

Reactive Oxygen Intermediate-Dependent NF- κ B Activation by Interleukin-1 β Requires 5-Lipoxygenase or NADPH Oxidase Activity

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We previously reported that the role of reactive oxygen intermediates (ROIs) in NF- κ B activation by proinflammatory cytokines was cell specific. However, the sources for ROIs in various cell types are yet to be determined and might include 5-lipoxygenase (5-LOX) and NADPH oxidase. 5-LOX and 5-LOX activating protein (FLAP) are coexpressed in lymphoid cells but not in monocytic or epithelial cells. Stimulation of lymphoid cells with interleukin-1 β (IL-1 β) led to ROI production and NF- κ B activation, which could both be blocked by antioxidants or FLAP inhibitors, confirming that 5-LOX was the source of ROIs and was required for NF- κ B activation in these cells. IL-1 β stimulation of epithelial cells did not generate any ROIs and NF- κ B induction was not influenced by 5-LOX inhibitors. However, reintroduction of a functional 5-LOX system in these cells allowed ROI production and 5-LOX-dependent NF- κ B activation. In monocytic cells, IL-1 β treatment led to a production of ROIs which is independent of the 5-LOX enzyme but requires the NADPH oxidase activity. This pathway involves the Rac1 and Cdc42 GTPases, two enzymes which are not required for NF- κ B activation by IL-1 β in epithelial cells. In conclusion, three different cell-specific pathways lead to NF- κ B activation by IL-1 β : a pathway dependent on ROI production by 5-LOX in lymphoid cells, an ROI- and 5-LOX-independent pathway in epithelial cells, and a pathway requiring ROI production by NADPH oxidase in monocytic cells.

The interaction of interleukin-1 β (IL-1 β) with its type 1 cell surface receptor initiates a cascade of intracellular reactions leading to the activation of transcription factors and the expression of target genes. One of the major transcription factors mediating IL-1 β biological activities is NF- κ B (for reviews, see references 2, 3, and 22). This factor is sequestered in the cytoplasm by an inhibitor from the I κ B family. IL-1 β cellular stimulation leads to a rapid phosphorylation and degradation of I κ B α , the most common NF- κ B inhibitor. This reaction allows NF- κ B to translocate to the nucleus, to bind DNA, and to activate the transcription of specific genes (2, 55).

Following its interaction with IL-1, the type 1 IL-1 receptor recruits the IL-1 receptor-associated kinase (IRAK) protein, which subsequently interacts with the TRAF6 adapter protein (15, 16, 30, 61, 62, 65). TRAF6 is required for IL-1 β -induced NF- κ B activation, as demonstrated in 293 cells (16).

However, the signaling pathways leading to NF- κ B activation from the IL-1 β receptors are still controversial. It has been demonstrated that TRAF6 interacts with a MAP kinase kinase (MAPKKK) known as NIK and that NIK is required for IL-1 β - or tumor necrosis factor alpha (TNF- α)-dependent NF- κ B activation (39, 56). Large (500 to 900 kDa) multimeric protein kinase complexes have been purified from HeLa cells and transmit the signal from the TNF receptor type 1 (TNFR-1) and type 1 IL-1 receptors to the NF- κ B/I κ B cytoplasmic complex (17, 20, 33, 41). From these complexes three I κ B kinases, IKK- α , IKK- β , and IKK- γ , have been purified, and their genes were cloned (20, 42, 49, 66). Other investiga-

tors have cloned IKK kinases by virtue of their association with the NIK protein kinase (47, 64). Moreover, inactivation of these kinases by dominant negative mutants suppresses IL-1 β and TNF- α induction of NF- κ B. These studies indicate that the activated NIK kinase phosphorylates and activates the IKK protein kinases. IKK protein kinases can in turn phosphorylate the I κ B α protein on serines located at positions 32 and 36, a reaction which targets I κ B α for ubiquitination and rapid degradation by the proteasome (12, 58, 59). These reactions are extremely rapid, and the cellular I κ B α protein is completely degraded within minutes following TNF- α or IL-1 β cell stimulation (4, 13).

