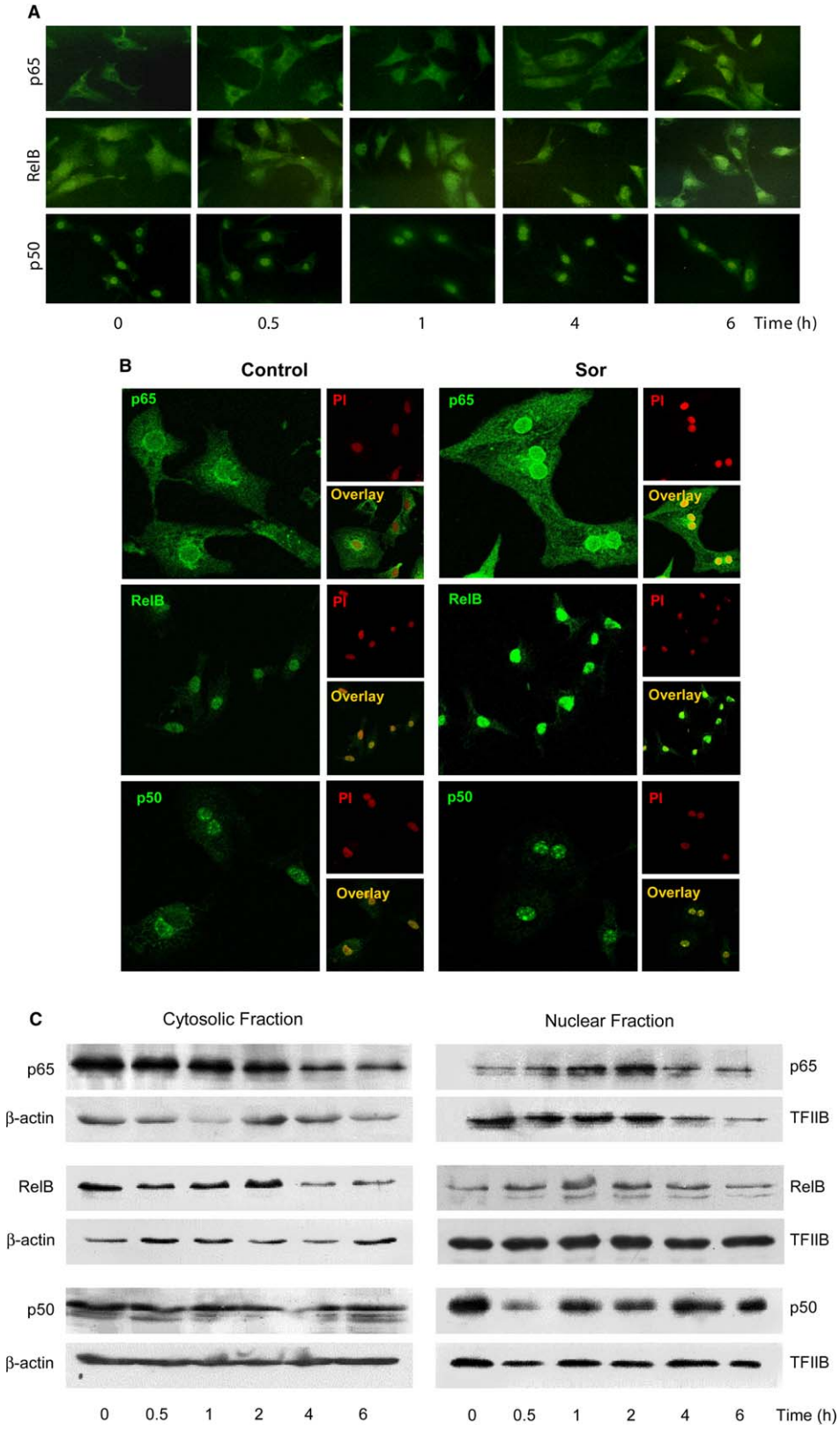

Fig. 2. Hyperosmotic stress induced p65 and RelB translocation to the nucleus. (A) Time-course of the effect of hyperosmotic stress on the NFκB isoform subcellular distribution assessed by epifluorescence microscopy. Cultured cardiomyocytes were incubated at indicated times and p65, RelB and p50 were detected. Antibody binding was detected with an anti-rabbit-FITC. Representative images of, at least, three independent experiments. (B) Confocal microscopy analysis of p65, RelB and p50 localization in cardiomyocytes exposed to hyperosmotic stress for 6 h. Antibody binding was detected with an anti-rabbit-FITC. Nuclei were stained with propidium iodide (PI). Representative images of, at least, three independent experiments. (C) Nuclear and cytosolic fractions were isolated from cardiomyocytes exposed to hyperosmotic stress with 600 mOsm sorbitol. p65, RelB, and 50 levels were detected by Western blotting. Gels are representatives of three independent experiments.

