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Nuclear factor NF-κB in myocardium: developmental expression of subunits and activation by interleukin-1β in cardiac myocytes in vitro

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Abstract

Objective: The aims of the study were to investigate the pattern of expression of the major subunits of the NF-κB transcription factor complex in human and rat heart development, and to characterise the timing of NF-κB activation by interleukin-1β (IL-1β) in rat neonatal cardiac myocytes. **Methods:** The expression of NF-κB subunits p65 and p50 and the inhibitory subunits IκB-α and IκB-β in human and rat myocardial samples was measured by immunoblotting, using antibodies specific to each subunit. The activation of NF-κB was measured in neonatal rat cardiac myocytes that were treated with IL-1β for different times (0–60 min). Depletion of the inhibitory factors IκB-α and IκB-β was assessed by immunoblotting. The presence of NF-κB DNA binding activity was measured directly in nuclear extracts by electrophoretic mobility shift assay (EMSA). **Results:** p65, p50, IκB-α and IκB-β are expressed at all stages of development analysed. In human myocardial samples, expression of p50, p65 and IκB-α show an apparent gradual decline relative to total protein. In contrast, the level of IκB-β remained relatively constant, suggesting a significant shift in the ratio of β and α subunits with development. In rat myocardium, p65, p50, IκB-α and IκB-β showed a gradual decline during development, with a particularly pronounced decrease between the ten day post-natal and adult samples. Treatment of neonatal rat cardiac myocytes with IL-1β (5 ng/ml) caused a rapid and transient depletion of IκB-α (reducing to 16±1.6% of initial levels within 5 min, returning to 82±10% within 60 min). A slower, less marked depletion is observed for IκB-β (24±6% by 30 min, returning to only 49±5% by 60 min). Rapid and transitory accumulation of NF-κB DNA binding activity was detected in the nucleus, with a pattern that correlated with the depletion of IκB-α. **Conclusions:** The principal NF-κB subunits p65, p50, IκB-α and IκB-β are present throughout development, suggesting that this transcription complex may participate in myocardial gene regulation throughout development and in the adult. Activation by IL-1β demonstrates that NF-κB probably plays a direct role in the regulation of gene transcription in response to cytokine activation in cardiac myocytes.

Keywords: Development; Cardiac myocyte; NF-κB; Interleukin-1β; Rat; Human

1. Introduction

Nuclear factor NF-κB is a transcription factor complex originally identified as a regulator of immunoglobulin genes but which has become recognised as being widely, and perhaps ubiquitously, expressed [1–3]. The importance of NF-κB in myocardium has been highlighted in a recent study where activation was abrogated using an NF-κB-binding decoy oligonucleotide [4], an effect that was shown to prevent myocardial infarction following coronary artery ligation in a rat experimental model. The detailed mechanisms involved in this cardioprotective effect remain to be determined, but specific observations included a decrease in interleukin-6 (IL-6) and VCAM gene expression, which may reduce lymphocyte infiltration. In other studies, direct NF-κB activation has been demonstrated in cardiac myocytes in response to IL-1β, [5,6] hydrogen peroxide [7] and myocardial ischemia [8]. The target genes activated by NF-κB in cardiac myocytes remain to be determined. Candidates include the inducible nitric oxide synthase (iNOS) gene, which is upregulated by IL-1β in cardiac myocytes e.g. [9–12] and which contains consensus binding sites that are positively regulated by NF-κB in

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other cell types [13] Similarly, the IL-6 gene promoter is activated by hypoxia in myocytes [14] and in other cells this is mediated by NF-κB [15].

The signals that can activate NF-κB have been widely studied in many other systems. Positive stimulatory factors include proinflammatory cytokines, bacterial lipopolysaccharides (LPS), oxidants, hypoxia and calcium overload [16–18]. Such factors have clear relevance to clinically important aspects of cardiac myocyte biology, including inflammatory myocardial disease, septic shock, cardiac ischemia and reperfusion injury. A further observation of interest is that NF-κB can interact with cardiac myotrophin [19], a protein isolated from hypertrophied hearts of SHR rats and from dilated cardiomyopathic human hearts and that is believed to contribute to the hypertrophic process by stimulating protein synthesis. NF-κB may be suppressed by cyclosporin A [20] and participate in the pathway leading to activation of IL-6 by FK506 [21], suggesting that immunosuppression (e.g. in cardiac transplantation) may influence cardiac myocyte NF-κB pathways.