Despite this simplified linear receptor-TRAF-NIK-IKK axis for I κ B α phosphorylation and degradation, other intermediates might be involved in NF- κ B activation by TNF- α or IL-1 β . First, several components of the large signaling complex remain to be identified, as the three IKK protein kinases do not account for the molecular weight of the whole complex. Second, a large number of studies, some of them being a matter of controversy, have identified other intermediates which seem to be required for TNF- α - or IL-1 β -mediated NF- κ B activation. These intermediates are Raf-1, MAP kinases, the PKC ζ and λ/ι isoforms, Rho and Rac proteins, and ceramide or reactive oxygen intermediates (ROIs) (19, 24, 25, 32, 33, 38, 46, 50–53, 57). Such a large number of controversial studies might be explained by cell type specificities. Indeed, most of these studies were performed with a single cell line, although as we reported the roles of sphingomyelinases, PKC λ/ι , and ROIs in NF- κ B activation by IL-1 β were cell specific (6–8).

We reported that an oxidative stress favored replication of the human immunodeficiency virus type 1 (HIV-1) containing a tandem κ B site in its long terminal repeat (LTR) (35). Later on, the authors of several studies proposed that ROIs were

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produced by all NF- κ B-activating agents and were required for NF- κ B activation (50–52). So far, however, the I κ B kinases activated by ROIs have not yet been identified. More recently, however, it was reported that the effects of oxidants and anti-oxidants on NF- κ B activation were dependent on the cell line and on the applied stimulus (10, 11). We also reported that IL-1 β induced NF- κ B through the production of ROIs in lymphoid cells but independently of any ROI production in epithelial cells (6). These data indicate that the role of ROIs in NF- κ B activation is probably cell specific.

The sources of ROIs following TNF- α or IL-1 β treatment are still largely unknown. CD28 stimulation of T lymphocytes induces ROI production through an increase of the activity of 5-lipoxygenase (5-LOX) (37), an enzyme which is also required for NF- κ B-dependent transcription induced by IL-1 β in endothelial cells (34). The 5-LOX enzyme catalyzes the production of leukotrienes and ROIs from arachidonic acid (29, 36). Its activity requires FLAP (5-LOX activating protein), which transports 5-LOX to the nuclear membrane (23, 43, 48, 63). Recently, we reported that the inhibition of 5-LOX in lymphoid cells blocked NF- κ B activation after IL-1 β or arachidonic acid stimulation (7). Similarly, according to our previous data, the absence of ROI induction in IL-1 β -treated epithelial cells might be due to low 5-LOX and high catalase activities (7). However, there are other sources of cellular ROIs, including the NADPH oxidase which is expressed not only in monocytes/macrophages and polymorphonuclear neutrophils but also in lymphocytes (54). In the present study, we investigated the roles of 5-LOX, FLAP, and NADPH oxidase in the IL-1 β -induced ROI production and NF- κ B activation in different cell types.

MATERIALS AND METHODS

Cell culture and biological reagents. The cell lines HL-60, U937, Jurkat, EL-4, Raji, OVCAR-3, SKOV-3, MCF7 A/Z, HCT-116, HT-29, and HTM-29 were grown in RPMI 1640 medium (Life Technologies) supplemented with 1% antibiotics, 1% glutamine, and 10% fetal bovine serum. The mouse pre-B-cell line 70Z/3 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% antibiotics, 1% glutamine, and 10 mM β -mercaptoethanol. Before stimulation, 70Z/3 cells were grown for one day in medium without β -mercaptoethanol. The breast cancer cell line MCF7 A/Z was a generous gift from M. Mareel (University of Ghent, Belgium).

IL-1 β treatment was carried out at a 50-U/ml concentration (specific activity, $>5 \times 10^7$ U/mg; Boehringer, Mannheim, Germany). *N*-Acetyl-cysteine (NAC) or pyrrolidine-9-dithiocarbamate (PDTC) was added to the medium 150 min before IL-1 β stimulation. Cells were incubated with the FLAP inhibitor MK-886, a generous gift from J. Evans (Merck Frosst Centre for Therapeutic Research, Quebec, Canada) (40), for 10 min before IL-1 β stimulation, with phenylarsine oxide (PAO; Sigma) for 1 h prior to IL-1 β treatment, or with diphenyleneiodonium chloride (DPI; Sigma) or eicosatetraenoic acid (ETYA; Sigma) for 40 min before IL-1 β stimulation.

