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Differential usage of IκBα and IκBβ in regulation of apoptosis versus gene expression

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Abstract

In this study we use the N-substituted benzamides declopramide (3-CPA) and *N*-acetyl declopramide (Na-3-CPA) to investigate the involvement of the transcription factor NF-κB in the induction of apoptosis and surface immunoglobulin κ (Igκ) expression in the mouse pre-B cell line 70Z/3. We first showed that 3-CPA-induced apoptosis at doses around 500 μM and that the 3-CPA-induced apoptosis could be suppressed by over-expression of the Bcl-2 protein. Na-3-CPA was shown to be non-apoptotic at doses up to 1–2 mM. On the other hand, Na-3-CPA inhibited LPS-induced Igκ expression while 3-CPA had no effect. Further analysis showed that while 3-CPA inhibited breakdown of IκBα, Na-3-CPA inhibited breakdown of IκBβ. In addition, we used a 70Z/3 cell line expressing a dominant negative IκBα (70Z/3$^{\Delta N I\kappa B\alpha}$). The 70Z/3$^{\Delta N I\kappa B\alpha}$ cell line was shown to be more sensitive to apoptosis and cytotoxicity induced by 3-CPA as well as by LPS, probably due to a defect in NF-κB rescue mechanism. Taken together, our data implicate distinct roles for IκBα and IκBβ in regulating various NF-κB activities.

Keywords: N-substituted benzamides; NF-κB; IκB; Apoptosis

Apoptosis, or programmed cell death, is a process that is important during the development of an organism as well as for adult tissue homeostasis. During apoptosis, the cells go through a series of morphological and biochemical changes, resulting in clearance of the apoptotic cells (reviewed in [1]). The biochemical events involved in apoptosis are to a large extent carried out by caspases, cysteine-aspartate proteases that cleave their substrates after specific aspartic acid residues [2]. Apoptosis can be induced via several pathways. One major pathway is through engagement of so-called death receptors on the cell surface. The two best-characterized death receptors are TNFR1 that binds TNFα, and Fas (CD95) that binds the Fas ligand (FasL, CD95L) [3]. Binding of the ligands to the respective receptors induces recruitment of death domain proteins such as TNF-associated death domain (TRADD), Fas-associated death domain protein (FADD), receptor-interacting protein 1 (RIP1), and TNF-receptor-associated factor 2 (TRAF2) [4,5]. In addition, the bacterial cell wall component LPS has been shown to induce apoptosis via FADD by binding to a death domain-containing membrane receptor [6]. Accumulation of the death domain proteins at the receptor intracellular region recruits pro-caspase-8 to the complex, and clustering of several pro-caspases-8 leads to their activation by self-cleavage [7–10]. The activated caspase-8 activates the effector caspases-3, -6, and -7 by cleavage [11] and the latter caspases subsequently cleave their protein substrates and execute the apoptotic process.

Apoptosis can also be initiated via the mitochondrial pathway. Several stimuli induce mitochondria-mediated apoptosis, including chemotherapeutic drugs, reactive oxygen species, UV- and γ-irradiation, and environmental stress (reviewed in [12]). In response to these signals, the mitochondria releases several apoptotic factors, one of which is cytochrome *c*. Cytochrome *c* forms together with APAF-1 and pro-caspase-9 a complex, the

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apoptosome, that converts pro-caspase-9 into active caspase-9 [13]. Activated caspase-9 subsequently activates the effector caspases and apoptosis is executed [14]. Triggering of apoptosis via the mitochondrial pathway is mainly regulated by members of the Bcl-2 family of proteins [1,15]. Anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-x_L, form homo- or heterodimers with other anti-apoptotic proteins or with pro-apoptotic members of the family, like Bax and Bid [16]. The balance between pro- and anti-apoptotic Bcl-2 proteins determines whether apoptosis will be triggered or not.

Apoptosis is also regulated by the NF-κB proteins. NF-κB is a family of ubiquitously expressed transcription factors that regulate multiple cellular processes [17,18]. In unstimulated cells, NF-κB is retained in the cytoplasm associated with the inhibitory IκB proteins. A wide variety of stimuli can induce NF-κB activity (reviewed in [19]). Most of the stimuli converge at the point where the IκB proteins, most notably IκBα and IκBβ, are phosphorylated at two N-terminal serine residues by the IκB kinases (IKKs) [19]. Phosphorylation of the IκB proteins targets them for ubiquitination and degradation by the proteasomes. Numerous studies have shown that inhibition of NF-κB activity renders cells more sensitive to apoptosis [20–25]. In general, rapid induction of NF-κB activity rescues the cell from apoptosis by the induction of anti-apoptotic genes that inhibit apoptosis at several levels.