In mammalian cells, NF-κB is composed of homo- or heterodimers of the rel/NF-κB family of proteins. These proteins share a common sequence domain, the Rel-homology domain, which is involved in DNA-binding, nuclear localization and dimerization. Members include p50, p52 (synthesised as p105 and p100 precursors, respectively), p65 (RelA), cRel and RelB, which bind specifically to κB sequence motifs located in the promoters of target genes [16]. Of the possible combinations, it is thought that only those that contain p65, c-Rel or RelB function as transcriptional activators, as other subunits do not contain recognised transactivating domains [16]. Most commonly investigated is the p50/p65 heterodimer, which is widely expressed and binds to DNA at the sequence motif (GGGRNNYYCC) [17].

In its inactive form, NF-κB is retained in the cytoplasm by inhibitor (IκB) molecules, which comprise a distinct family of proteins that bind to NF-κB and inhibit nuclear localization and DNA-binding. Inhibition is relieved by a process that involves phosphorylation and ubiquitination of IκB, and its subsequent degradation via the proteosome pathway. Five IκB isoforms have been described to date. Of these, two (IκB-γ and IκB-δ) are in fact precursors of the p50 and p52 mature subunits [3]. The others, IκB-α, IκB-β and IκB-ε, are separate gene products. IκB-α is rapidly depleted in many cell types following stimulation. IκB-β shows different characteristics in its response time and may differ in its mode of activation [22], leading to speculation that it may play a role in the longer-term responses observed in some cell types [23,24]. IκB-α, IκB-β and IκB-ε [25,26] are widely expressed and are probably ubiquitous. One of the genes that NF-κB acts upon is the IκB-α gene. Depletion of IκB-α following cell stimulation therefore acts to reinitiate its own synthesis via activation of the IκB-α gene promoter by NF-κB. This overall process is rapid such that, following stimulation, IκB can be completely degraded within 5 min and subsequently restored to initial levels within 60 min. This feedback loop forms an essential part of the rapid transient response of NF-κB.

To date, no study has specifically addressed the expression of subunits in myocardium in adult or at different developmental stages or determined the characteristics of the response of IκB-α and IκB-β following activation. Here, we demonstrate the presence of NF-κB subunits p50 and p65, and of the inhibitory subunits IκB-α and IκB-β, in ventricular myocardial samples from fetal, neonatal and adult rat and human heart, as well as in neonatal rat cardiac myocytes that were cultured in vitro. We demonstrate that both IκB-α and IκB-β are subject to rapid transitory degradation following IL-1β stimulation of rat neonatal cardiac myocytes in vitro, with subsequent re-synthesis of IκB-α within 1 h. There is a concomitant rapid and transitory accumulation of NF-κB DNA-binding activity in the nucleus. These data demonstrate that NF-κB is present during fetal, neonatal and adult life and that it contributes to the response of cardiac myocytes to cytokine activation.

2. Methods

2.1. Materials

Pancreatin was obtained from Gibco. Collagenase was from Worthington Biochemical Corporation. Primaria plates and 60 mm dishes were from Falcon. Cytosine β-D-arabinofuranoside (Ara-C), cycloheximide, DMEM, Medium 199, horse serum (HS), fetal calf serum (FCS), penicillin/streptomycin, glutamine, HEPES, insulin–transferrin–selenium (ITS), phosphate-buffered saline (PBS), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin were all from Sigma. Human recombinant interleukin-1β (IL-1β) was kindly provided by Dr. Tim Wells, Wellcome–Glaxo. The primary antibodies anti-IκB-α (sc-371) and anti-p65 (sc-372) were from Santa Cruz and have been widely used in the analysis of these proteins (e.g. [27,28], respectively). Anti-p50 was a kind gift from Dr. Nancy Rice [29] and anti-desmin was from Sigma (Cat≠ D8281). In each case, these antibodies revealed a single band of the expected molecular mass, as determined by comparison with standard markers (Rainbow markers, Amersham). Anti-IκB-β antibody was a kind gift from Dr. Alain Israel [22] and revealed a single major band of the molecular mass predicted for IκB-β with a minor non-specific band of about 66 kDa. Secondary antibodies were from Dako.

