II) Eigene Originalarbeiten

A) Spezifische Transkriptionsfaktoren bei entzündlichen Lungenerkrankungen

Bindungsstellen für den Transkriptionsfaktor NF-κB finden sich in den Promotorregionen verschiedener, am Entzündungsprozess beteiligter Zytokine. Im Rahmen der angeborenen Immunreaktion kommt diesem Transkriptionsfaktor eine zentrale, koordinierende Rolle zu (Hatada et al., 2000). Darüber hinaus weisen jüngste Befunde auf eine entscheidende Rolle dieses Transkriptionsfaktors bei der Zellzyklusregulation sowie der Apoptose hin. NF-κB ist ein Heterodimer, welches sich aus Polypeptiden der rel-Familie zusammensetzt. Nach vorliegenden Untersuchungen erscheint die Regulation dieses Transkriptionsfaktors von entscheidender Bedeutung für eine Vielzahl pathogenetisch relevanter zellulärer Stoffwechselvorgänge in so verschiedenen Erkrankungen wie dem Asthma bronchiale, der Arteriosklerose sowie der Herzinsuffizienz.

Dem Asthma bronchiale liegt eine chronische Entzündung der Atemwege zu Grunde, die auf zellulärer Ebene durch die Einwanderung und Aktivierung von eosinophilen Granulozyten, Mastzellen, Monozyten/Makrophagen und Lymphozyten gekennzeichnet ist (Barnes and Karin, 1997). Die Einwanderung und Aktivierung dieser Zellen wird durch Zytokine und Enzyme z.B. des Arachidonsäurestoffwechsels reguliert. Diese werden zu einem großen Teil innerhalb der Zellen neu synthetisiert. Als *in vitro* Modell dieser Aktivierung kann die Stimulation einer Zelle durch die Zytokine Tumor necrosis factor-α (TNF-α) und Interleukin-1β (IL-1β) dienen, die eine Schlüsselstellung in der Regulation der inflammatorischen Reaktion einnehmen. Die Regulation der induzierbaren Genexpression kann in einer groben Einteilung auf drei Ebenen stattfinden. Nach Bindung der Liganden an spezifische Rezeptoren kommt es zur Aktivierung mehrerer Signalübertragungswege. Diese induzieren die nukleäre Translokation und Aktivierung einer Reihe von Transkriptionsfaktoren wie z.B. dem „nuclear factor κB“ (NF-κB) (Schema 1). Nach Bindung an die spezifischen Promotorelemente wird durch Transkriptionsfaktoren die basale Transkriptionsmaschinerie zum Transkriptionsstartpunkt rekrutiert und die messengerRNA (mRNA)-Synthese des entsprechenden Gens beginnt. Die mRNA wird anschließend aus dem Zellkern exportiert und es beginnt die Translation. Die Rate der Proteinneusynthese wird auch

entscheidend auf dieser posttranskriptionellen Ebene moduliert. Insbesondere die mRNA-Stabilität sowie die Rate an mRNA-Translation werden stimulusabhängig gesteuert.

Die Therapie des Asthma bronchiale mit Kortikosteroiden kann den langfristigen Verlauf der Erkrankung positiv beeinflussen. Dies gelingt über die hochpotente anti-inflammatorische Wirkung. Deren molekulare Grundlage ist der *in vitro* nachweisbare hemmende Effekt auf viele pro-inflammatorische Zytokine (Barnes and Karin, 1997). Welcher intrazelluläre Mechanismus liegt dieser Hemmung zu Grunde? Die Bindung des physiologischen Liganden an den Glukokortikoidrezeptor (GR) führt zur Translokation des Komplexes in den Kern. Über lange Zeit wurde die direkte Bindung dieses Komplexes an das nukleäre GRE (Glukokortikoid-response element, spezifische Gensequenz zur Bindung des GR) für die positiv und negativ regulierenden Eigenschaften der Glukokortikoide verantwortlich gemacht. Dieses Modell war jedoch unzureichend, um das breite Spektrum der anti-inflammatorischen Wirkung der Glukokortikoide zu erklären. Zum einen fanden sich in den meisten untersuchten Promotorregionen keine GRE, zum anderen war bei *in vitro* Versuchen deutlich geworden, dass die DNA-Bindung des GR nicht notwendige Voraussetzung für die Hemmung der Genexpression ist. Vielmehr scheint die hemmende Wirkung durch eine direkte Protein-Protein Interaktion des GR mit Transkriptionsfaktoren wie z.B. NF-κB (nuclear factor -κB) oder AP-1 zustande zu kommen (Newton, 2000).