We have previously shown that the N-substituted benzamides metoclopramide (MCA), and declopramide (3-CPA) induce apoptosis in the mouse pre-B cell line 70Z/3, and that the level of apoptosis can be reduced by addition of caspase inhibitors or by over-expression of the Bcl-2 protein [26,27]. We have also shown that N-substituted benzamides can inhibit NF-κB activity monitored by Igκ expression on 70Z/3 cells or as TCR/CD28-induced NF-κB-reporter activity in Jurkat cells [27,28]. In this study we investigated the effects of N-substituted benzamides and LPS on NF-κB activities in wild-type 70Z/3 cells and 70Z/3 cells expressing a N-terminal truncated form of IκBα (70Z/$3^{\Delta NI\kappa B\alpha}$). This dominant negative IκBα sequesters NF-κB in the cytoplasm and thereby inhibits NF-κB activity and consequently suppresses the NF-κB-driven apoptotic rescue pathway [21,29,30]. We have also used a 70Z/3 cell line that over-expresses the anti-apoptotic gene Bcl-2 and hence is less sensitive to induction of apoptosis [26]. By using these cell lines we could show that 3-CPA and Na-3-CPA affect NF-κB activity and apoptosis at distinct levels and that this phenomenon can be explained by a differential usage of IκBα and IκBβ.

Materials and methods

Chemicals. Metoclopramide (MCA; Neu-Sensamide, metoclopramide hydrochloride monohydrate), 3-chloroprocainamide (3-CPA; Declopramide, 3-chloroprocainamide hydrochloride), *N*-acetyl-3-chloroprocainamide (Na-3-CPA), and procainamide (PA) were custom synthesized by Oxigene Europe AB (Lund, Sweden) and prepared as hydrochloride salt solutions in phosphate buffered NaCl (0.9%). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue) and 7AAD (7-amino-actinomycin D) were purchased from Sigma–Aldrich (St. Louis, MO). Annexin V was purchased from Molecular Probes (Leiden, The Netherlands). The Bcl-2 antibody was purchased from BD Pharmingen (San Diego, CA, USA), anti-IκBα and anti-IκBβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-Igκ and anti-actin antibodies were from Sigma–Aldrich.

Cells and cell culturing. The murine 70Z/3 pre-B-cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, gentamicin (50 μg/ml), glutamine (2 mM), Na-pyruvate (2 mM), β-mercaptoethanol (50 μM), and Hepes-buffer (20 mM) (all from Life Technologies AB, Täby, Sweden) at 37 °C, 5% CO_2, and 80% humidity. The mutant 70Z/3 cells were stably transfected with cDNA for mouse Bcl-2 (70Z/$3^{Bcl\text{-}2}$) as previously described [26] or with cDNA for IκBα (with truncated aa 1–36) cloned into a pCMV4 plasmid containing a CMV promoter and a neomycin (G418) resistant site (70Z/$3^{\Delta NI\kappa B\alpha}$) [29]. Electroporation (960 μF and 240 V) was performed on 10×10^6 cells and stable 70Z/$3^{\Delta NI\kappa B\alpha}$ transfectants were cloned by limiting dilution under selection by 1.0 mg/ml G418.

Flow cytometry analysis. The 70Z/3 cell line contains a silent but functionally rearranged Igκ locus that is transcribed when the NF-κB transcription factor is translocated to the nucleus [31,32]. Both Igκ expression and apoptosis were measured by flow cytometry (FACScan, BD Pharmingen). Apoptotic cells were identified as cells that excluded the vital dye 7AAD but were positive to Annexin V (Alexa Flour 488). For apoptosis and Igκ measurements, the cell density was adjusted to 150,000 cells/well at the day before the experiment.

MTT assay. The anti-proliferative and cytotoxic potencies of the N-substituted benzamides were examined by MTT staining (1 μg/ml) for 2 h in 100 μl cell cultures grown in 96-well round bottomed microtiter plates, three days after addition of the different drugs [33]. Cell growth in drug-treated cells was expressed as percent of untreated control cells.

SDS–PAGE/Western blot analysis. SDS–PAGE and Western blot analysis were performed on the 10,000*g* supernatant fraction of total soluble proteins prepared from 5×10^6 cells as previously described [34]. Equal amounts of protein (30–60 μg) were loaded in each lane. Electrophoresis was performed on a 12% polyacrylamide gel (150 V/60 mA). Protein transfer to a nylon membrane (Hybond-C Extra; Amersham Pharmacia, Uppsala, Sweden) was performed at 15 V/200 mA for 30 min. After blocking overnight in 5% dried fat-free milk in TBST (10 mM Tris–HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20), incubation with a primary antibody for 2 h was performed. The membranes were washed three times in TBST, whereafter incubation with either a secondary (HRP)-conjugated anti-mouse (Santa Cruz) or anti-rabbit antibody (Amersham Pharmacia) was performed, followed by detection of chemiluminescence using ECL-reagent (Amersham Pharmacia).

