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Inhibition of interleukin-12 expression in diltiazem-treated dendritic cells through the reduction of nuclear factor-κB transcriptional activity

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Abstract

Diltiazem is a calcium channel blocker that suppresses the activation of a variety of immune cells, such as T and B cells, NK cells, monocytes and dendritic cells (DCs). It has been used in the treatment of cardiovascular disorders and has been widely included in clinical protocols to prevent rejection after kidney transplantation. In line with these data, we previously showed that diltiazem directly affects maturation of human DCs and the production of IL-12. Here, we extended our analysis studying the effect of diltiazem on the transcription of IL-12 p35 and p40 subunits focusing on the activity of nuclear factor-κB (NF-κB). A marked reduction of NF-κB binding to the κB sequences present within the p35 and p40 subunit promoters was observed in diltiazem-treated DCs following the stimulation with lipopolysaccharide (LPS) or CD40L. In order to examine the mechanisms by which NF-κB binding activity is reduced by diltiazem, we analyzed the NF-κB inhibitor, IκBα. No significant differences were observed in the phosphorylation and/or the degradation of IκBα. On the other hand, the subcellular distribution of NF-κB subunits was clearly affected in diltiazem-treated DCs following LPS stimulation, with a reduced nuclear translocation of p65, and RelB, and a nuclear accumulation of p50 subunit. Thus, all together, our data provided evidence that in addition to the inhibition of p65/p50 nuclear translocation, the selective induction and translocation of p50/p50 homodimers is an important mechanism by which diltiazem inhibits NF-κB activity, and in turn, IL-12 expression.

Keywords: NF-κB; Diltiazem; Dendritic cells; IL-12; CD40L; Lipopolysaccharide

1. Introduction

Nuclear factor-κB (NF-κB) is a transcriptional factor required for the gene expression of many inflammatory mediators and its activation is finely regulated at multiple levels. Various pharmacological agents inhibit NF-κB at different stages, including up-regulation of the inhibitory proteins IκBs, and nuclear translocation [1–3]. To date, five proteins, belonging to this family, have been identified in mammalian cells: p65 (Rel-A), c-Rel, RelB, p50 (NF-κB1) and p52 (NF-κB2). In non-stimulated cells, NF-κB dimers are maintained in the cytoplasm through interaction with IκBs. In response to cell stimulation, a multi-subunit protein kinase, the κB kinase (IKK), is rapidly activated and phosphorylates two serines in the N-terminal regulatory domains of the IκBs, causing a dissociation of the NF-κB–IκBα complex. NF-κB dimers translocate into the nucleus to activate gene transcription binding to specific DNA sequence elements (κB motifs) [4]. The classical NF-κB heterodimer, composed of p50 and p65 subunits, is a potent activator of gene expression for many costimulatory proteins and pro-inflammatory cytokines, including IL-12.

IL-12 is a potent pro-inflammatory cytokine that plays a central role in the initiation and control of cell mediated immunity [5–7]. IL-12 produced by accessory cells during early antigenic stimulation has been reported to be a powerful inducer of Th1 responses. Th1 cytokines promote both cytotoxic T lymphocytes and delayed-type hypersensitivity responses, which are considered to be the principal effector mechanisms of allograft rejection. Therefore, the

Abbreviations: DCs, dendritic cells; RPA, RNase protection assay; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; IκB, inhibitor of NF-κB

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inhibition of IL-12 production consequently blocks Th1 polarization and prolongs transplant survival [8].

Dendritic cells (DCs) are among the major producers of IL-12 and referred to as professional antigen-presenting cells, possessing the unique ability to induce primary immune responses [9,10]. During their life cycle, DCs undergo phenotypical and funtional changes corresponding to maturation. Following their activation, through their recognizing patterns from pathogens, such as lipopolysaccharide (LPS) or double stranded RNA, or inflammatory cytokines, such as TNF-α and IL-1, DCs migrate out of the tissues to reach secondary lymphoid organs, where they stimulate naive T cells. In this anatomical site, most likely, they can be further stimulated by CD40L expressed on activated T helper cells. DCs are not only critical for the induction of primary immune responses, but may also be important for the induction of immunological tolerance as well as the regulation of the type of T-cell mediated immune response [10,11]. The down-regulation of costimulatory molecules by biological agents has been shown to be beneficial in autoimmune diseases and allograft rejection [12,13]. NF-κB activation has been shown to relate to DC maturation and function [14]. Manipulation of DCs via the control of their maturation and differentiation, or the genetic engineering of these cells to express immunosuppressive molecules, offers potential for therapy of allograft rejection and autoimmune disease [15].