Immunoblots. Protein extracts (30 μ g) obtained by sodium dodecyl sulfate (SDS) lysis were separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After transfer to a nylon membrane (Immobilon-P; Millipore, Bedford, Mass.) and overnight blocking at 4°C with Tris-buffered saline-Tween (20 mM Tris [pH 7.5], 500 mM NaCl, 0.2% Tween 20 plus 5% dry milk), the membranes were incubated for 1 h with specific antibodies directed against human 5-LOX and FLAP (Merck Frosst Centre for Therapeutic Research), washed, and then incubated with the second peroxidase-conjugated antibody. The reaction was revealed by the enhanced chemiluminescence detection method (ECL kit; Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

Fluorescence measurement of intracellular ROIs. Formation of ROIs was measured using dichlorofluorescein diacetate (DFCH) (6). The cells were incubated in 175-cm² flasks with 20 μ M DCFH in phenol red-free medium 199. After 15 min, the medium was removed and the cells were washed and incubated with IL-1 β or H₂O₂ for 15 min. Fluorescence was measured by spectrofluorometry with excitation at 505 nm and detection of light emission at 525 nm. ROI concentrations in cytoplasmic extracts were measured as previously described (6) and expressed as a function of the protein content in nuclear extracts from the same cells. The measurements were performed on 3×10^6 cells for all cell types with the exception of selected CD20-positive cells, for which measurements were made on 1.2×10^6 cells.

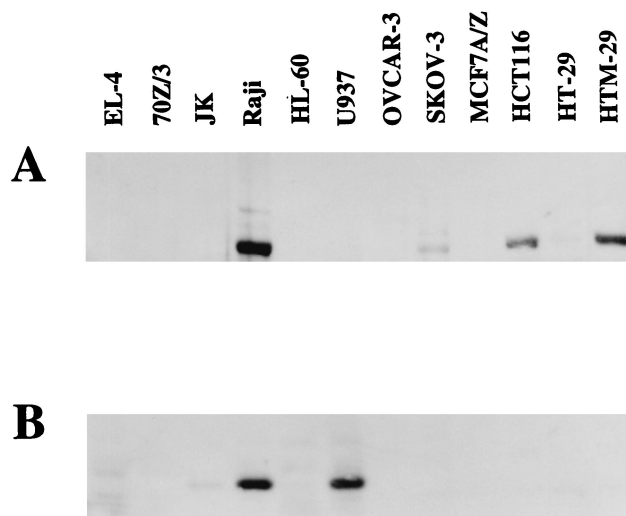


FIG. 1. 5-LOX and FLAP expression in cell lines. Protein extracts from various cell lines were prepared and analyzed for 5-LOX (A) and FLAP (B) expression by immunoblots revealed with specific antibodies. The 5-LOX antibody was directed against the human protein and could not detect the murine enzyme in EL-4 and 70Z/3 cells. The FLAP antibody could recognize both the human and the murine proteins, and the murine protein gave a faint and more slowly migrating band in EL-4 and 70Z/3 cells, which is visible after longer exposures of the gel.

Nuclear protein extraction and EMSA. Nuclear protein extracts were prepared as described (7). Briefly, the pelleted nuclei were resuspended in nuclear buffer (20 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 0.2 mM EDTA, 0.63 M NaCl, 25% glycerol, protease inhibitors [protease inhibitor kit; Boehringer]), incubated for 20 min at 4°C, and centrifuged for 30 min at 14,000 rpm in an Eppendorf 5415C centrifuge. Protein amounts were quantified with the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, Ill.). Electrophoretic mobility shift assays (EMSA) were performed as described (26). The palindromic (PD)- κ B probe was 5'-TTGGCAACGGCAGGGGAATCCCTCTCCTTA-3'.

Plasmids. The reporter plasmid HIV- κ B-CAT contained the two κ B sites from the HIV LTR cloned upstream of a chloramphenicol acetyltransferase (CAT) reporter gene (9). Expression vectors for wild-type and mutant RhoA, Rac1, and Cdc42 were generous gifts from L. Cross (National Institutes of Health, Bethesda, Md.) (18, 46). The 5-LOX and FLAP expression vectors were received from J. Evans (Merck Frosst Centre for Therapeutic Research). The pCMV-CD20 (cluster of differentiation 20 antigen) plasmid was kindly provided by Jim Koh (Laboratory of Molecular Oncology, Massachusetts General Hospital and Harvard Medical School, Cambridge, Mass.).

Transfections. Expression vectors (1 μ g) and the HIV- κ B-CAT reporter plasmid (4 μ g) were transfected into HCT-116 and MCF7 A/Z cells by using the DOTAP liposomal transfection reagent (Boehringer Mannheim). After 6 h, the DNA-containing medium was removed and replaced by fresh medium. Cells were then left untreated or were stimulated with 50 U of IL-1 β /ml for 6 h. Cellular extracts were prepared and CAT activity was determined as described previously (9).