Results

Inhibition of NF-κB enhances 3-CPA-induced apoptosis and cell death while over-expression of Bcl-2 protects only from early apoptosis

We have previously shown that the N-substituted benzamides MCA and 3-CPA-induced apoptosis in the mouse pre-B cell line 70Z/3 [27]. We have also shown that benzamide-induced apoptosis could be reduced by addition of caspase inhibitors or by over-expression of the

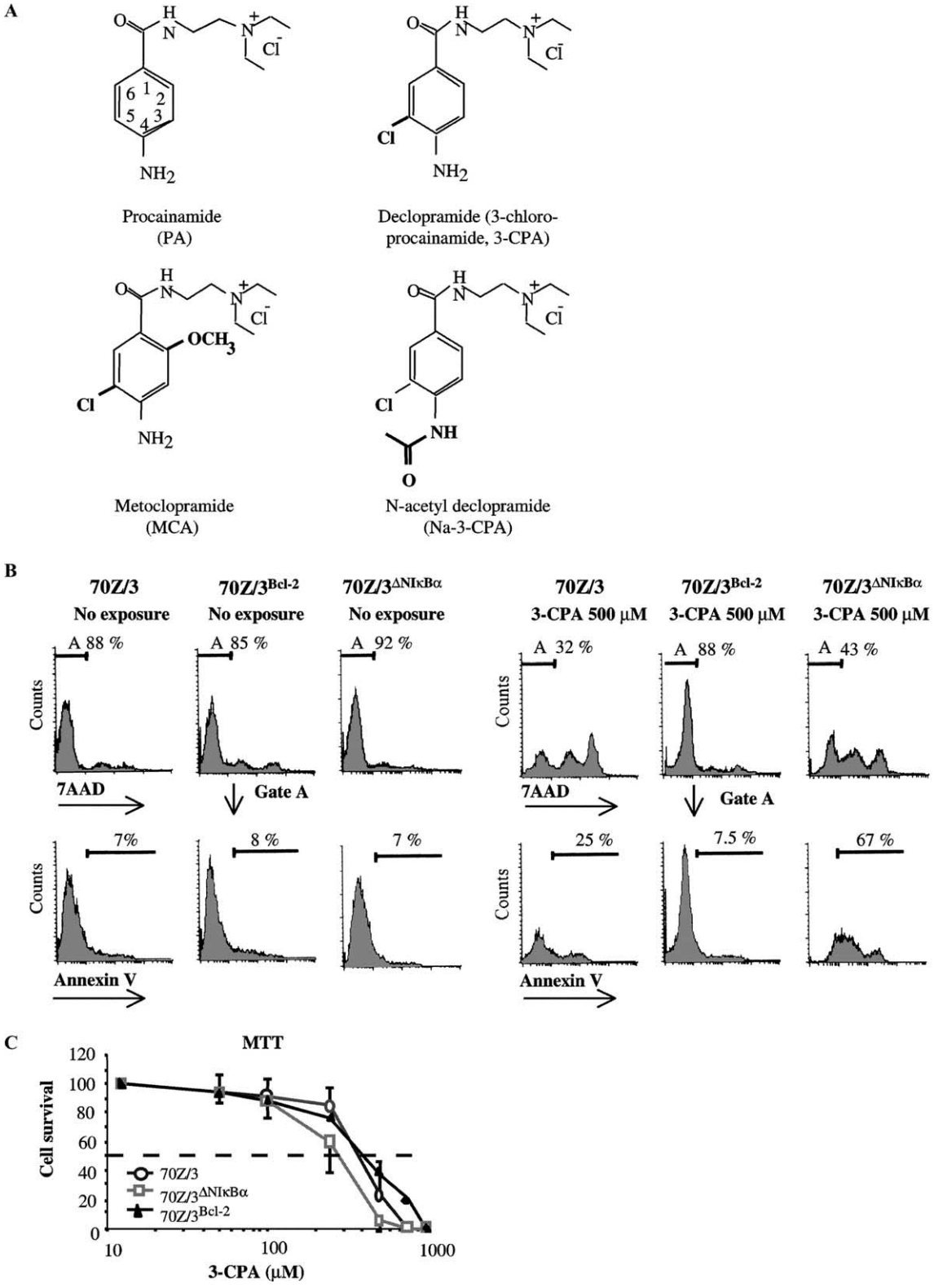

Fig. 1. 3-CPA-induced apoptosis and cell death in wild-type 70Z/3 cells compared to 70Z/3^{Bcl-2} and 70Z/3$^{\Delta NI\kappa B\alpha}$ transfectants. (A) Structures of the different benzamides used in this study. (B) 3-CPA (500 mM) was added to wild-type 70Z/3 cells or to 70Z/3$^{\Delta NI\kappa B\alpha}$ or 70Z/3^{Bcl-2} cells. Apoptosis was determined as percent Annexin V^{+} cells out of gated 7AAD-cells after 18 h of exposure to 3-CPA. (C) Cell survival after three days of exposure to 10–1000 mM of 3-CPA was determined for the different cell lines by the MTT assay as described in Materials and methods. Cell survival for each cell line is presented as percent live cells of unexposed control cells.

Bcl-2 protein [26]. Furthermore, 3-CPA and Na-3-CPA have been shown to inhibit NF-κB activity [27,28]. Since NF-κB activation and apoptosis are coupled events, we decided to use N-substituted benzamides (Fig. 1A) to probe these processes. To this end we generated a NF-κB variant cell line by introducing a dominant negative IκBα [29,30] into the 70Z/3 cells (70Z/$3^{\Delta NI\kappa B\alpha}$). Expression of the truncated IκBα has been shown to prevent the cytoplasmic p65/Rel-unit from nuclear localization, thereby inhibiting NF-κB activation and the NF-κB-driven apoptotic rescue pathway [21]. As a reference, we used a 70Z/3 cell line that over-expressed the Bcl-2 protein (70Z/$3^{Bcl\text{-}2}$), which was previously shown to be resistant against 3-CPA-induced apoptosis [26]. The variant cell lines were incubated with 500 μM of 3-CPA for 18 h and then analyzed for induction of apoptosis by staining with the vital dye 7AAD and Annexin V followed by FACS analysis. Under these conditions apoptosis was induced in 70Z/3 