Pharmacological agents inhibit NF-κB at one of its activation steps, and several classes of drugs are well-known NF-κB inhibitors. Such agents include glucocorticoids, like dexamethasone and prednisone; immunosuppressants, such as cyclosporine, tacrolimus, and deoxyspergualin; and some nonsteroidal anti-inflammatory drugs, such as aspirin, sodium salicylate, and tepoxalin [16].

Diltiazem is a calcium channel blocker, which promotes the relaxation of cardiac and smooth muscle cells by inhibiting calcium influx through the channels and calcium release from intracellular stores. Several studies have shown in vitro that calcium channel blockers also suppress the activation of a variety of immune cells, such as T and B cells, NK cells, monocytes and DCs, hence proving that they act as immunosuppressant [17–22]. To this regard, diltiazem, besides its common use in the treatment of cardiovascular disorders, has been widely included into clinical protocols to prevent rejection after kidney transplantation, in association with cyclosporin A and corticosteroids [23]. In line with these data, we have previously shown that diltiazem directly affects maturation of human DCs by reducing their antigen-presenting capacity, and by decreasing their production of IL-12 [22]. Similar effects are exerted by other immunosuppressive drugs used routinely in organ transplantation, like corticosteroids, cyclosporin A and mycophenolate mofetil [3,24–26].

The aim of our study was to investigate whether the previously observed inhibitory effect of diltiazem on IL-12 production by DCs could be ascribed to the action of the drug on DNA binding and nuclear translocation of NF-κB. We found that diltiazem inhibits IL-12 production induced by LPS or CD40L, and impairs the NF-κB binding to the promoters of IL-12 p35 and p40 subunits. Furthermore, diltiazem inhibits the nuclear translocation of RelB and p65 NF-κB subunits but not that of p50.

2. Materials and methods

2.1. Chemicals and reagents

Diltiazem hydrochloride (Dilzene, Sigma-Tau) was supplied as a powder, dissolved in culture medium and used at different concentrations. Diltiazem has been used at the concentration of 10^{-4} M and added at the first day of culture.

2.2. Generation of DCs from peripheral blood of healthy donors

The method for in vitro culture of human DCs has been already described [22]. Briefly, monocytes were isolated from PBMC using CD14 microbeads and magnetic activated cell sorting (MACS; Miltenyi Biotec). The recovered monocytes, that were >95% pure as shown by flow cytometry with an anti-CD14 antibody (Pharmingen, San Diego, CA), were cultured at 5×10^5/ml in RPMI-10% FCS supplemented with 50 ng/ml GM–CSF and 1000 U/ml IL-4 (R&D System) at 37 °C at 5% CO_2 for 6 days before use. Diltiazem at 10^{-4} M was added at day 0 of culture. To induce DC maturation, 1 μg/ml of LPS from *E.coli* (Sigma Chemicals) or CD40L-transfected J558 cells (CD40L–J588/DC ratio of 1:5) were added to the cultures.

2.3. IL-12 detection

Supernatants of DC cultures were harvested at different times after the indicated treatments and stored at −80 °C. IL-12 production was detected in the culture supernatants using sandwich ELISA (R&D Systems), and results were expressed as ng/ml. Supernatants from four to five separate experiments were analyzed. The ELISA assay was carried out according to manufacturer's instructions.

2.4. RNA isolation and RNase protection assay (RPA)

RNA was extracted from DCs with RNeasy kit (Qiagen Inc.) according to the manufacturer's instructions that includes a DNaseI treatment. Five micrograms of each target RNA was then analyzed by RPA using the hCK-2 multiprobe template set (Riboquant, PharMingen). RPA was performed as previously described [27].