U937 and THP-1 cells were transfected with DEAE-dextran (Amersham). Cells (8×10^5 cells) were incubated for 90 min at 37°C in 1 ml of Tris-buffered saline (pH 7.6) containing 5 μ g of total DNA and 400 μ g of DEAE-dextran. After 10% dimethyl sulfoxide was added for 2 min, 15 ml of Tris-buffered saline was added. Cells were centrifuged and resuspended in 1 ml of culture medium for 24 h. The IL-1 β stimulation was then performed after the addition of 2 ml of fresh medium.

For stable transfections, MCF7 A/Z cells were transfected with the linearized pDNA3 (10 μ g) empty vector or with the 5-LOX expression vector (10 μ g) (MCF7 A/Z LOX) by using the DOTAP system (Boehringer Mannheim). Forty-eight hours later, transfected cells were selected in a medium containing G418 at 0.5 mg/ml. After 15 days of selection, 10 G418-resistant colonies were isolated, grown separately, and selected for 5-LOX expression by immunoblotting.

Positive selection of CD20-positive cells. Cells transfected with the CD20 expression vectors were washed, incubated in the presence of a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 monoclonal antibody (Sanver Tech, Boeuhout, Belgium) for 30 min, washed again, and then incubated with magnetic beads coated with a monoclonal anti-FITC antibody (Miltenyi Biotec, Germany). The beads were loaded on columns in a magnetic field, and positive cells were finally eluted in culture medium.