wild-type cells, while the cell line with a perturbed NF-κB activation pathway (70Z/$3^{\Delta NI\kappa B\alpha}$) was even more sensitive to 3-CPA-induced apoptosis at 18 h compared to wild-type 70Z/3 cells (Fig. 1B). In contrast, the level of apoptosis was reduced in 70Z/3 cells where the Bcl-2 protein was over-expressed (70Z/$3^{Bcl\text{-}2}$ cells) (Fig. 1B).

Extending the analysis and measuring cell death also after three days of exposure to 3-CPA, the 70Z/$3^{Bcl\text{-}2}$ cells could not be significantly distinguished from wild-type 70Z/3 cells (IC_{50}-values: 402 ± 91 mM vs. 494 ± 220 mM for 70Z/3 and 70Z/$3^{Bcl\text{-}2}$, respectively; Fig. 1C and Table 1). However, the 70Z/$3^{\Delta NI\kappa B\alpha}$ cells showed significantly increased cell death compared to wild-type 70Z/3 cells after three days of incubation with 3-CPA (IC_{50}-value: 274 ± 80 μM) (Fig. 1C and Table 1); indicating that the NF-κB signaling pathway is involved in the regulation of both early and delayed cell death after an apoptotic stimulus.

Similar effects on induction of apoptosis and survival as observed with 3-CPA were also observed after the addition of MCA, whereas Na-3-CPA and PA were poor inducers of apoptosis and did effect only the survival of 70Z/$3^{\Delta NI\kappa B\alpha}$ cells at high doses (Table 1). We conclude from these results that early apoptosis induced by N-substituted benzamides can be modulated by both Bcl-2 and NF-κB signals, while delayed cytotoxicity is modulated only by interference with NF-κB signaling.

Table 1
IC_{50} values for wild-type 70Z/3 70Z/$3^{\Delta NI\kappa B\alpha}$ cells after addition of the different benzamides as indicated

	IC_{50}-values		
	70Z/3	70Z$3^{Bcl\text{-}2}$	70Z/$3^{\Delta NI\kappa B\alpha}$
3-CPA	402 ± 9	$494 \pm 220^{**}$	$274 \pm 80^{*}$
MCA	600 ± 237	$604 \pm 204^{**}$	$354 \pm 140^{*}$
Na-3-CPA	950 ± 269	$878 \pm 256^{**}$	$525 \pm 85^{*}$
PA	1900 ± 141	1800 ± 141	1200 ± 12

The benzamides were added in concentration of 10–200 μM for three days before MTT assays were performed as described in Materials and methods. Values are calculated as 50% growth inhibition compared to untreated control cells.