2.2. Cell culture

Cardiac myocytes were isolated from the ventricles of one–two-day-old Sprague-Dawley rats by successive digestions with pancreatin (0.6 mg/ml)/collagenase (0.48

mg/ml), according to published protocols [30]. Cardiac myocytes were enriched by selective preplating for 45 min on plastic culture dishes (Primaria), during which time, the majority of non-myocytes adhered to the bottom of the dish. The resulting suspension of cardiac myocytes was plated on pre-gelatinised 60 mm plates at a density of 10^6 cells per dish in complete medium (DMEM, Medium 199, 10% HS, 5% FCS, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin, HEPES, pH 7.4, 2 mM glutamine). Ara-C was added to the medium to a final concentration of 10^{-5} M, to prevent the proliferation of non-myocytes. Following overnight incubation, the medium was changed to either serum-containing (i.e. complete medium as above) or serum-free medium (DMEM, Medium 199, 0.1 U/ml penicillin, 0.1 mg/ml streptomycin, HEPES, pH 7.4, 2 mM glutamine, supplemented with ITS) and incubated for at least 24 h before being used in an experiment. Using this method, cultures containing at least 95% cardiac myocytes were routinely obtained, as determined by FACS analysis using an anti-sarcomeric actin antibody. The investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986, published by HMSO, London.

2.3. IL-1β stimulation, protein preparation and immunoblot assay

Cardiac myocytes were washed and fed fresh medium before the addition of 5 ng/ml IL-1β at time 0. After 5, 15, 30, 45 and 60 min, cells were washed in ice-cold PBS before being lysed by the addition of 200 μl of lysis buffer (1% SDS, 0.5 mM PMSF, 2.5 μg/ml each of aprotinin and leupeptin, 1 μg/ml pepstatin). Chromosomal DNA was sheared by drawing the sample through a 26.5G needle. The final protein concentration was measured using the BCA Assay (Pierce). Protein (25 μg) was mixed with sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M 2-mercaptoethanol, 0.0025% bromophenol blue), heated to 100°C for 2 min and loaded onto a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel (25 mM Tris, 192 mM glycine and 1% SDS). Samples were electrophoresed for 3.5 h at 35 mA before being transferred onto Hybond-C super nitrocellulose membranes (Amersham) by Western blotting for 2.5 h at 1 A in 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were immersed in PBS–Tween 20 (PBS-T) and 3% milk protein overnight at 4°C, to block non-specific binding. Primary antibody, diluted 1:1000 (v/v) in PBS-T/3% milk protein, was added to the membranes for 60 min (room temperature); the membranes were rinsed (2×20 min) with PBS-T and subsequently incubated with the appropriate horseradish-peroxidase-conjugated secondary antibody, diluted 1:2000 (v/v) in PBS-T/3% milk protein for 30 min (room temperature). Membranes were rinsed for 2×20 min before the immunoreactive bands were visualised using Amersham ECL reagents, according to the manufacturer's protocol. Immunoreactive bands produced by immunoblot analysis were scanned using Image Analysis 1000 software (Alpha Innotech). Data were from five experiments, each experiment performed in duplicate, and the result were expressed as an average±S.E.M.

2.4. Electrophoretic mobility shift assay (EMSA)

IL-1β stimulation of cardiac myocytes was performed as for total cell lysate before the cells were treated to extract nuclear and cytosolic fractions [31]. After incubation, the cells were washed with ice-cold PBS and detached using trypsin solution (0.5 mg/ml) for 5 min at 37°C. Digestion was stopped by the addition of complete medium and the cells were pelleted by centrifugation for 5 min at 1000 rpm (room temperature). The cell pellet was resuspended in buffer A without Nonidet-P 40 (NP-40) (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 2.5 μg/ml each of aprotinin and leupeptin, 1 μg/ml pepstatin). The cells were pelleted by centrifugation for 5 min at 1000 rpm (4°C) and the pellet was resuspended in 200 μl of buffer A with 0.1% NP-40 added. After incubation on ice for 15 min, the suspension was passed through a 26.5G needle ten times and the nuclei were pelleted by centrifugation for 1 min at 12,000 *g* and 4°C. The supernatant (cytosolic fraction) was transferred to a fresh tube. The nuclear pellet was washed with buffer A twice before lysis by osmotic shock in 40 μl of a solution containing 20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF, 2.5 μg/ml each of aprotinin and leupeptin, and 1 μg/ml pepstatin and then was incubated at 4°C for 30 min with rotation. Nuclear debris was pelleted by centrifugation for 10 min at 4°C and 12,000 *g*, and the supernatant of nuclear proteins was transferred to a fresh tube. Protein concentrations were determined as for total cell extract.