2.5. Cell extracts

Nuclear cell extracts were prepared as previously described [28]. Briefly, cell pellets (5×10^6) were resuspended in 1 ml of buffer A (0.5% Nonidet P-40, 10 mM EDTA, 10 mM EGTA, 10 mM KCl, 10 mM HEPES (pH 7.9) to which 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml trypsin inhibitor, and 1 μg/ml antipain were freshly added) and incubated on ice for 10 min. Nuclei were sedimented by centrifuging the lysates at $1200 \times g$ for 10 min. The nuclear pellets were resuspended in 30–40 μl of buffer C (1 mM EDTA, 1 mM EGTA, 0,4 M NaCl, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 25% glycerol, with fresh addition as above) and incubated for 10 min on ice with occasional mixing. The suspensions were clarified by centrifuging at $15{,}000 \times g$ for 10 min. The supernatants were recovered as nuclear extracts and were rapidly frozen on crushed dry ice and stored at −80 °C. Whole cell extracts were prepared as previously described [29]. Briefly, cells (10^7) were lysed in 30–50 μl of ice-cold whole cell extraction buffer (20 mM Hepes (pH 7.9), 50 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 10 mM EDTA and 2 mM EGTA, 10 μg/ml leupeptin, 100 mM NaF, 0.5 mM PMSF, 10 mM sodium orthovanadate and sodium molybdate). The lysate was incubated 30 min on a shaker at 4 °C, and insoluble debris was removed by centrifugation ($13{,}000 \times g$ at 4 °C for 10 min), and the lysate was stored at −80 °C.

2.6. Electrophoretic mobility shift assay (EMSA)

To measure the association of DNA-binding proteins with different DNA sequences, synthetic double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. For the analysis of NF-κB complexes, nuclear cell lysates (5 μg) were used in EMSA experiments. Binding reaction mixture (20 μl final volume) contained labelled oligonucleotide probes (30,000 cpm) in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), 1 μg poly(dI)–poly(dC)). Nuclear lysates were added and the reaction mixture was incubated for 30 min at room temperature. For supershift analysis, 1 μg of anti-p50 and 1 μg of anti-p65 (Santa Cruz Biotechnology Inc.) were added to the reaction. Samples were analyzed on 5% polyacrylamide gels with 0.5× TBE (1× TBE is 50 mM Tris–borate (pH 8.2), 1 mM EDTA) for 1.5 h at 200 V at 18 °C.

The oligonucleotides used were as follows: NF-κB p35 (5′-GTCCCGGGAAAGTCCTGCCG-3′) [30] and NFκB-p40 (5′-TTCTTGAAATTCCCCCAGAA-3′) [31].

2.7. Western blot analysis

Cell extracts (30 μg) were separated by 7% SDS–PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit polyclonal Abs against p50, p65, RelB and IκBα (Santa Cruz Biotechnology Inc.) and reacted with anti-rabbit horseradish peroxidase-coupled secondary antibody (Amersham) using an ECL system. The phosphorylation of IκBα was detected by immunoblotting with Ab against to Ser32 phosphorylated IκBα (Cell Signaling Technology Inc.), and after stripping, with the specific IκBα Abs to evaluate loading.

3. Results

3.1. Inhibition of IL-12 expression induced by LPS or CD40L

We have previously shown that diltiazem can reduce the production of IL-12 p70 in DCs stimulated with LPS or CD40L [32]. Here, we extended our studies investigating the mechanisms involved in the inhibition of diltiazem on the IL-12 expression. First, we performed kinetic studies to identify the timing of the inhibitory effect exerted by diltiazem. DCs were generated in GM–CSF and IL-4 in presence or absence of diltiazem for 5 days. Cell culture supernatants were collected 24 and 48 h after LPS or CD40L-transfected J588 cells. IL-12 p70 levels were determined by ELISA (Fig. 1A). A reduced IL-12 production was observed in the supernatants of diltiazem-treated cell cultures following 24 and 48 h of LPS or CD40L stimulation. This result was also investigated at the mRNA level. DCs were collected 6 h after LPS or CD40L stimulation. Total cellular RNA was isolated and cytokine gene expression was analyzed by RPA (Fig. 1B). A clear reduction of both IL-12 p35 and p40 transcripts was observed following LPS and CD40L stimulations in diltiazem-treated cells compared to the untreated cell cultures. This result indicates that the impaired secretion of IL-12 p70 observed in the supernatants of diltiazem-treated DCs is related to the inhibition of IL-12 p35 and p40 gene transcription.