Die epitheliale Lungenzelllinie A549 diente für die hier vorgestellten Untersuchungen als Modell der den Entzündungsprozess über eine Reihe von Zytokinen steuernden bronchoepithelialen Barriere. Die *in vitro* Stimulation dieser Zelllinie mit Il-1β und TNF-α führt zur Aktivierung von NF-κB, des für die Prostaglandinsynthese entscheidenden Enzyms Cyclooxygenase-2 (COX-2) sowie des auf eosinophile Granulozyten chemotaktisch wirkenden Zytokins „granulocyte-macrophage colony- stimulating factor“ (GM-CSF). Die Aktivierung von COX-2 und GM-CSF in A549 ließ sich durch das synthetische Glukokortikoid Dexamethason hemmen. Darüber hinaus wurde GM-CSF nach Stimulation mit Mitogenen wie Phorbol Ester (PMA), Lektinen wie Phytohaemagglutinin (PHA) oder Zytokinen wie IL-1β oder TNF-α von T-Lymphozyten synthetisiert. Auch in T-Zellen ließ sich die Freisetzung von GM-CSF durch Glukokortikoide hemmen (Schema 2).

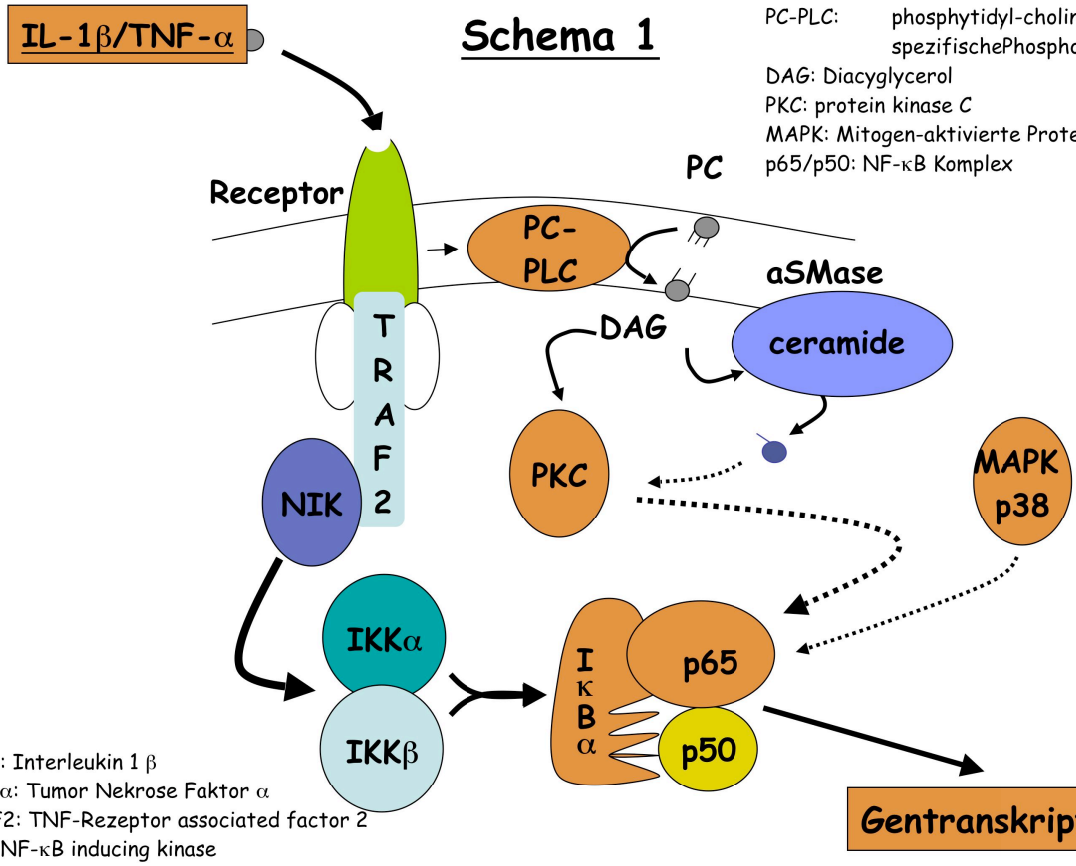
1.) Signaltransduktionswege der Zytokin-induzierten NF-κB Aktivierung