For EMSA studies, nuclear protein extracts (2 μg) were added to a total volume of 20 μl of reaction mix (40 mM NaCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 10% glycerol and 2.5 μg poly-dIdC) in the presence of 10 fmol of NF-κB consensus oligonucleotide cassette, 5′-AGTTGAG*GGGACTTTCC*CAGGC (Promega), labelled with [^{32}P]-γdATP (Amersham) according to the manufacturer's protocols. These were incubated for 20 min at room temperature before half of the volume was loaded onto a 10% non-denaturing polyacrylamide gel and electrophoresed for 1 h 45 min at 160 V. Supershift analysis was performed by incubating 2 μg of nuclear protein with 1 μg of anti-p65, in a total volume of 20 μl as for the EMSA, for 20 min at room temperature before the addition of the labelled NF-κB cassette and a further 20 min incubation. The gel was dried and exposed to film (Kodak) at −70°C between intensifying screens.

2.5. Developmental series

Human fetal and post-natal cardiac muscle was obtained from elective terminations and at autopsy and placed in liquid nitrogen before treatment for Western blot analysis. Adult heart samples were from explanted hearts taken at the time of transplantation. Samples of late fetal (i.e. post 20 weeks) and of post-natal and adult heart were of ventricular muscle only. Earlier samples were predominantly ventricular, although contamination with atrial muscle could not be excluded. Fetal and neonatal samples were judged to be non-pathological and without cardiovascular developmental abnormality. The age of fetal samples was determined by anatomic measurement and is given as the calculated gestational age derived from standard normograms.

Samples of rat (Sprague-Dawley) heart were obtained from fetuses of time-mated females, neonates and adults. For fetal samples, pregnant mothers were sacrificed by cervical dislocation, fetuses were rapidly removed and placed immediately in ice-cold, sterile, calcium-free PBS, pH 7.4. Hearts were dissected and rinsed in ice-cold PBS and were frozen in liquid nitrogen: 12.5 day fetal samples were essentially whole heart, 15 day fetal and older samples were ventricular myocardium (apical 2/3rds). Neonatal samples were obtained by decapitation, the hearts were placed immediately in ice-cold PBS as above, the ventricular myocardium (apical 2/3rds) was removed, rinsed in PBS and frozen in liquid nitrogen. Adult heart samples were obtained by cervical dislocation; the ventricular muscle (apical 2/3rds) was removed, rinsed immediately in ice-cold PBS as above and frozen in liquid nitrogen.

For Western blot analysis, total protein extracts were prepared by homogenising heart tissue or tissue cultured cells in lysis buffer, and the protein content of each sample was determined using the Pierce BCA kit, as for cardiac myocyte cell lysates. A 30-μg amount of protein was prepared, electrophoresed, transferred to membrane and treated with antibodies as previously described.

3. Results

3.1. Expression of NF-κB subunits in myocardium

In order to investigate the expression of NF-κB subunits in myocardium, total protein extracts were prepared from different stages of human and rat heart development. Equal amounts were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis with the commonly found members of the NF-κB family; p65, p50, IκB-α and IκB-β. Fig. 1 shows the pattern of expression during human heart development from 12 weeks fetal life to adult. All subunits were expressed at all of the stages of development analysed. The expression of p50 and IκB-α showed an apparent gradual decline relative to total protein, an effect that may relate to the overall developmental increase in cell mass (see Section 4). In contrast, the level of IκB-β remained relatively constant, suggesting a significant shift in the ratio of β and α subunits with development. The level of p65 observed in the 17- and 24 week fetal-, nine month post-natal- and adult samples showed an apparent decrease with developmental age, in line with that observed for p50 and IκB-α. The level detected in the 12- and 33-week fetal samples appeared to be unexpectedly low. Analysis of a second series of immunoblots using the same tissue extracts and antibodies resulted in the same pattern. In the rat heart series (Fig. 2), the abundance of all subunits, including IκB-β, showed a gradual decline during development, with a particularly pronounced decrease between the ten day post-natal and adult (60 day) samples.