3.2. Impairment of NF-κB binding to the promoters of IL-12 p35 and p40 subunits in diltiazem-treated DCs

Having found that the inhibition of both IL-12 subunit mRNAs was rapidly observed in diltiazem-treated cells, we sought to investigate its effect on NF-κB activity that represents a key transcription factor for IL-12 p40 and p35 subunit expression induced in DCs by either LPS or CD40L engagment [5]. In particular, the p50/p65 heterodimer is known to bind to the −64 to −60 region of the p35 promoter and to the −118 to −108 region of p40 promoter [30,31]. To study whether diltiazem was able to inhibit binding of NF-κB to the p35 and p40 κB regulatory sequences, DCs were prepared in the presence or absence of diltiazem and then stimulated for 1 h with LPS or CD40L. Nuclear extracts were prepared and

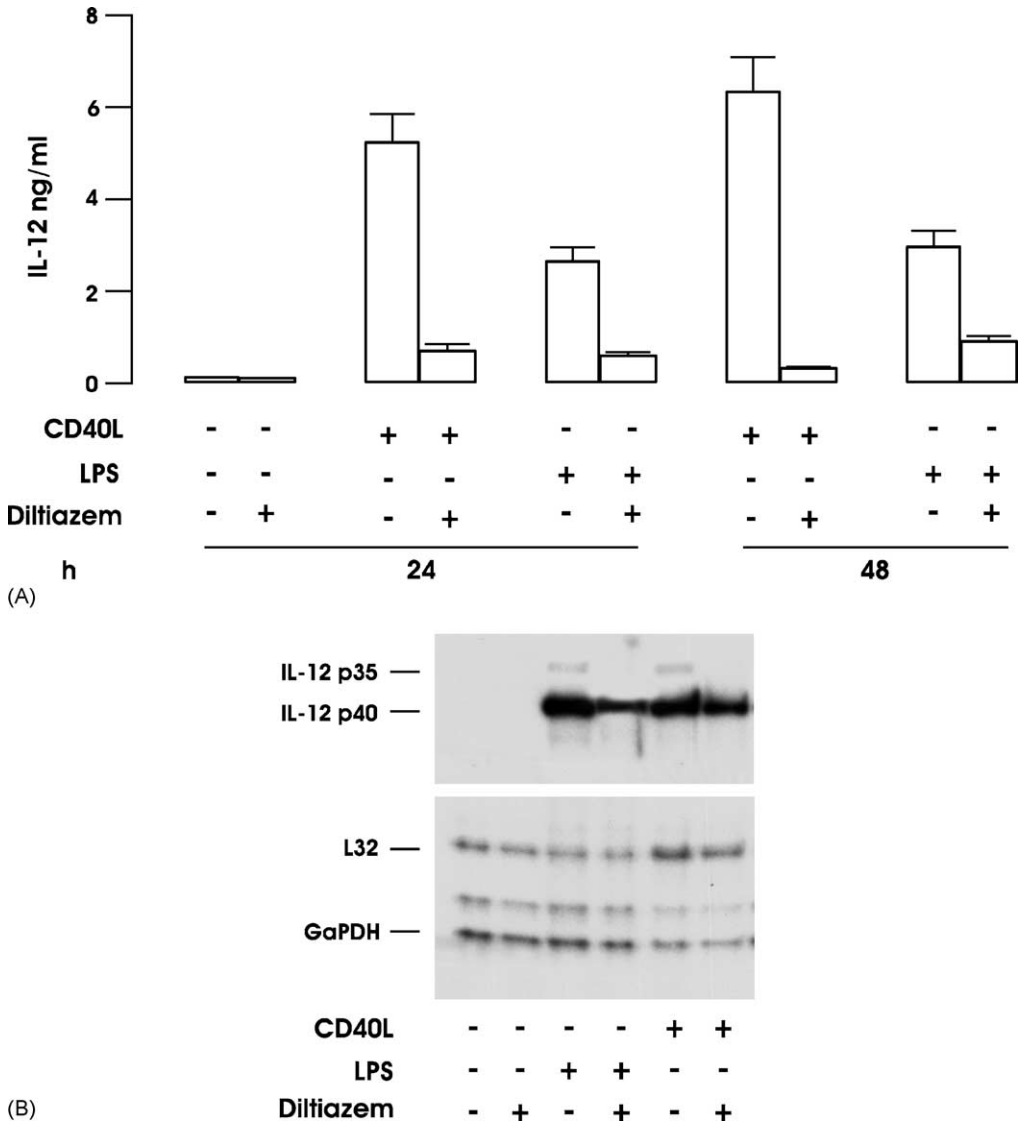

Fig. 1. Effect of diltiazem on IL-12 production in LPS or CD40L treated DCs: (A) DCs were prepared in the presence or absence of diltiazem. Cell culture supernatants were collected 24 and 48 h after LPS (1 μg/ml) or CD40L-transfected J558 cells (CD40L-J558/DC ratio of 1:5) treatment and the levels of IL-12 p70 were determined by ELISA. (B) DCs were collected 6 h following LPS or CD40L stimulation. Total cellular RNA (5 μg) was isolated and analyzed by RPA. This is a representative RPA experiment, which was repeated for an additional three times with RNA extracted from different DC preparations.