Die Aktivierung des Transkriptionsfaktors NF-κB erfolgt durch Phosphorylierung und anschließende Degradierung der inaktivierenden Untereinheit IκBα. Die Signalübertragungswege, die nach Il-1β oder TNFα Stimulation zur Phosphorylierung von IκBα führen, sind zum großen Teil noch unklar. Ausgehend von der Analyse mitogen wirkender Botenstoffe konnte in jüngster Zeit gezeigt werden, dass offensichtlich ein gemeinsames Grundprinzip der Signalübertragung von der Zellmembran zum Zellkern besteht. Dabei spielt die spezifische Phosphorylierung von Tyrosin beziehungsweise von Serin/Threonin (dual-spezifische Kinase) eine entscheidende Rolle. Die Aktivierung von membranständigen Tyrosin-Kinasen, die die intrazelluläre Domäne der spezifischen Zytokinrezeptoren darstellen, führt über eine Kaskade von zytoplasmatischen Kinasen zur Aktivierung von Transkriptionsfaktoren wie NF-κB, die in den Kern wandern und dort die Gentranskription im Zusammenspiel mit anderen Transkriptionsfaktoren induzieren (Schema 1). Viele Schritte dieser Signalübertragung sind dabei phylogenetisch bewahrt worden, selbst in Hefen lassen sich den Säugerzellen verwandte Kinasen nachweisen. Um einen genaueren Einblick in die Signalübertragungswege zu gewinnen, wurde ein Zellmodell entwickelt, bei dem die Aktivierung des stabil transfizierten Luziferase-Reporter Gens ausschließlich durch multiple NF-κB-Bindungsstellen kontrolliert wird. An diesem Modell konnten interessante Einblicke in die Signaltransduktionswege der IL-1β und TNF-α induzierten NF-κB Aktivierung gewonnen werden.

Um die an der Aktivierung des Transkriptionsfaktors NF-κB beteiligten Signalübertragungswege zu untersuchen, wurde zunächst eine Genkassette mit der 6-fachen Wiederholung des NF-κB Konsensus-Motivs in den Luziferase-Vektor pGL3-basic (Promega, UK) kloniert. Anschließend wurde ein Neomycinresistenzgen in den Vektor kloniert, um nach transienter Transfektion mit Hilfe des Lipides Tfx50 (Promega, UK) stabil transfizierte Zellen selektieren zu können. In stabil transfizierten Zellen kam es nach IL-1β Stimulation zu einer 4-7 fachen Induktion des Luziferase Reportergens.

Neben den Mitogen-aktivierten Protein Kinasen (MAPK) spielt offensichtlich die Familie der Protein Kinasen C (PKC), ursprünglich vor allem im Zusammenhang mit der Zellstimulation durch Mitogene wie Phorbol Myristate Acetate untersucht, eine entscheidende Rolle in der Signalübertragung von proinflammatorischen Zytokinen wie TNF-α und IL-1β. Die PKC-Familie lässt sich in 3 Gruppen unterteilen: „klassische“, „neue“ und „atypische“ PKC. Wir untersuchten daher zunächst mit Hilfe verschiedener selektiver Inhibitoren der Tyrosinkinasen (Herbimycin A), der MAPK ERK (PD 098059) und p38 (SB 203580), der Phosphatidylcholin spezifischen Phospholipase C (PC-PLC; D609) sowie aller PKC-Isoenzyme (RO 31-8220) die Beteiligung dieser Pfade (Bergmann et al., 1998). Sowohl der Tyrosin-Kinasen Inhibitor Herbimycin A als auch der PKC-Inhibitor RO 31-8220 und der PC-PLC-Inhibitor D609 haben einen dosisabhängigen Effekt auf die Aktivierung des stabil transfizierten NF-κB Promotor-Konstruktes (Schema 1).

Im nächsten Schritt wurde der Effekt der im Reportergen-assay identifizierten NF-κB Inhibitoren auf die NF-κB DNA-Bindung untersucht. Überraschenderweise fand sich eine unvermindert starke DNA-Bindung in Gegenwart dieser Inhibitoren (Bergmann et al., 1998). Die Diskrepanz dieser beiden Ergebnisse kann nicht durch unspezifische Effekte auf die Luziferase-Expression zurückgeführt werden, da RO31-8220 und D609 auch die Luziferase mRNA Expression schon 1h nach Koinkubation hemmen. Weiterhin lassen sich diese Ergebnisse auf Protein-Ebene validieren, da sich auch kein Effekt der Inhibitoren auf die IκBα -Degradation nachweisen läßt. Diese Ergebnisse definieren daher einen zusätzlichen NF-κB-Aktivierungspfad nach Zytokin-Stimulation, der unabhängig von IκBα-Degradation und NF-κB DNA-Bindung die Transaktivierung durch NF-κB steuert (Schema 1).



IL-1β: Interleukin 1 β
TNF-α: Tumor Nekrose Faktor α
TRAF2: TNF-Rezeptor associated factor 2
NIK: NF-κB inducing kinase
IKK: IκB-kinase
IκBα: inhibitor of NF-κB

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IκBα Degradation and Nuclear Factor-κB DNA Binding Are Insufficient for Interleukin-1β and Tumor Necrosis Factor-α-induced κB-dependent Transcription*

REQUIREMENT FOR AN ADDITIONAL ACTIVATION PATHWAY

(Received for publication, December 29, 1997, and in revised form, January 28, 1998)