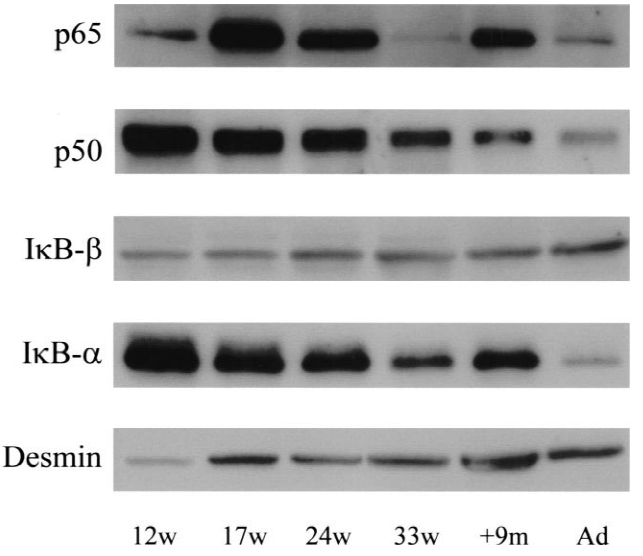

Fig. 1. Expression of p65, p50, IκB-β and IκB-α during human heart development. Total cell extracts were prepared from ventricular samples of 12, 17, 24 and 33 week fetal (12w, 17w, 24w and 33w), nine months postnatal (+9m) and adult (Ad) human myocardium. Following SDS–PAGE and immunoblotting, membranes were probed with antibodies for p65, p50, IκB-β and IκB-α subunits and visualised using ECL. Anti-desmin antibody was used as an internal control.

3.2. Stimulation of neonatal cardiac myocytes with IL-1β results in transitory depletion of IκB-α and IκB-β

In order to analyse the activation of NF-κB in cardiac myocytes, we tested the ability of a variety of agents to deplete IκB-α, as assessed by immunoblot analysis. Preliminary experiments confirmed that the addition of IL-1β to cultures of primary rat neonatal cardiac myocytes resulted in IκB-α depletion after a 5-min incubation (e.g. see Fig. 3) and initial experiments showed that there was no significant difference in magnitude of IκB-α depletion using IL-1β concentrations ranging from 1 to 100 ng/ml. For all subsequent experiments, 5 ng/ml was used and a

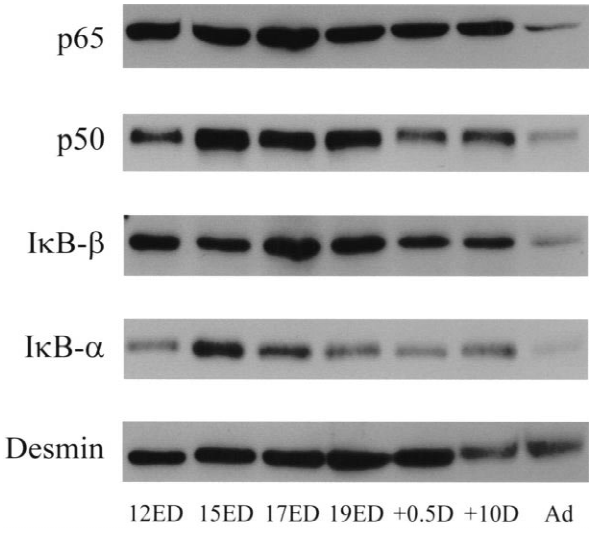

Fig. 2. Expression of p65, p50, IκB-β and IκB-α during rat heart development. Total cell extracts were prepared from ventricular samples of 12, 15, 17 and 19 day embryonic (12ED, 15ED, 17ED and 19ED), neonatal (+0.5D), ten days postnatal (+10D) and adult (Ad) rat myocardium. Following SDS–PAGE and immunoblotting, membranes were probed with antibodies for p65, p50, IκB-β and IκB-α subunits and visualised using ECL. Anti-desmin antibody was used as an internal control.

prolonged time course at this concentration resulted in the production of iNOS mRNA and protein (within 6 h, reaching a maximum at 24 h), as determined by reverse transcriptase polymerase chain reaction (RT-PCR) and immunoblot analysis (data not shown), in agreement with previous studies [10,11,32]. Other preliminary experiments further suggested little difference between the response of cells maintained in defined (serum-free) media compared to those cultured in the presence of serum.