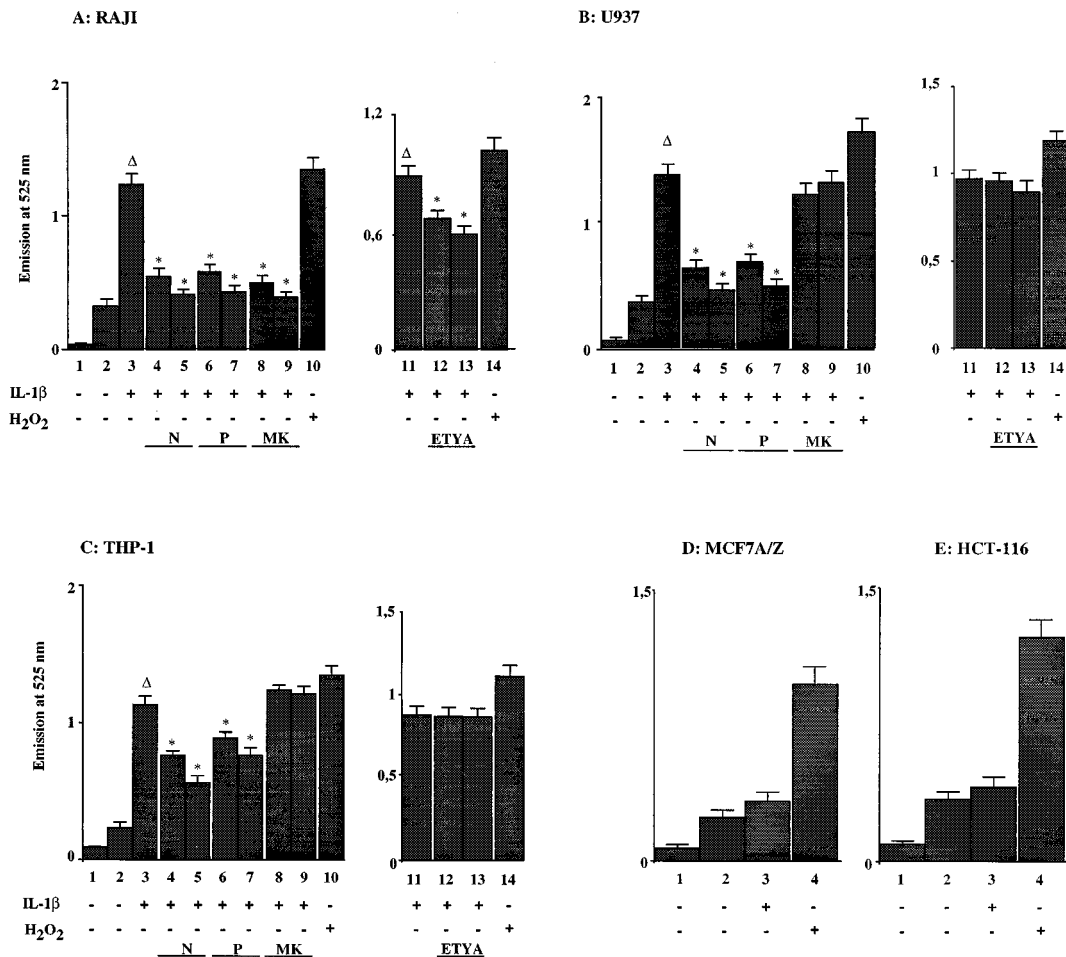


FIG. 2. Production of ROIs following IL-1 β stimulation. Formation of ROIs was measured using a DFCH probe in unstimulated and IL-1 β -stimulated cells. Panels A, B, and C show the relative fluorescence emission at 525 nm of the DFCH probe in Raji, U937, and THP-1 cells. Columns 1, background (no DFCH probe); columns 2, unstimulated cells; columns 3, stimulation with IL-1 β (50 U/ml); columns 4, as in columns 3 plus NAC (10 mM); columns 5, as in columns 3 plus NAC (20 mM); columns 6, as in columns 3 plus PDTC (60 μ M); columns 7, as in columns 3 plus PDTC (100 μ M); columns 8, as in columns 3 plus MK886 (0.5 μ M); columns 9, as in columns 3 plus MK886 (1 μ M); columns 11, stimulation with IL-1 β (50 U/ml); columns 12, as in columns 11 plus ETYA (35 μ M); columns 13, as in columns 11 plus ETYA (65 μ M); columns 10 and 14, stimulation with H₂O₂ (250 μ M). Panels D and E show the relative fluorescence emission at 525 nm of the DFCH probe in MCF7 A/Z and HCT-116 cells. Columns 1, background (no DFCH probe); columns 2, unstimulated cells; columns 3, stimulation with IL-1 β (50 U/ml); columns 4, stimulation with H₂O₂ (250 μ M). Asterisks indicate that the values were statistically different from the reference values (Δ).

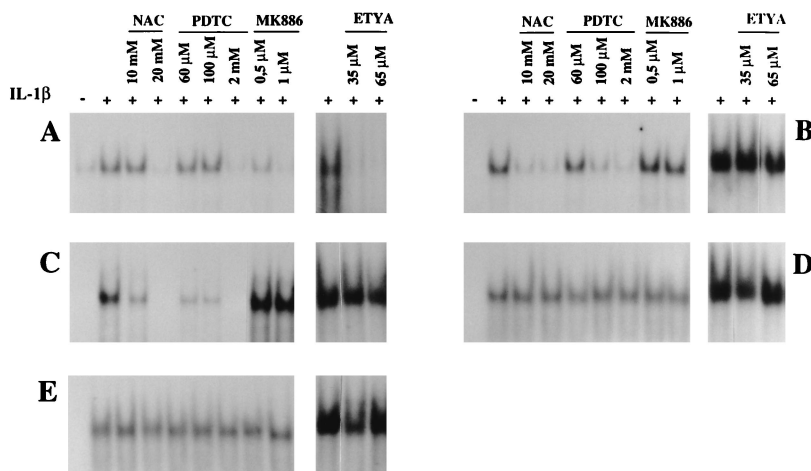


FIG. 3. ROI or 5-LOX inhibitors blocked NF- κ B activation by IL-1 β in a cell-specific manner. Nuclear extracts were prepared from Raji (A), U937 (B), THP-1 (C), HCT116 (D), or MCF7 A/Z (E) cells, either untreated or stimulated with IL-1 β (50 U/ml). The same cells were preincubated prior to IL-1 β stimulation with increasing concentrations of NAC, PDTC, MK886, or ETYA, as indicated in the figure. These extracts were analyzed by EMSA for binding to a specific κ B probe.