analyzed by EMSA using oligonucleotides corresponding to the κB sites present within the p35 and p40 promoters (Fig. 2A and B). A clear induction of NF-κB DNA binding activity to the p35 and p40 κB regulatory sequences was observed in LPS- or CD40L-treated DCs. A reduced formation of p50–p65 heterodimer was found in diltiazem-treated DCs, whereas a discrete complex, corresponding to p50–p50 homeodimer as demonstrated by supershift experiments using antibodies raised against the p50 subunit, was induced in diltiazem-treated DCs following LPS or CD40L stimulation. No binding of RelB subunit was observed since the addition of antibodies against this subunit did not modify the binding pattern in EMSA (data not shown).

3.3. Reduced nuclear translocation of RelB and p65 NF-κB subunits but not that of p50 in diltiazem-treated DCs

In order to investigate the mechanisms involved in the inhibition of NF-κB binding in diltiazem-treated cells, we focused our attention on the inhibitor IκBα, which keeps NF-κB in an inactive state by sequestring it in the cytoplasm [33]. After inducer-mediated stimulation, IκBα is phosphorylated within the N-terminal domain at Ser-32 and Ser-36 [34–36] by the IKK [37–40], ubiquitinated, and subsequently degraded by the 26S proteasome [41] leading to the release and translocation of NF-κB to the nucleus, and binding to κB sites. To experimentally address the question whether diltiazem inhibits nuclear translocation