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Two closely related IκBα kinases as well as the upstream kinase, NIK, which integrates interleukin-1β (IL-1β)- and tumor necrosis factor (TNF)-α-dependent activation of the transcription factor NF-κB have recently been described. However, in this emerging pathway the role of previously identified components of cytokine-induced NF-κB activation, namely phosphatidylcholine-specific phospholipase C and protein kinase C, remains unclear. We now show that, in A549 human alveolar epithelial cells, the activation of a stably transfected NF-κB-dependent reporter gene by TNF-α and IL-1β is completely blocked by the phosphatidylcholine-specific phospholipase C inhibitor D609 and the protein kinase C inhibitor RO31-8220. However, IL-1β-induced IκBα degradation as well as NF-κB nuclear translocation and DNA binding, as determined by Western blot and electro-mobility shift assay, respectively, are not affected by these inhibitors. A similar effect, although less pronounced, is observed with the p38 mitogen-activated protein kinase inhibitor SB 203580. On the basis of these data we propose the existence of a second signaling pathway induced by IL-1β and TNF-α that is activated in parallel to the cascade leading to IκBα degradation and is specifically required for NF-κB-dependent transcriptional competency.

The transcription factor nuclear factor-κB (NF-κB)[1] plays a key role in the transcriptional regulation of adhesion molecules, enzymes, and cytokines involved in chronic inflammatory diseases (reviewed in Ref. 1). In epithelial cells, which play a major role in inflammation, pro-inflammatory cytokines, such as interleukin (IL)-1β, rapidly induce NF-κB DNA binding and cause up-regulation of NF-κB-dependent genes, including cyclooxygenase-2 (2) and inducible nitric-oxide synthase (3). Because the potent anti-inflammatory effects of glucocorticoids have been linked to a functional antagonism between the NF-κB subunit p65 and the activated glucocorticoid receptor (4–6), NF-κB activation pathways have attracted much attention as potential targets for new anti-inflammatory strategies.

In resting cells, the inhibitory subunit IκBα is bound to the p50/p65 heterodimer of NF-κB in the cytoplasm. Treatment of cells with IL-1β or tumor necrosis factor (TNF)-α results in the specific phosphorylation of two serine residues on IκBα (7) followed by the ubiquitination (8) and degradation of this subunit (9). This releases active NF-κB, which then translocates to the nucleus and activates transcription. Recently, two closely related kinases that directly phosphorylate IκBα have been described (10–12). In addition, the upstream kinase, where the IL-1β and TNF-α signaling pathways converge prior to IκBα phosphorylation, has been identified as a mitogen-activated protein kinase kinase kinase and named NF-κB-inducing kinase (13).

At present, it remains unclear where other previously identified pathways activated by IL-1β and TNF-α feed into this emerging signal transduction cascade. For example, the phosphatidylcholine-specific phospholipase C (PC-PLC) as part of the sphingomyelin pathway upstream of the second messenger ceramide has been implicated in TNF activation of NF-κB (14). However, the signal transduction pathway leading to nuclear translocation of NF-κB after TNF stimulation was found to be intact in acidic sphingomyelinase-deficient mice (15). Protein kinase C (PKC) isoforms have also been implicated in NF-κB activation. Transfection of a dominant negative mutant of the atypical isoform PKC-ζ severely impaired the activation of a NF-κB-dependent reporter gene plasmid by sphingomyelin (16), implicating a role of PKC-ζ downstream of the sphingomyelin pathway. In addition, PKC-ζ was shown to phosphorylate IκBα *in vitro* (17). In contrast, the expression of highly purified PKC isoenzymes α, β, γ, δ, ϵ, and ζ *in vivo* failed to induce IκBα phosphorylation (18). However, *in vivo* studies with constitutively active isoforms demonstrated novel PKC-ϵ to be a potent inducer of a NF-κB-dependent reporter gene (19).

In addition to PC-PLC and PKC, the mitogen-activated protein kinase (MAPK), p38, has been implicated in NF-κB activation, because the selective inhibitor, SB203580, was able to inhibit the activation of a NF-κB-dependent reporter gene by TNF-α. However, NF-κB nuclear translocation and DNA binding was unaffected (20). A similar effect has been observed with the protein-tyrosine kinase (PTK) inhibitor genistein, which was able to inhibit lipopolysaccharide-induced activation of NF-κB-dependent transcription (21).

We have used human type II A549 pneumocyte cells to

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; EMSA, electromobility shift assay; ERK, extracellular regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, IκB kinase; IL, interleukin; MAPK, mitogen-activated protein kinase; PC-PLC, phosphatidylcholine-specific phospholipase C; PKC, protein kinase C; PTK, protein-tyrosine kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; TNF, tumor necrosis factor; PMSF, phenylmethylsulfonyl fluoride.

provide evidence of a second signaling pathway that is distinct from IκBα degradation and NF-κB nuclear translocation but is required for NF-κB transcriptional activation by IL-1β and TNF-α.