[*] Significantly different from 70/Z3 wild-type cells, $p < 0.05$ (Student's t test).

[**] Significantly different from 70/Z3 cells, $p < 0.05$.

3-CPA and Na-3-CPA inhibit distinct functions of LPS-induced NF-κB activation

Na-3-CPA has previously been shown to inhibit LPS-induced cell surface Igκ expression by preventing breakdown of IκBβ and subsequent NF-κB activation [27]. Since NF-κB is important for cell survival after stimulation with TNFα, we wanted to investigate the effects of 3-CPA and Na-3-CPA on NF-κB-mediated salvage from apoptosis after LPS stimulation of 70Z/3 cells. The cells were co-incubated with LPS and respective compound for 18 h, after which they were stained with 7AAD and Annexin V to measure apoptosis, and with anti-κ antibodies to measure NF-κB activity as previously described [31,32]. Addition of Na-3-CPA at doses up to 1 mM did not affect LPS-induced apoptosis and cytotoxicity after 18 h defined as Annexin V^+/$7AAD^-$ cells under these conditions (Fig. 2B), even though Igκ expression, and hence NF-κB activation, was completely inhibited in the same cultures ($Ig\kappa^+$/$7AAD^-$, Fig. 2A). In contrast, LPS-induced Igκ expression on total or gated live 70Z/3 cells ($7AAD^-$) cells was marginally affected by addition of 3-CPA at 1 mM (Fig. 2A), whereas LPS-induced apoptosis and cytotoxicity was significantly increased (Fig. 2B). These results suggest that Na-3-CPA and 3-CPA may both

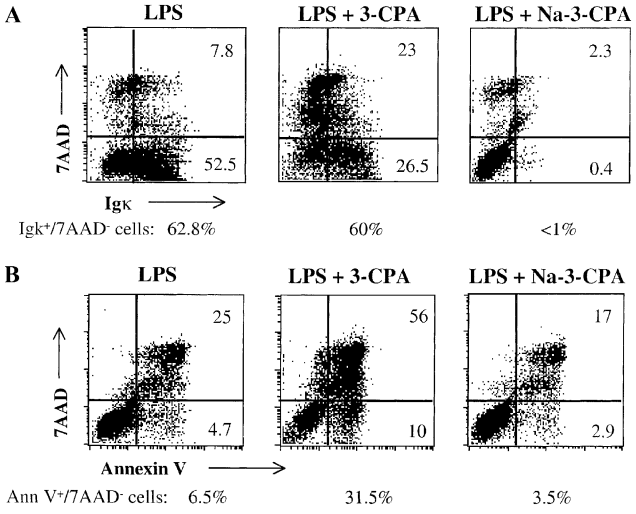

Fig. 2. The effects of 3-CPA and Na-3-CPA on LPS-induced Igκ expression and apoptosis in 70Z/3 cells. 70Z/3 cells were pre-incubated with 1 mM of 3-CPA or Na-3-CPA for 2 h before stimulation with LPS (25 μg/ml) for 18 h. (A) Igκ expression on the surface, detected by staining with an antibody against Igκ. 7AAD was included to detect dead cells. (B) Apoptotic cells, defined as cells positive for Annexin V but negative for 7AAD.

influence NF-kB activation but at discrete steps in the activation pathway.

3-CPA inhibits breakdown of IκBα while Na-3-CPA inhibits breakdown of IκNβ

To investigate the differences between Na-3-CPA and 3-CPA in NF-κB activation in detail we prepared cytosolic extracts from 70Z/3 cells exposed to LPS for 0.5–6 h, with or without the addition of 1 mM 3-CPA or Na-3-CPA. These extracts were subsequently analyzed by Western blotting using antibodies towards IκBα or IκBβ. The results showed that while 3-CPA inhibited IκBα breakdown (Fig. 3A), Na-3-CPA did not (Fig. 3B). On the other hand, while 3-CPA had no effect on IκBβ breakdown, Na-3-CPA inhibited breakdown of IκBβ at 1 mM and even more profoundly at 2 mM (Figs. 3C and D). Thus, these biochemical data support the conclusion from the cellular experiment above that the N-substituted benzamides interfere with NF-κB activation at distinct levels. While Na-3-CPA inhibits breakdown of IκBβ and LPS-induced Igκ expression, 3-CPA inhibits breakdown of IκBα and the transient initial activation of NF-κB that leads to rescue of 70Z/3 cells from apoptosis after LPS stimulation.