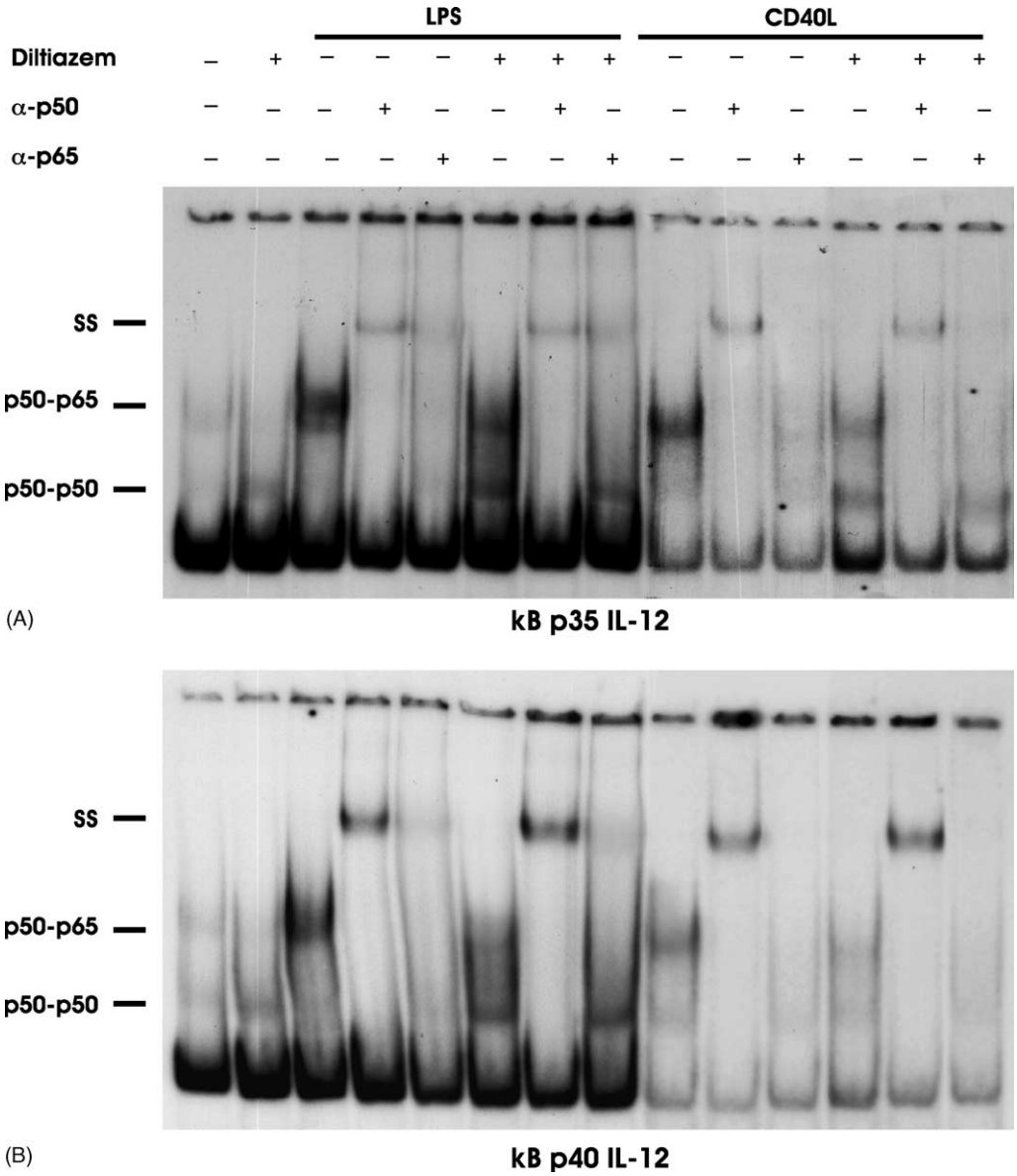

Fig. 2. Diltiazem reduces NF-κB binding to the promoters of IL-12 p35 and IL-12 p40 subunits. DCs, prepared in the presence or absence of diltiazem, were treated with LPS or CD40L-transfected J558 and collected after 1 h. Nuclear extracts (15 μg) were analyzed by EMSA using a specific radiolabeled oligonucleotide corresponding to the κB motif present within the IL-12 p35 promoter (A) or within IL-12 p40 promoter (B). Supershift assay (SS) was performed with anti-p50 (α-p50) and anti-p65 (α-p65), as indicated.

of NF-κB by modulating the phosphorylation and/or the degradation of IκBα, DCs were prepared in presence or absence of diltiazem and then stimulated with LPS at the indicated time points. The extent of IκBα phosphorylation and degradation was, therefore, analyzed in LPS-treated DCs. A short kinetic of treatment from 15 min to 3 h (Fig. 3) was performed, and total extracts were analyzed by immunoblot using specific antibodies recognizing the native and phosphorylated IκBα forms (Fig. 3). No differences were observed in the kinetic of phosphorylation and degradation of IκBα following LPS stimulation both in control and diltiazem-treated cells. Beginning at 30 min–1 h post-LPS treatment, the phosphorylated IκBα was detected (Fig. 3, upper panel), while at 3 h, the phosphorylated form decreased both in control and diltiazem-treated cells. A similar pattern of phosphorylation-dependent degradation of IκBα was observed in control or in diltiazem-treated cells (Fig. 3, lower panel) indicating that diltiazem does not affect the events leading to IκBα phosphorylation/degradation.