EXPERIMENTAL PROCEDURES

Cell Culture— A549 cells obtained from ECACC (code 86012804) were cultured as previously described (22). Prior to transfection, cells were grown in T-75 culture flasks to 50–60% confluency.

Plasmid Construct—The NF-κB-dependent reporter, 6NF-κBtkluc, contains three tandem repeats of the sequence 5′-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3′, which harbors two copies of the NF-κB binding site (underlined) upstream of a minimal thymidine kinase promoter (−105 to +51) driving a luciferase gene as described before (23). Neomycin resistance was conferred by ligating a *Hinc*II (blunted)/*Pvu*I fragment from pMC1neoPoly(A) (Stratagene, Cambridge, UK) into the *Pvu*I site of 6NF-κBtkluc downstream of the luciferase gene. The resulting plasmid was named 6NF-κBtkluc.neo.

Stable Transfection and Luciferase Assay— Cells were washed with serum-free medium and incubated with medium containing 8 μg of plasmid and Tfx50 (Promega, UK) for 2 h. Subsequently, cells were cultured in fresh medium for 16 h before adding 0.5 mg/ml G-418 (Life Technologies, Inc.). Foci of stable transfected cells developed after approximately 14 days of culture in the presence of G-418. To create a heterogeneous population with regard to integration site, multiple clones were then harvested and used for experiments for another eight passages while maintained in medium containing 0.5 mg/ml G-418. Cells were stimulated with IL-1β and TNF-α (R & D Systems, Oxon, UK) at 1 and 10 ng/ml, respectively. Where used, RO31-8220 (Alexis, Nottingham, UK), SB203580, herbimycin A, and D609 (Calbiochem, Nottingham, UK) were added 5 min prior to stimulation. Cells were harvested 6 or 24 h later and assayed for luciferase activity using a commercially available luciferase reporter gene assay (Promega).

Semi-quantitative RT-PCR—RNA isolation, reverse transcription, PCR primers, conditions, and cycling parameters for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as described previously (24). Luciferase primers were: 5′-GAG AGC AAC TGC ATA AGG CTA-3′ (forward) and 5′-TAC ATC GAC TGA AAT CCC TGG-3′ (reverse) (accession number M15077). Cycling parameters were: 94 °C, 20 s; 60 °C, 30 s; 72 °C, 30 s. The number of amplification cycles used was the number necessary to achieve exponential amplification where product formation is proportional to starting cDNA, and in each case this was determined as described (24). Following amplification, products (10 μl) were run on 2.0% agarose gels stained with ethidium bromide. After densitometry, data were expressed as the ratio of luciferase/GAPDH as a percentage of IL-1β treated as means ± S.E. Because the transfected luciferase gene has no introns, identical control amplifications were performed from similar reverse transcriptions in which the reverse transcriptase had been omitted. In these cases no product was visible, indicating that any genomic contamination was below detectable levels (data not shown).

Nuclear Extract Preparation and Assay for DNA Binding of Transcription Factor—A549 cells were grown to confluency in 6-well plates and incubated in serum-free medium for 24 h prior to treatment. Nuclear protein was isolated 1 h after stimulation with 1 ng/ml IL-1β or 10 ng/ml TNF-α as described previously (22). Where used, inhibitors were added 5 min prior to stimulation. The consensus NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG-3′) and Oct-1 (TGT CGA ATG CAA ATC ACT AGA) probe were obtained from Promega. Specificity was determined by prior addition of 100-fold excess unlabeled consensus oligonucleotide. Reactions were separated on 7% native acrylamide gels before vacuum drying and autoradiography.

Western Blot Analysis—Confluent A549 cells grown in 6-well plates were stimulated for the indicated times and harvested in 200 μl of lysing buffer (1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM Tris-base, 75 mM NaCl, 10 mM EDTA, pH 7.4, supplemented with 0.5 mM PMSF, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate).

Prior to loading onto 10% SDS polyacrylamide gels, samples were denatured by boiling for 5 min. Gels were run at 200 mA for 40 min at 25 °C. Proteins were transferred onto Hybond-ECL nitro-cellulose paper (Amersham, Buckinghamshire, UK) in blotting buffer (20 mM Tris-base, 192 mM glycine, 20% methanol) at 400 mA for 1 h at 25 °C. Membranes were blocked for 1 h with a 5% (w/v) nonfat dry milk solution in TBS/T (10 mM Tris-base, 150 mM NaCl, 0.1% Tween-20) before incubating the filter for 1 h with rabbit polyclonal anti-human IκBα (clone C21, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000. Membranes were washed five times with TBS/T and incubated for a further hour with goat anti-rabbit horseradish peroxidase-linked IgG (Dako, Bucks, UK) diluted 1:4000. After another five washes, antibody-labeled proteins were detected by ECL as described by the manufacturer (Amersham, Buckinghamshire, UK).