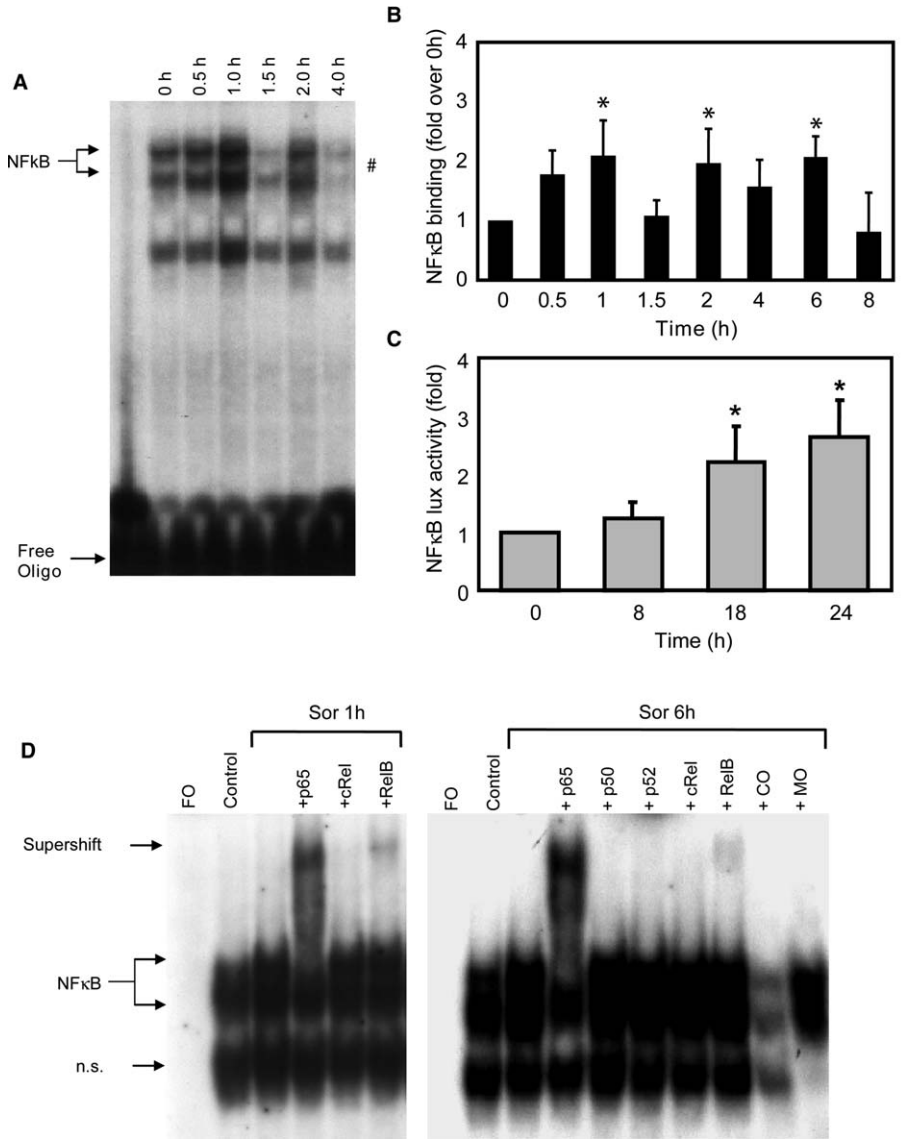

Fig. 3. NFκB binding to DNA, NFκB reporter gene expression and identification of NFκB isoforms by supershift assays in hyperosmotically-stressed cardiomyocytes. (A) Time course of the NFκB binding to the κB probe induced by hyperosmotic sorbitol. Nuclear extracts from cardiac myocytes were incubated with a ^{32}P-labeled consensus NFκB oligonucleotide, resolved in a 6% polyacrylamide gel and detected by autoradiography. The gel is representative of six different experiments. (B) Quantitation of the binding of upper NFκB band (#). Values are means ± S.E; $^{*}P < 0.05$ versus time 0 min. (C) NFκB-lux reporter gene activation by hyperosmotic stress. Cardiac myocytes were cotransfected with 5 μg of NFκB-lux and β-galactosidase genes, incubated for 24 h and exposed to hyperosmotic sorbitol for 8, 18 and 24 h. Cell extracts were prepared and luciferase and β-galactosidase activities were determined. Results are expressed as ratios of the increase of Luc/β-galactosidase activity relative to controls (n = 2–5) ± S.E.; $^{*}P < 0.05$ versus control. (D) NFκB supershift assay. Nuclear extracts from cardiac myocytes exposed to hyperosmotic stress for 1 or 6 h were incubated with a ^{32}P-labelled consensus NFκB oligonucleotide and antibodies to NFκB isoforms, resolved in a 4% polyacrylamide gel and detected by autoradiography. Gels are representative of three independent experiments; FO = free oligonucleotide; CO, 1000-fold excess cold NFκB oligonucleotide; MO, 100-fold excess cold mutated NFκB oligonucleotide.

4. Discussion

We report here that hyperosmotic stress activates the canonical NFκB pathway in cardiomyocytes. Others, using various stimuli [12,20–22], have only described activation of some of the steps of the canonical NFκB pathway in cardiomyocytes. We have shown that hyperosmotic stress stimulates the activation of NFκB in these cells. The kinetic IκBα degradation resemble the one reported in Li et al. [21]. Courtois et al. [15] demonstrated that hyperosmotic stress induces IκBα degradation in preB cells, although they did not examine NFκB activation. Our results do not exclude the existence of the non-canonical NFκB activation pathways.

Although all five NFκB proteins occur in cardiomyocytes, only p65 and RelB were involved in the response to hyperosmotic stress. We found that hyperosmotic stress induced translocation of p65 to the nucleus more slowly than observed in cardiomyocytes exposed to cardiotrophin 1 [23]. The limited ability of RelB to form heterodimers with p50 or p52, relative to p65 or c-Rel, together with its inability to homodimerize, identifies RelB as an unusual member of the NFκB family [24,25]. The p65/RelB heterodimer is transcriptionally inactive