To elucidate whether diltiazem affected the subcellular distribution of NF-κB p50 and p65 subunits, Western blot analysis was performed with cytoplasmic and nuclear extracts of DCs prepared in the presence or absence of diltiazem that had been treated with LPS for 1 h (Fig. 4). We extended our analysis also to RelB, since it has been demonstrated that this subunit is important for DC maturation [42,43]. In untreated cells, RelB, p65, and p50 were

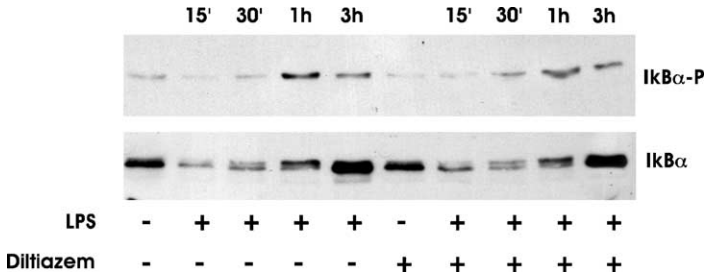

Fig. 3. Diltiazem does not affect the phosphorylation and/or the degradation of IκBα. DCs were stimulated with LPS in the presence or absence of diltiazem and collected at the indicated time points. Whole cell lysates (30 μg) were separated on a 12% SDS–PAGE and blotted sequentially with the indicated antibodies to evaluate both IκBα phosphorylation (upper panel) and protein content (lower panel).

predominantly localized in the cytoplasm (Fig. 4, right panels in whole extracts). Moreover, the expression of cytoplasmic RelB, p65, and p50 was not visibly affected by diltiazem, LPS alone, or in combination with diltiazem (Fig. 4, left panels in whole extracts). As expected, cells stimulated with LPS showed an increase in nuclear translocation of RelB, p65, and p50 (Fig. 4, right panels in nuclear extracts). In contrast, while LPS alone and in combination with diltiazem clearly augmented nuclear p50, it did not visibly induce nuclear translocation of p65, and RelB even though degradation of IκBα had been observed. Moreover, diltiazem treatment alone clearly induced nuclear translocation of p50 in DCs. These results well correlated with the increased binding of p50 homodimers observed in EMSA experiments shown in Fig. 2 and suggested that diltiazem inhibits LPS-induced nuclear translocation of p65 and RelB while inducing the nuclear accumulation of p50.

4. Discussion

Diltiazem is a drug that exerts its biological effect through the capability to block calcium channels in different cell types. Due to its anti-nephrotoxic and anti-hypertensive effects, it has been often included in clinical protocols for preventing acute graft rejection after kidney transplantation [23]. A lot of evidences has shown diltiazem's ability to induce direct immunosuppression on different immune cells [17–19]. The latter also modulates in vitro alloreactivity when used in combination with cyclosporin A and corticosteroids, besides exibiting modulatory effect on antigen presenting cells [20]. In particular, we have previously demonstrated that DC functions are modified by diltiazem through its marked down-regulation of antigen presenting and costimulatory molecules, and inhibition of IL-12 production [22]. Given the role played by IL-12 in host defense, the comprehension of IL-12 regulation may lead to the development of strategies for the treatment of inflammatory diseases and several immune disorders. In this paper we, therefore, studied the effect of diltiazem on transcriptional events leading to the production of IL-12 by human DCs stimulated by two different inducers, such as the Toll-like receptor agonist LPS and the co-stimulatory molecule CD40L, the best characterized and potent inducers of proinflammatory and immunoregulatory cytokines from DCs [5].