70Z/3$^{\Delta NI\kappa B\alpha}$ cells are more sensitive to LPS-induced apoptosis but do not show reduced Igκ expression

To further study the ability of NF-κB to exert different functions depending on breakdown of different IκB proteins, we decided to explore the 70Z/3$^{\Delta NI\kappa B\alpha}$ cell line. We started by comparing breakdown of IκBα in wild-type 70Z/3 cells and 70Z/3$^{\Delta NI\kappa B\alpha}$ cells after stimulation with LPS at different time points (Fig. 4). Western blot analysis of cytosolic fractions from the different cell lines showed that a partial LPS-induced breakdown of

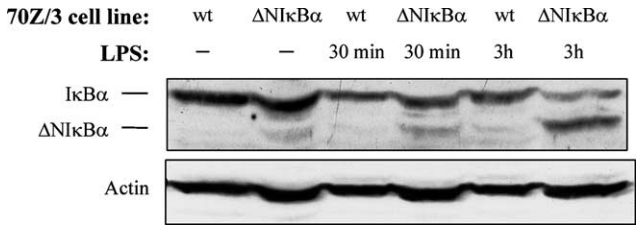

Fig. 4. Western blot on IκBα in wild-type 70Z/3 and 70Z/30$^{\Delta NI\kappa B\alpha}$ cell lines after LPS stimulation for different time points. IκBα was detected by Western blot in cytosolic 70Z/3 or 70Z/30$^{\Delta NI\kappa B\alpha}$ extracts after no stimulation or LPS-stimulation (25 μg/ml) for 30 min or 3 h. The membranes were re-probed with an antibody against actin for loading control.

endogenous IκBα occurred within 30 min both in wild-type 70Z/3 and 70Z/3$^{\Delta NI\kappa B\alpha}$ cells. However, while resynthesis of endogenous IκBα was similar in wild-type 70Z/3 cells and 70Z/3$^{\Delta NI\kappa B\alpha}$ cells after 30 min, it was shifted towards the truncated form after 3 h of incubation in 70Z/3$^{\Delta NI\kappa B\alpha}$ cells. The N-terminal truncated IκBα was expressed at rather low levels in unstimulated 70Z/3$^{\Delta NI\kappa B\alpha}$ cells, and the levels seen after LPS stimulation were significantly higher, suggesting that the dominant negative IκBα protein might influence LPS stimulated 70Z/3$^{\Delta NI\kappa B\alpha}$ cells more than unstimulated controls.

We subsequently proceeded to analyze functionally NF-κB dependent Igκ expression on wild-type 70Z/3 and 70Z/3$^{\Delta NI\kappa B\alpha}$ cells after LPS stimulation. Igκ expression on viable (7AAD$^-$) 70Z/3$^{\Delta NI\kappa B\alpha}$ cells was only marginally reduced by stimulation with LPS (Fig. 5A), whereas LPS-induced apoptosis and cytotoxicity was increased around threefold (Fig. 5B). Thus, 70Z/3$^{\Delta NI\kappa B\alpha}$ cells were more sensitive to LPS-induced apoptosis. Furthermore, the fraction of 7AAD$^+$/Igκ$^+$ cells was increased threefold in 70Z/3$^{\Delta NI\kappa B\alpha}$ cells after LPS stimulation compared to wild-type controls (Fig. 5A). This observation confirms the data in Fig. 4 that the domi-

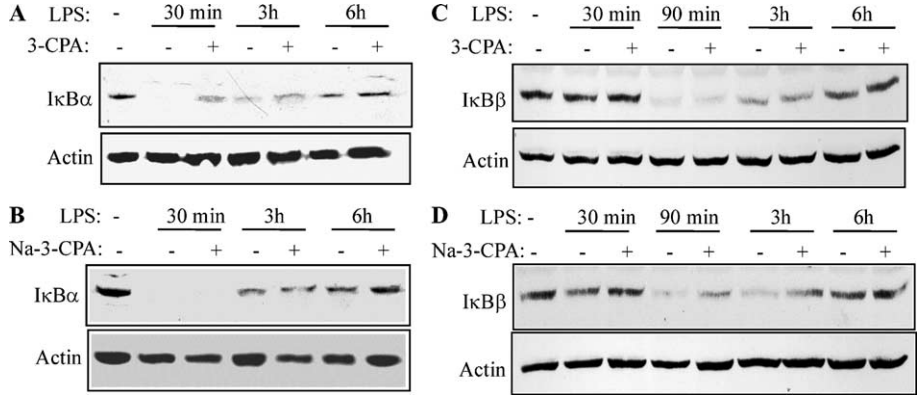

Fig. 3. 3-CPA inhibits LPS-induced IκBα breakdown while Na-3-CPA inhibits LPS-induced IκBβ breakdown. IκBα was detected by Western blot in 70Z/3 cytosolic extracts after LPS-stimulation (25 μg/ml) for 30 min, 3 or 6 h and (A) pre-incubation with 1 mM of 3-CPA or (B) pre-incubation with 1 mM Na-3-CPA for 2 h before LPS-stimulation. IκBβ was detected by Western blot in 70Z/3 cytosolic extracts after LPS-stimulation (25 μg/ml) for 30 min, 90 min, 3 h or 6 h and (C) pre-incubation with 1 mM of 3-CPA or (D) pre-incubation with 2 mM Na-3-CPA for 2 h before LPS-stimulation. Each membrane was re-probed with an antibody against actin for loading control.