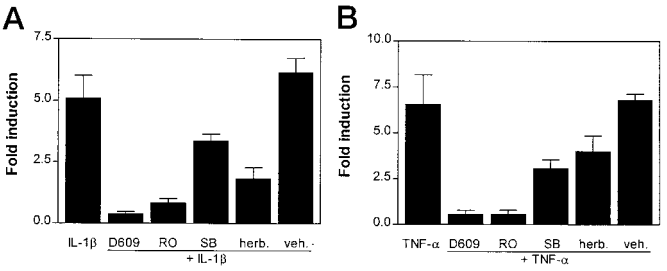

FIG. 1. **Effect of kinase inhibitors on IL-1β and TNF-α stimulated NF-κB-dependent transcription.** Luciferase activity after stimulation of 6NF-κBtkluc.neo stably transfected A549 cells with 1 ng/ml IL-1β (*A*) or 10 ng/ml TNF-α (*B*). Inhibitors D609 (100 μg/ml), RO31-8220 (10 μM), SB203580 (10 μM), herbimycin A (10 μM), or vehicle (0.1% Me_2SO) were added 5 min prior to stimulation. Data represent averages of four independent experiments.

RESULTS

Induction of two p50/p65 NF-κB DNA binding complexes by IL-1β and TNF-α has previously been shown in these cells (2, 22). To investigate the effects on κB-dependent transcription, a NF-κB-dependent reporter, 6NF-κBtkluc.neo, was stably transfected into A549 cells. As shown in Fig. 1, the PTK inhibitor herbimycin A (25), the PC-PLC inhibitor D609 (14), the PKC inhibitor RO31-8220 (26), and the p38 MAPK inhibitor SB203580 (27) inhibited both the IL-1β and TNF-α stimulation of NF-κB-dependent luciferase activity at concentrations previously shown to be selectively effective. The MAPK/extracellular regulated kinase kinase-1/2 inhibitor, PD 098059, at 10 μM, which potently inhibits downstream activation of the extracellular regulated kinase (ERK)1 and ERK2 (28), and the PTK inhibitor, genistein (21), at 100 μM had no effect on luciferase activity induced by IL-1β or TNF-α (data not shown). Table I depicts the EC_{50} for the various inhibitors; results are in the range of previously reported selectively active concentrations (see references cited above). Vehicle, 1 μl/ml Me_2SO, had no effect on luciferase activity after IL-1β or TNF-α stimulation. Inhibitors alone had no effect on luciferase activity (data not shown).

To examine the effect of these inhibitors on NF-κB nuclear translocation and DNA binding electro mobility shift assays (EMSA) were performed. IL-1β-induced NF-κB DNA binding was only slightly altered by the nonselective PTK inhibitor herbimycin A, whereas the potent inhibitors of NF-κB-dependent transcription, RO31-8220, SB203580, and D609, had no effect (Fig. 2*A*). Specificity of the complex was shown by competing out the signal with a 100-fold excess of cold competitor (data not shown). Neither IL-1β nor any of the inhibitors under investigation had an effect on DNA binding activity of the noninducible transcription factor Oct-1 (data not shown). Likewise induction of NF-κB DNA binding by TNF-α was also unaffected by RO31-8220, D609, and SB203580 (Fig. 2*B*). Because the changes in luciferase activity were observed at 24 h and the lack of change in NF-κB DNA binding was after a 1-h treatment, there remained the possibility that these drugs exert their effects by changing the longer term levels of active NF-κB. However, because the reporter assay produced identical data after a 6-h treatment, this possibility seemed remote (data not shown). Semi-quantitative RT-PCR was used to further examine this question. Consistent with the luciferase activity data, both RO31-8220 and D609 showed total repression of IL-1β-induced luciferase mRNA following a 1-h incubation

TABLE I
EC_{50} of different inhibitors on IL-1β and TNF-α induced NF-κB-dependent transcription

6NF-κBtkluc.neo stably transfected A549 were stimulated with IL-1β (1 ng/ml) and TNF-α (10 ng/ml) for 24 h. D609, RO31–8220, SB203580, and herbimycin A were added 5 min prior to stimulation. The cells were lysed and assayed for luciferase activity. Results were expressed as fold induction. The EC_{50} was calculated on the basis of at least three independent experiments with four different concentrations over a 100-fold dilution range of each inhibitor.