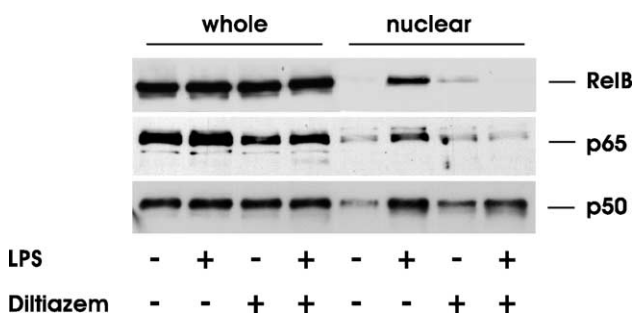

Fig. 4. Diltiazem inhibits nuclear translocation of RelB and p65 but not that of p50. DCs were prepared in the presence or absence of diltiazem and treated with LPS for 1 h. Whole and nuclear extracts were subjected to immunoblot analysis with anti RelB, α-p65 and α-p50.

Once evaluated that diltiazem inhibits the mRNA expression of both p35 and p40 IL-12 subunits, we investigated whether this inhibitory effect was exerted on the binding of NF-κB, since both subunits are regulated by this transcription factor [30,31]. We found that the binding of p65–p50 heterodimer was significantly reduced in DCs treated with LPS or CD40L in the presence of diltiazem. On the contrary, we observed a slight increase in the binding of p50–p50 homodimer to IL-12 p35 and p40 κB sequences.

A variety of drugs can inhibit the activation of NF-κB at different steps. These agents include many immunosuppressants, such as corticosteroids, cyclosporine, tacrolimus and deoxyspergualin [2]. Two different mechanisms have been proposed for the inhibition of NF-κB by glucocorticoids. The first possible mechanism is that glucocorticoids inhibit NF-κB activation by enhancing the production of IκBα; the second feasible one is that glucocorticoids inhibit NF-κB through a direct protein–protein interaction. The mechanism of action of cyclosporine and tacrolimus is known to involve the inhibition of nuclear translocation of the nuclear factor of activated T cells, while deoxyspergualin appears to exert its immunosuppressive activity, at least in part, by inhibiting NF-κB nuclear translocation

[16]. Moreover, the inhibition of IL-12 production by 1,25-dihydroxyvitamin D_3, a hormone with efficient immunosuppressive properties, has been reported in monocytes and DCs [44].

Conversely, the mechanism underlying the inhibitory effect of diltiazem on IL-12 expression has not been identified so far. Although possible alterations of the IκBα posphorylation and degradation in diltiazem-treated DCs were investigated, no differences were found. We also observed that the transient degradation of IκBα correlated with a preferential nuclear translocation of p50 subunit, indicating that diltiazem may favor the formation of p50–p50 homodimers. Indeed, a reduced nuclear translocation of RelB and p65 subunits was observed suggesting that the transcription of genes dependent from these factors could be significantly impaired in diltiazem-treated DCs. In addition, considering the published data that the homodimeric complexes of p50–p50 or p52–p52 are associated with transcriptional repression [45–49], one may assume that in DCs diltiazem might reduce IL-12 expression, as well as the expression of other genes, promoting the formation of the inhibitory p50–p50 complex. Furthermore, taking into account that the subunit p40 together with the subunit p19 forms IL-23, one may hypothesize that diltiazem could also inhibit the production of this new cytokine that has been described to modulate the proliferation of memory T cells and their IFN-γ production [50]. Studies are in progress to evaluate this aspect in view of the role that memory T cells specific for alloantigens play in early and late graft rejection [51].

A possible involvement of IL-10 was also considered in view of a recent report by Driessler et al. describing the ability of this anti-inflammatory cytokine to induce selectively the nuclear translocation and DNA-binding of the repressive p50–p50 homodimers [52]. In our experimental system, no effect on IL-10 production was observed following diltiazem treatment (data not shown), thus suggesting that the inhibition of IL-12 expression is not a result of a general dampening of cellular activation induced generally by IL-10 [53].

Taken together, our results are of particular interest in light of the observations that the most important procedures of allograft rejection, including ischemia/reperfusion injury, recipient T cell activation and DC maturation, are regulated by NF-κB [54]. The identification of NF-κB as a key factor for the pathogenesis of allograft rejection suggests that NF-κB-targeted therapeutics, such as diltiazem, might be effective in transplantation.

Acknowledgments

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