The precise timing of IκB-α depletion was determined in cultures of rat neonatal cardiac myocytes that were stimulated with 5 ng/ml IL-1β for 5, 15, 30, 45 and 60 min. Total cell extracts were prepared and analysed by immunoblot analysis using an IκB-α-specific antibody. As shown in Fig. 3a, the addition of IL-1β resulted in a significant drop in IκB-α levels within 5 min (16±1.6% of the initial level). Levels remained low for 30 min, but by 60 min, they returned to 82±10% of the initial values. Neonatal rat cardiac myocytes therefore exhibit a transient, reversible depletion of IκB-α in response to IL-1β, in line with the response noted for other cell types [23,33]. In order to determine whether the level of IκB-α detected at 5 and 15 min was due to residual (undegraded) protein or to rapid re-synthesis, cells were treated with IL-1β in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 4). This resulted in a drop in the level of IκB-α to less than 5% of initial values, indicating that rapid re-synthesis accounts for the major part of the protein level detected at 5 and 15 min.

The time course of IκB-β depletion was followed in the

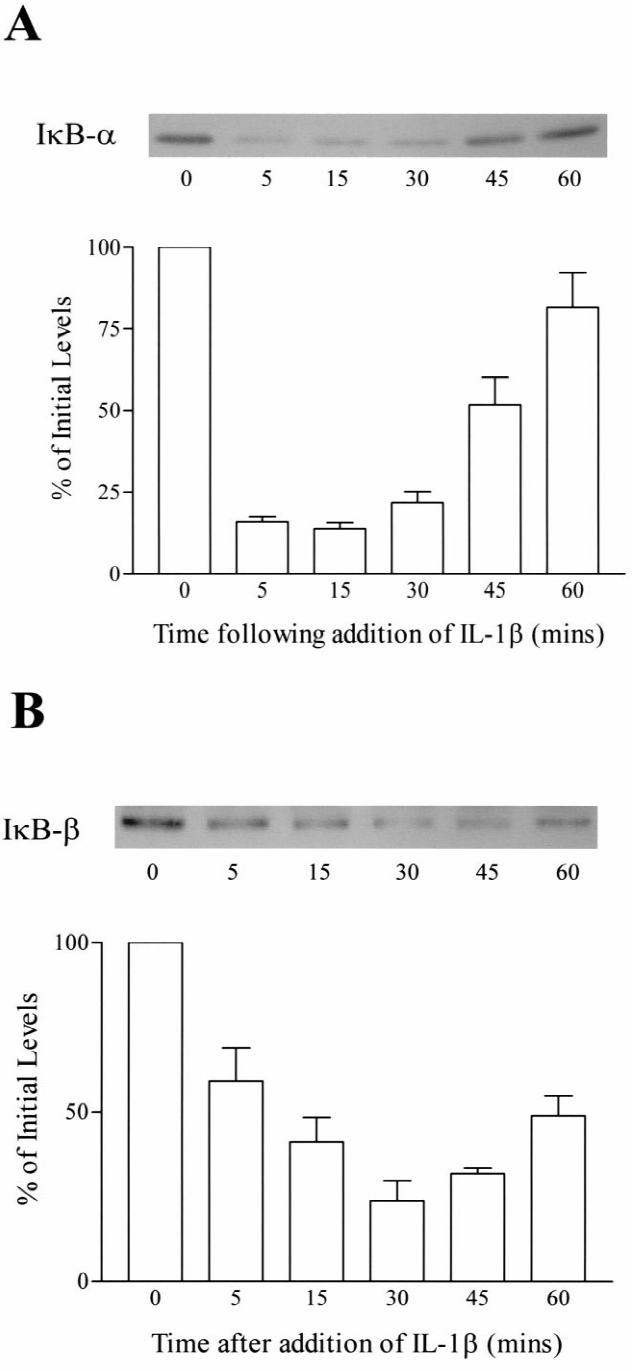

Fig. 3. (A and B) Depletion of IκB-α and IκB-β in rat neonatal cardiac myocytes in response to IL-1β. Cardiac myocytes were incubated for varying times with 5 ng/ml IL-1β before preparation of total cell extracts. Following SDS–PAGE and immunoblotting, membranes were probed using antibodies that were specific for IκB-α (panel A) or IκB-β (panel B) and the resulting complexes visualised by ECL. Each time point was performed in duplicate. Top panels show representative Western blots. Bottom panels show pooled data for five independent experiments. Error bars=S.E.M.

same experimental series using an antibody that was specific for this isoform, as shown in Fig. 3b. Following stimulation, the level fell more slowly than seen for IκB-α,