Stimulus	RO31–8220	D609	SB 203580	Herbimycin A
		μM		
IL-1β (1 ng/ml)	1.03	112 (25.5 μg/ml)	1.3	1.01
TNF-α (10 ng/ml)	0.86	76 (17.4 μg/ml)	1.83	3.93

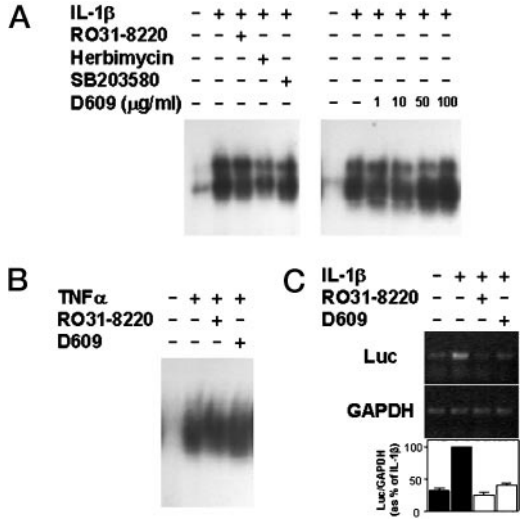

FIG. 2. **Effect of kinase inhibitors on NF-κB nuclear translocation and DNA binding.** *A*, EMSA analysis of A549 nuclear extracts after stimulation with IL-1β (1 ng/ml) plus RO31-8220 (10 μM), herbimycin A (10 μM), SB 203580 (10 μM), and D609 (1–100 μg/ml) for 1 h. *B*, EMSA analysis of A549 nuclear extracts after stimulation with TNF-α (10 ng/ml) plus RO31-8220 (10 μM) and D609 (50 μg/ml) for 1 h. Data are representative of at least three independent experiments. *C*, cells were treated with D609 (100 μg/ml) or RO-31-8220 (10 μM) for 5 min and then IL-1β as indicated and harvested after 1 h for RNA and semi-quantitative RT-PCR analysis of luciferase (*Luc*) and GAPDH mRNA. Representative ethidium bromide-stained agarose gels are shown, and data from four such experiments are plotted below as percentages of IL-1β treated as means ± S.E.

(Fig. 2*C*). These data indicate that the changes in luciferase expression were the result of immediate changes in κB-dependent transcription and not due to effects on p50 or p65 expression or luciferase translation.

Because NF-κB DNA binding was unaffected, these data suggest that phosphorylation and subsequent degradation of IκBα would also be unaffected by these compounds. The time course of IL-1β-induced IκBα degradation and resynthesis is shown in Fig. 3. The IκBα signal is lost by 15 min post-stimulation except for a retarded band indicating phosphorylated but as yet undegraded IκBα. RO31-8220 and SB203580 appeared to have little effect on loss of IκBα, whereas D609 seemed to result in a marginally reduced loss of IκBα. These data are consistent with the EMSA data indicating no substantial effect of these compounds on NF-κB activation. By contrast both D609 and RO31-8220 delayed, by 30 and 60 min, respectively, the reappearance of IL-1β-induced IκBα, whereas SB203580 had no obvious effect.

DISCUSSION

A significant step toward understanding the mechanism of NF-κB activation was the recent identification of two related

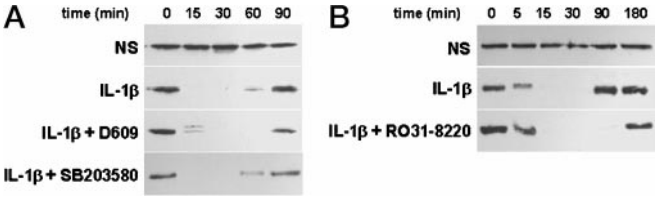

FIG. 3. **Effect of kinase inhibitors on cytoplasmic IκBα degradation after IL-1β treatment.** A549 cells were stimulated with IL-1β (1 ng/ml) as indicated. The effect of D609 (100 μg/ml) and SB203580 (10 μM) (*A*) and RO31-8220 (10 μM) (*B*) added 5 min prior to stimulation on cytoplasmic IκBα immunoreactivity was studied by Western blot analysis. IκBα was detected as a 40-kDa protein. Data are representative of at least four independent experiments. *NS*, no stimulation.

IκBα kinases (IKKα and IKKβ) and the upstream kinase, NF-κB-inducing kinase, integrating IL-1β- and TNF-α-induced NF-κB activation (reviewed in Refs. 11, 29, and 30). However, because the pathways leading to IκBα phosphorylation and subsequent degradation are characterized in some detail now, the role of other previously identified components of cytokine-mediated NF-κB activation is becoming less clear. By employing a NF-κB-dependent luciferase reporter gene stably transfected into the human alveolar epithelial cell line A549, the compounds D609, RO31-8220, and SB203580 selective for the sphingomyelin, PKC, and p38 MAPK pathways, respectively, were shown to be potent inhibitors of NF-κB-dependent transcription after IL-1β or TNF-α stimulation. However, neither of these inhibitors had an effect on IκBα degradation or NF-κB nuclear translocation and DNA binding. We therefore propose the existence of a second pathway triggered by TNF-α and IL-1β in parallel to IκBα degradation, which confers NF-κB transcriptional competency.