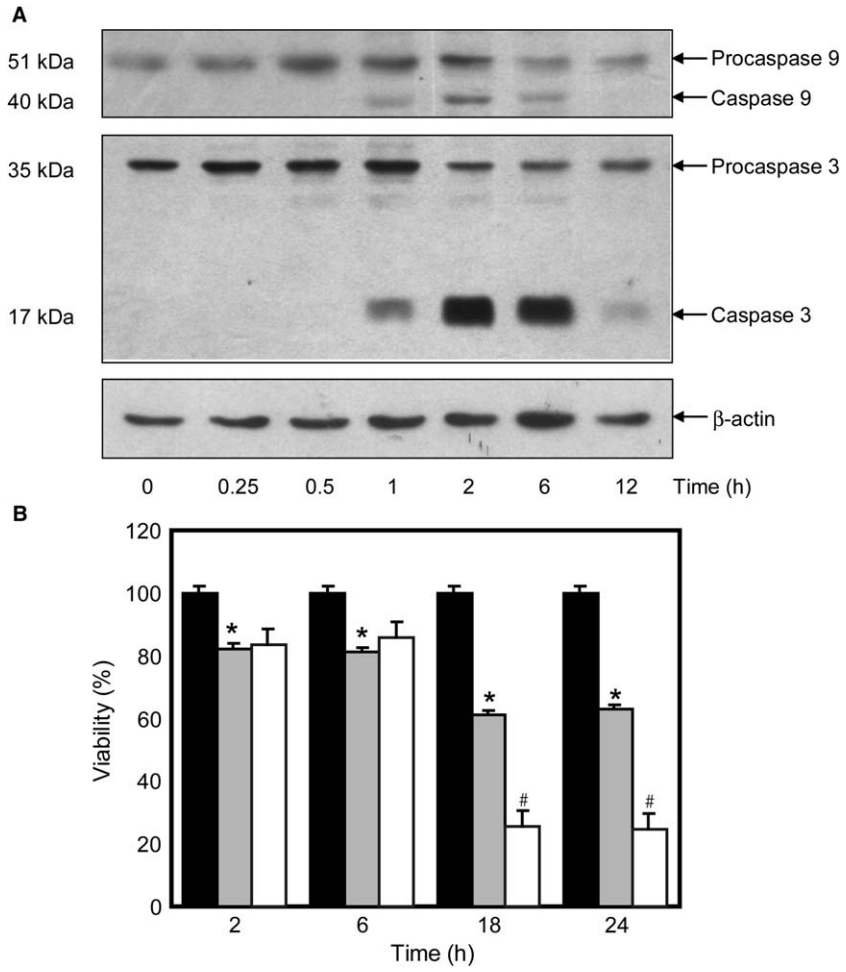

Fig. 4. Caspase activation and hyperosmotic stress induced cardiomyocyte cell death. (A) Time course evaluation of caspase 9 and 3 degradation induced by sorbitol. Western blot analysis of procaspase and caspase. (B) Cardiomyocytes were incubated with culture media (control, black bars), hyperosmotic sorbitol (gray bars) or sorbitol + Z-VAD-FMK (10 μM, white bars). At different times viable cells were quantified using cell titer ($n = 3$). $^{*}P < 0.05$ versus control and $^{\#}P < 0.05$ versus sorbitol.

in vitro [26]. Several reports emphasize the importance of RelB when complexed with NFκB2 (p52 or its precursor p100) as the basis of an "alternative" NFκB pathway (reviewed in [27]). Our data show that expression of the NFκB-lux reporter gene by hyperosmotic stress may be consistent with the p65/RelB heterodimer being transcriptionally active. Our results could be, however, equally consistent with a coexistence of p65/RelB heterodimer with p65/p65 homodimer. This homodimer has been described as an activator of gene transcription [4]. The previous failure to detect p65/RelB by others may be because this heterodimer does not bind efficiently to conventional κB sites or because its amount in the cell is low [28–30]. The activation level of NFκB reporter gene found in cardiac myocytes exposed to hyperosmotic stress could be considered low. However similar activations for this NFκB-lux reporter gene have been reported for TNF-alpha in GH3 cells [31] or for angiotensin II in vascular smooth muscle cells [32]. Morover, the 2×NFκB reporter gene used in our studies has been mainly used to monitor gene expression driven by p50 and p65 NFκB. In contrast, p65 and RelB, our main NFκB isoforms activated by hyperosmotic stress could not have the same affinity and bind efficiently to conventional κB sites [30]. In hyperosmotically-stressed cardiomyocytes, the oscillation of NFκB DNA binding might, on the other hand, be the result of a dynamic equilibrium between p65/p65 and p65/RelB. Such an interconversion between p65/p65 and p65/RelB is plausible because changes in the composition of the NFκB subunits have been shown to occur in resting mature B cells treated with anti-Ig antibodies or a CD40 ligand [33].

The hyperosmotic stress induced cardiomyocyte death with apoptotic hallmarks [1,2]. Here, we demonstrated caspases 9 and 3 degradation between 2 and 6 h. Previously, Morrison et al. [34] demonstrated that caspase 3 is activated after 8 h of hyperosmotic stress in cardiomyocytes. Caspase inhibition by Z-VAD did not prevent hyperosmotic stress induced cardiomyocyte death. This result demonstrates that the programmed cardiomyocyte death induced hyperosmotic stress is caspase independent. In the last years, many reports have demonstrated such condition [reviewed in 35]. Caspase 3 and 9 were transiently activated in the cardiomyocyte, indicating that they participate in other caspase-dependent biological process or in a more complex cell death mechanism. The activation of caspases 2, 3 and 8 without cell death has been described in bone morphogenic protein osteoblasts differentiation [36]. Caspase 3 transient activation was also associated with erythroid differentiation and muscle differenti-