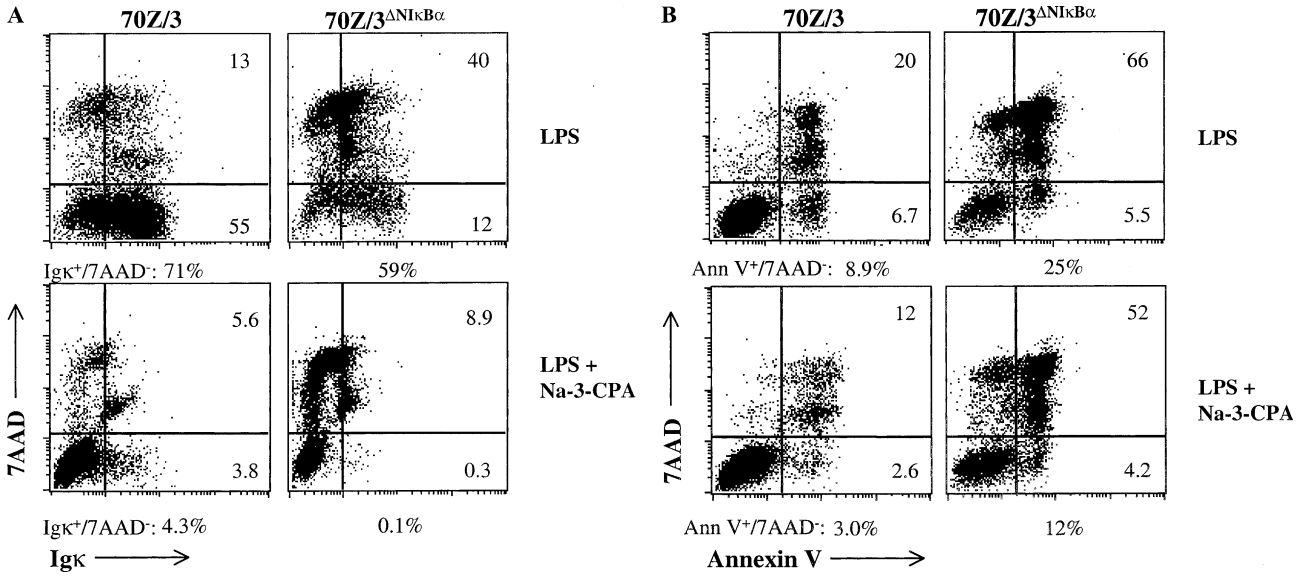

Fig. 5. Z/30$^{\Delta NI\kappa B\alpha}$ cells show increased cell death without inhibition of Igκ expression after LPS-stimulation. (A) Igκ expression was detected by FACS on wild-type and 70Z/30$^{\Delta NI\kappa B\alpha}$ cells after 18 h of LPS-stimulation (25 μg/ml). Na-3-CPA (1 mM) was added 2 h before stimulation. (B) Apoptosis detected by staining with Annexin V and 7AAD after 18 h of LPS-stimulation (25 μg/ml) on wild-type and 70Z/30$^{\Delta NI\kappa B\alpha}$ cells. Na-3-CPA (1 mM) was added 2 h before stimulation.

nant negative IκBα protein shows a higher level of expression in LPS-stimulated 70Z/3$^{\Delta NI\kappa B\alpha}$ cells. Thus, NF-κB-mediated rescue from apoptosis can be inhibited, while induction of Igκ expression mediated by the same transcription factor complex is intact in the same target cells. To further investigate the interrelationship between the two NF-κB activation pathways we treated LPS-stimulated 70Z/3 and 70Z/3$^{\Delta NI\kappa B\alpha}$ cells with Na-3-CPA. As shown in Figs. 5A and B, Na-3-CPA suppressed both the induction of Igκ surface expression and apoptosis in wild-type 70Z/3 cells as well as in 70Z/3$^{\Delta NI\kappa B\alpha}$ cells. We conclude that LPS-induced Igκ expression utilizes a NF-κB activation pathway that primarily is under the control of IκBβ while being only marginally influenced by IκBα. The reverse relationship seems to be involved in NF-κB-mediated rescue from apoptosis. Furthermore, these two NF-κB activation pathways seem to separate rather early after LPS surface signaling since the different phenotypes are easily distinguishable.