In the same assay system the PTK inhibitor herbimycin A was able to partially inhibit both the luciferase activity and DNA binding as determined by EMSA. The PTK inhibitor genistein, previously reported to inhibit NF-κB transactivation induced by lipopolysaccharide in a pro-monocytic cell line (21), had no effect on NF-κB-dependent transcription in our model. The PTK $p59^{fyn}$ is a possible target for these inhibitors, because it was shown to be involved in NF-κB-mediated activation of the HIV long terminal repeat promoter (31). Discrepant findings with different PTK inhibitors as shown here for genistein and herbimycin A were recently described for the regulation of inducible nitric-oxide synthase mRNA in primary rat hepatocytes involving the PTK $pp60^{c\text{-}src}$ (25), suggesting different target proteins for each inhibitor.

In contrast to the partial effect exercised by herbimycin A and SB203580, the PC-PLC inhibitor D609 completely abolished NF-κB-dependent reporter gene activation by IL-1β and TNF α at doses of 50 μg/ml. The sphingomyelin pathway with its second mediator ceramide has previously been implicated in TNF-α-induced NF-κB activation (14). At doses of 100 μg/ml, D609 completely inhibited PC-PLC; however, doses of 250 μg/ml only partially affected NF-κB DNA binding (14). In contrast, the EC_{50} for the D609 effect on NF-κB-dependent transcription in this study is similar to the one calculated from the dose response curve for TNF-α-activated PC-PLC (14). Taking the data concerning the time course of IκBα degradation presented here into account, we conclude that the inhibitory effect of D609 on NF-κB activation is only marginally due to the inhibition of IκBα degradation and nuclear translocation. More importantly, D609, at doses that abolish PC-PLC activity completely, inhibits NF-κB-dependent transcription induced by IL-1β and TNF-α. The delayed resynthesis of IκBα observed here supports this view, because the IκBα promoter contains multiple NF-κB sites responsible for conferring TNF-α inducibility (32, 33). Importantly, this hypothesis would also explain

recent findings in sphingomyelinase-deficient mouse embryonic fibroblasts, where TNF-α-induced IκBα degradation and NF-κB nuclear translocation was unaffected (15). Based on these findings, Zumbansen and Stoffel (15) questioned any role for acidic sphingomyelinase, which is downstream of PC-PLC (14). However, NF-κB transactivation competency was not investigated and may be the crucial step mediated by PC-PLC and sphingomyelinase in response to TNF-α. The specificity of D609 for PC-PLC has recently been challenged by data showing the inhibition of platelet-derived growth factor-activated phospholipase D as well as PC-PLC (34). However, TNF-α-induced phopholipase D activity was not affected by D609 (14), raising the possibility of a platelet-derived growth factor-specific effect.

Data concerning the role of PKC isoforms in TNF-α- or IL-1β-induced NF-κB activation have been conflicting. Here we find that the PKC-inhibitor RO31-8220 was able to completely block NF-κB-dependent transcription yet failed to block NF-κB nuclear translocation and DNA binding or IκBα degradation. The marked delay in IL-1β-dependent IκBα resynthesis further supports the hypothesis of a selective effect on NF-κB transcriptional competency. However, evidence for the involvement of PKC isoforms is only indirect, because RO31-8220 is not selective for PKC isoenzymes (36, 37). Both MAPK-activated protein kinase 1β and p70 S6 kinase, which are activated in response to growth factors and phorbol esters, are also inhibited (36). Whether other kinases activated by cytokines are inhibited remains unclear. The cross-reactivity toward MAPK-activated protein kinase 1β was examined by testing the MAPK/extracellular regulated kinase kinase-1/2 specific inhibitor PD 098059, which blocks the upstream activation of ERK1 and ERK2. PD 098059 had no effect on NF-κB-dependent transcription.

In summary, PC-PLC and PKC isoforms appear to be involved in a TNF-α- and IL-1β-induced pathway of NF-κB transcriptional activation that is distinct from the signaling pathway leading to IκBα degradation, NF-κB nuclear translocation, and DNA binding. Because cytokine-mediated phosphorylation of NF-κB subunits is shown to occur for p65 after TNF-α stimulation of HeLa cells (38) and a serine kinase, which specifically phosphorylates NF-κB subunits and not IκBα, has been described (39), we speculate that both D609 and RO31-8220 may prevent p65 phosphorylation and lead to reduced transcriptional activity. Furthermore, Mercurio *et al.* (12) identified a RelA kinase activity that was associated with the IKK signalsome and supports the hypothesis that cytokine-dependent phosphorylation of p65 (RelA) may be required for transcriptional activity.

However, regulated phosphorylation of NF-κB subunits has been tested for the p38 inhibitor SB203580 (20), and no change in NF-κB subunit phosphorylation was detected. Because the inhibition of NF-κB-dependent transcription by SB 203580 was the least pronounced effect observed in this study, future work needs to specifically address the effect of D609 and RO31-8220 on NF-κB subunit phosphorylation.

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