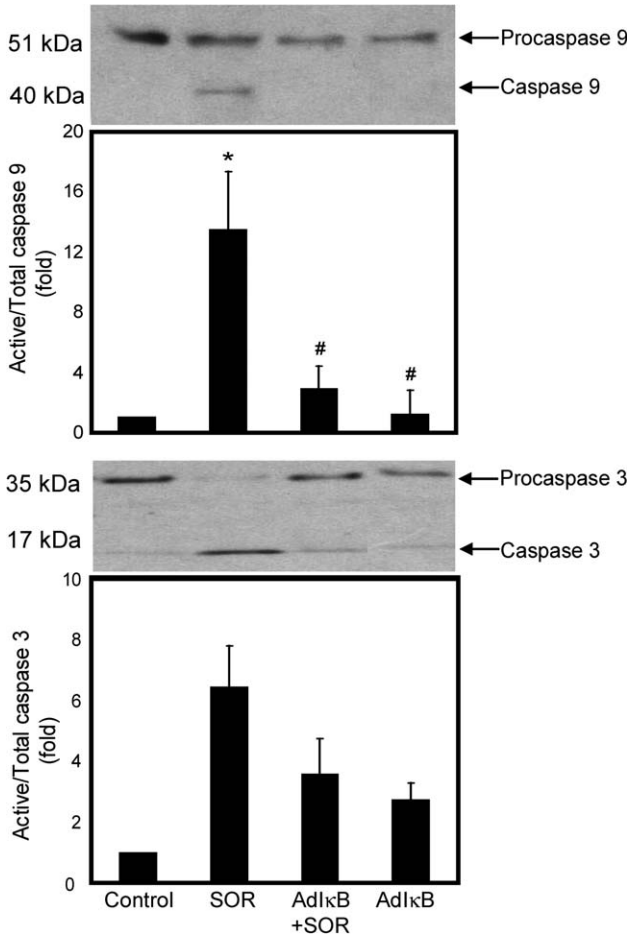

Fig. 5. Effect of NFκB inhibition on caspase activation in hyperosmotically-stressed cardiomyocytes. Cells were infected with AdIκBα (MOI = 300) 24 h before 2 h exposure to hyperosmotic stress and caspase 9 and 3 degradation levels were evaluated by Western blot ($n = 4$) and expressed as ratio active caspase/total caspase. $^{*}P < 0.05$ versus control and $^{\#}P < 0.05$ versus sorbitol.

Table 1
Effect of NFκB inhibition on cardiomyocyte death induced by hyperosmotic stress

	% Annexin V positive cells	% PI positive cells
Control	7.3 ± 2.6	2.8 ± 1.4
SOR	18.0 ± 3.1**	3.7 ± 2.4
AdIκBα + SOR	22.3 ± 2.4**	4.1 ± 2.5
AdIκBα	9.7 ± 0.6	5.2 ± 2.7
AdLacZ + SOR	17.2 ± 3.7*	1.8 ± 1.1
AdLacZ	5.4 ± 0.7	2.2 ± 0.9

Data show mean values ± S.E. (n = three independent experiments). $^{*}P < 0{,}05$ and $^{**}P < 0{,}01$ versus control.
After 24 h AdIκBα transduction, cardiomyocytes were exposed to hyperosmotic stress for 6 h. AdLacZ was applied as infection control under the same conditions. Flow cytometry analysis was performed to evaluate phosphatidylserine externalization by annexin V binding and propidium iodide (PI) incorporation in cardiomyocytes.

ation without cell death [37,38]. Moreover, other authors reported that cell death is not blocked by caspase inhibitors, that render apoptosis like [35,39] or autophagic cell death [40]. Our model probably represents some of these possibilities. ZVAD also increased cell death above the effect of hyperosmotic stress by sorbitol at later time points. Although this effect on cell viability remains to be determined, Li et al. have shown that caspase 8 inhibition stimulated autophagic death [40].

Finally, when we inhibited NFκB, the hyperosmotic stress-dependent activation of caspase 9, more than the observed for caspase 3, was attenuated. These results suggest that this transcriptional factor may control genes associated with regulation of initiator caspase 9 than executioner caspase 3. However future experiments will be necessary to define which genes are controlled by NFκB in relation to caspase expression.

In this work, we demonstrated that the functional meaning of NFκB activation by hyperosmotic stress was not related to a significant anti-cell death role, as it is documented in our experiments with phosphatidylserine externalization/PI incorporation. NFκB has been involved in the direct regulation of both pro- and anti-apoptotic genes [4] and in cardiomyocytes under other stress conditions, NFκB acts as an antiapoptotic factor [41,42].

In summary, our results showed that hyperosmotic stress activates NFκB pathway that involved stimulation of IKK phosphorylation, IκBα degradation, p65/Rel B NFκB translocation to the nucleus and transcriptional activation of NFκB target genes. Hyperosmotic stress also resulted in transient activation of caspases 9 and 3 that was not related with cardiomycyte death. In this scenario, NFκB shows dichotomical actions, regulating caspase activation without altering cardiomyocyte survival.

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