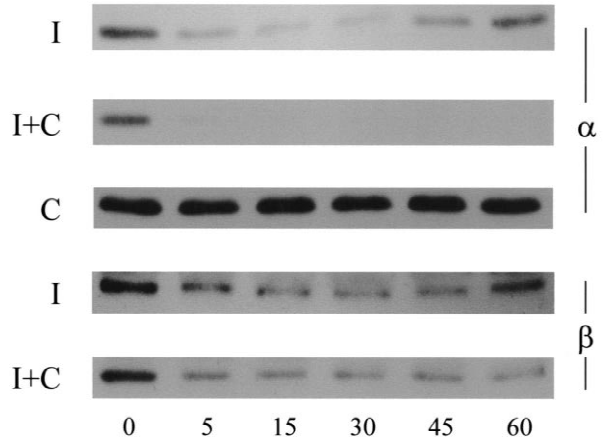

Fig. 4. Re-synthesis of IκB-α and IκB-β after depletion in response to IL-1β is prevented by treatment with cycloheximide. Cardiac myocytes from neonatal rats were incubated for varying times with 5 ng/ml IL-1β (I), 5 ng/ml IL-1β+5 μM cycloheximide (I+C) or 5 μM cycloheximide (C) before the preparation of total cell extracts. Following SDS–PAGE and Western blotting, membranes were probed using antibodies that were specific for IκB-α and IκB-β and the resulting complexes were visualised by ECL.

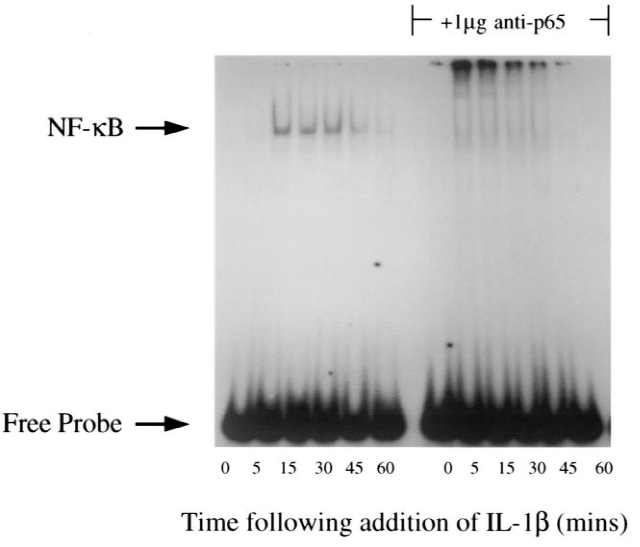

Fig. 5. Transient accumulation of NF-κB DNA binding activity in rat neonatal cardiac myocytes in response to IL-1β. Cardiac myocytes were incubated for varying times with 5 ng/ml of IL-1β and nuclear extracts were prepared. An electrophoretic mobility shift assay was performed in the absence (left panel) or presence (right panel) of anti-p65 antibody (Santa Cruz).

reaching a minimum by 30 min (24±6% of initial levels). Moreover, after 60 min, only half of the initial observed level (49±5%) was restored. The addition of cycloheximide did not cause any further reduction in the minimum levels of IκB-β, suggesting the existence of a significant residual, undepleted pool of IκB-β (Fig. 4). Overall, it is apparent from these experiments that the IκB-β response differs from that of IκB-α, being slower to reduce in level (reaching a minimum at 30 min compared to 5–15 minutes for IκB-α), having a less marked overall reduction (to 24±6% of initial levels compared to 16±1.6%) and being slower to increase following the initial reduction.

3.3. Accumulation of nuclear NF-κB DNA-binding activity

As a direct measurement of NF-κB activation, we assayed the ability of NF-κB to translocate from the cytoplasm into the nucleus in response to IL-1β. Nuclear extracts were prepared from rat cardiac myocytes that were stimulated with IL-1β (5 ng/ml) for the same time intervals as above. As shown in Fig. 5, no DNA binding activity was detected in unstimulated cells. DNA-binding activity was readily detectable within 5 min of the addition of IL-1β, gradually declining over the duration of the 60 min stimulus. On adding anti-p65 antibody, the band was super-shifted, confirming the identity of the complex as NF-κB.