Discussion

During the last few years, numerous reports have demonstrated a role for NF-κB in inhibition of apoptosis (reviewed in [25]). For example, ligation of TNFα to TNFR1 can induce both apoptosis and NF-κB activation [35]. NF-κB activation rescues the cells and initiates inflammation, while inhibition of NF-κB, on the other hand, leads to apoptosis. We have previously shown that N-substituted benzamides can inhibit NF-κB activity in B and T cells [27,28]. We have also shown that N-substituted benzamides can induce apoptosis via caspase-9 activation and cytochrome *c* release [26]. In this study, we wanted to investigate the connection between N-substituted benzamides and NF-κB and if this might be coupled to the NF-κB rescue pathway and apoptosis. We used the 70Z/3 pre-B cell line as a model system and looked at apoptosis and also at Igκ expression as a reflection of NF-κB activity. We generated a 70Z/3 cell line containing a dominant negative IκBα that had a defect in NF-κB activation. We also used a 70Z/3 cell line which over-expressed the anti-apoptotic gene Bcl-2 and which was less sensitive to apoptosis [26]. As shown in Figs. 1B and C, over-expression of Bcl-2 decreased apoptosis induced by 3-CPA, but that did not lead to increased long-time cell survival compared to wild-type cells. Inhibition of NF-κB, on the other hand, rendered the cells more sensitive to both initial apoptosis as well as to delayed cell death after three days of incubation with 3-CPA. This suggests that NF-κB might rescue cells from 3-CPA-induced apoptosis and cell death. We further studied apoptosis and NF-κB activation, measured by Igκ expression, on wild-type 70Z/3 cells. As shown in Figs. 2A and B, despite inhibiting NF-κB-dependent Igκ expression, Na-3-CPA did not enhance apoptosis while 3-CPA, that did not inhibit Igκ expression, induced apoptosis. In a previous study we showed that 3-CPA could inhibit LPS-induced Igκ expression by about 50% [27]. The discrepancy between the data in this paper and the data in the previous one might be due to differences in the duration of pre-incubation with 3-CPA. In the previous study, we

pre-incubated the cells for 5 h, compared to only 2 h in this paper. Considering the toxic effects by 3-CPA, the longer pre-incubation time might result in more cell death, and the observed inhibition of Igκ expression in live cells may thus be due to the fact that the Igκ-expressing cells are already dead and not visible in the viable fraction.

In the previous study, we showed that Na-3-CPA inhibited NF-κB by interfering with breakdown of IκBβ. In the present study we confirmed those data (Fig. 3D) and we also investigated the effects of Na-3-CPA and 3-CPA on IκBα breakdown. After stimulation of cells, IκBα is degraded faster than IκBβ and IκBα is thought to regulate transient and rapid NF-κB responses, while IκBβ is thought to regulate persistent responses [36]. We studied the degradation of IκBα after LPS-treatment and could show that 3-CPA but not Na-3-CPA could inhibit breakdown of IκBα (Figs. 3A and B). This suggests that 3-CPA may target IκBα and thus inhibit the rapid NF-κB activation that is responsible for rescue from apoptosis. Furthermore, we could show that the IκBα defective 70Z/3 cell line 70Z/3$^{\Delta NI\kappa B\alpha}$ showed a similar phenotype as cells treated with 3-CPA. The 70Z/3$^{\Delta NI\kappa B\alpha}$ cells were more sensitive to LPS-induced apoptosis (Fig. 5B) but the live cells had no significantly decreased Igκ expression (Fig. 5A). Na-3-CPA, on the other hand, was shown to target IκBβ (Fig. 3D) and also to inhibit Igκ expression (Figs. 2A and 5A). Na-3-CPA also protected the cells from apoptosis and may thus block upstream signaling pathways that are common for NF-κB activation by IκBβ and apoptosis.

In conclusion, we show that 3-CPA induces apoptosis itself by activation of caspases as well as by enhancing LPS-induced apoptosis through inhibition of IκBα breakdown and the NF-κB rescue pathway. Na-3-CPA, on the other hand, inhibits IκBβ breakdown and Igκ expression but does not affect the NF-κB rescue pathway. Interestingly, this difference in the mode of action of the two N-substituted benzamides is caused by only a minor structural modification by N-acetylation of the N-3 position of the benzamide structure.

Acknowledgments

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