4. Discussion

In this study, we have shown that the major NF-κB subunits (p50 and p65) and the NF-κB inhibitory subunits, IκB-α and IκB-β, are readily detectable in ventricular myocardial extracts throughout cardiac development and in isolated neonatal cardiac myocytes in culture. Cardiac myocytes occupy over 80% of the volume of the ventricular wall [34] and probably contribute over 90% of protein in whole tissue extracts as used here. We therefore propose that the developmental pattern observed reflects the level of expression in cardiac myocytes. The decrease in abundance of p50 and p65 subunits observed between neonatal and adult samples in both rat and man may be explained in part by the transition from hyperplastic to hypertrophic growth during heart development [35]. This results in a significant increase in cell volume following birth, including deposition of myofibrillar and associated proteins, which may act to mask the effective amounts of p50 and p65 available for translocation to the nucleus. Similarly, the inhibitory molecules IκB-α and IκB-β are present throughout development. In the human heart series, the ratio of IκB-β to IκB-α showed a developmental increase that was not seen in rat.

The activation of NF-κB by cytokines and other factors has been widely investigated in a number of cell systems [1,3]. However, few studies have examined the situation in cardiac myocytes [5–8]. Our data show the activation of NF-κB by IL-1β. Stimulation resulted in a rapid and transitory depletion of IκB-α and a slower, partial, transitory depletion of IκB-β. The effects of IL-1β on cardiac myocytes are varied and include direct negative inotropy

[36], induction of cardiac hypertrophy [37,38], initiation of cardiac myocyte DNA synthesis [38] and NO production [39]. Other specific alterations in gene expression have been noted, including activation of atrial natriuretic peptide (ANP) gene and depression of genes encoding sarcoplasmic calcium ATPase, calcium release channel, voltage-dependent calcium channel and phospholamban [37,40,41]. It is not currently known if these alterations in gene expression are mediated via an NF-κB dependent pathway. In the case of cytokine-induced NO production, this has been linked directly to the activation of the inducible nitric oxide synthase (iNOS) gene [42] by NF-κB, as blocking NF-κB activity using pyrrolidine dithiocarbamate (PDTC) eliminates iNOS gene activation [5,42,43], in agreement with studies in other cells where NF-κB binding was shown to be required for activation of the iNOS gene promoter [13]. Further studies are now required to determine if similar direct mechanisms operate on the other genes influenced by IL-1β-stimulated myocytes.

The depletion of IκB-α in cardiac myocytes as demonstrated here follows the rapid, transitory response noted for most cell types [33,44,45]. Furthermore, the accumulation of NF-κB DNA-binding activity in the nucleus appears to mirror this depletion, suggesting that IκB-α may be the principal regulator of NF-κB activity, at least in response to IL-1β. The precise role of IκB-β remains to be determined. However, a recent study has identified a number of potentially important functional differences between IκB-α and IκB-β [46]. In particular, IκB-α is probably a stronger inhibitor of NF-κB and can act to remove bound NF-κB from the nucleus, whereas IκB-β cannot. IκB-β activity may also be influenced by phosphorylation and interaction with other nuclear factors, including HMGI. Our data show that IκB-α and IκB-β respond differently to IL-1β stimulation in cardiac myocytes. This is in line with previous studies in lymphocytes, which demonstrated a slower, more persistent depletion of IκB-β in response to a variety of stimuli including IL-1β [23], suggesting that it is involved in more long-term NF-κB activation. The precise signal transduction pathways that result in IκB activation remain to be determined but are probably different for IκB-α and IκB-β. For example, while tumour necrosis factor-α (TNF-α) elicits a strong depletion of IκB-α in both endothelial and Jurkat cells, IκB-β is only responsive in Jurkat cells [23,47]. In our experiments, IL-1β resulted in only a partial depletion of IκB-β in cardiac myocytes. Further experiments are now required to determine the functional significance of IκB-β in NF-κB regulation in these cells.

Recent studies have shown that inhibiting NF-κB activity through the use of a decoy oligonucleotide results in a significant reduction in myocardial damage following coronary artery ligation [4]. The mechanisms involved in this are currently unknown but may involve direct inhibition of NF-κB in cardiac myocytes. In this context, it is of interest to note that NF-κB activation has been demonstrated in isolated cardiac myocytes in response to hydrogen peroxide [7]. Moreover, as it has been shown that hypoxia alone does not activate NF-κB [6], suggesting that reperfusion injury and not ischemia is the primary trigger for activation. Given the potential therapeutic value of these observations, detailed experiments are now required to examine the precise signal transduction pathways involved as well as the downstream targets of NF-κB.

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