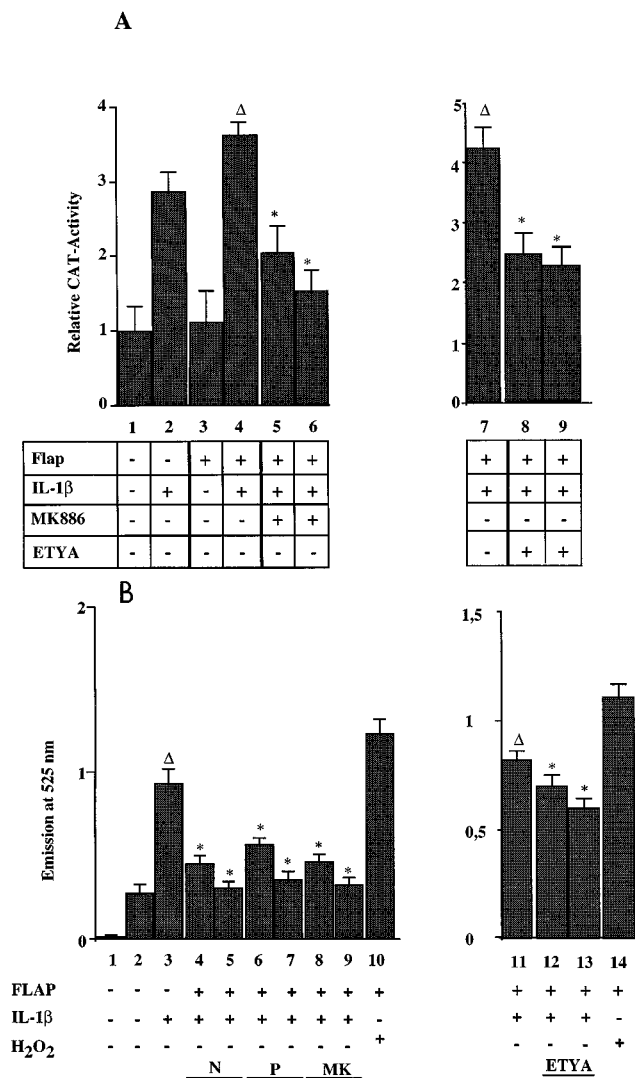


FIG. 5. Transfection of the FLAP expression vector restored ROI production and ROI-dependent NF- κ B activity in HCT-116 cells. (A) HCT-116 cells were transfected with the HIV- κ B-CAT reporter plasmid alone or together with a FLAP expression vector, and CAT activities were measured in unstimulated cells or in cells stimulated with IL-1 β for 6 h (50 U/ml), as indicated in the figure. The IL-1 β treatment was performed in the absence or in the presence of either the FLAP inhibitor MK886 at 0.5 μ M (column 5) and 1 μ M (column 6) or the 5-LOX inhibitor ETYA at 35 μ M (column 8) and 65 μ M (column 9). Each column represents the mean of three independent experiments (\pm SD). Asterisks indicate that values were statistically different from the reference values (Δ). (B) Formation of ROIs in HCT-116 cells transiently transfected with the FLAP expression vector. Column 1, background (no DFCH probe); column 2, unstimulated, FLAP transfected cells; column 3, stimulation with IL-1 β (50 U/ml); column 4, as in column 3 plus NAC (10 mM); column 5, as in column 3 plus NAC (20 mM); column 6, as in column 3 plus PDTC (60 μ M); column 7, as in column 3 plus PDTC (100 μ M); column 8, as in column 3 plus MK886 (0.5 μ M); column 9, as in column 3 plus MK886 (1 μ M); column 11, stimulation with IL-1 β (50 U/ml); column 12, as in column 11 plus ETYA (35 μ M); column 13, as in column 11 plus ETYA (65 μ M); columns 10 and 14, stimulation with H₂O₂ (250 μ M). Asterisks indicate that values are statistically different from the reference values (Δ).

29) (Fig. 1A). Using an antibody directed against the human 5-LOX, the protein was detected in Raji, SKOV-3, HCT-116, HT-29 (faint band), and HTM-29 cells but not in the murine cell lines. Moreover, we could not detect any 5-LOX expression in the THP-1 monocytic cells (data not shown). 5-LOX

mRNA expression was also detected by reverse transcription (RT)-PCR in the murine lymphoid cell lines 70Z/3 and EL-4 (reference 7 and data not shown). In other words, we could not observe any 5-LOX expression, either by immunoblotting or by RT-PCR, in monocytic cells or in breast or ovarian carcinoma cells.

Similarly, the FLAP protein was detected by Western blotting in the four analyzed lymphoid cell lines (Fig. 1). Its expression was high in Raji cells and considerably lower in Jurkat cells. A faint and more slowly migrating specific band was also detected with the human antibody in the murine cell lines EL-4 and 70Z/3. The expression of FLAP was not observed in any of the adenocarcinoma cell lines. Among monocytic cell lines, FLAP was expressed in U937 and THP-1 cells but not in HL-60 cells (Fig. 1 and data not shown). We could thus conclude that only the lymphoid cells expressed simultaneously 5-LOX and FLAP proteins and could therefore demonstrate 5-LOX enzymatic activity.

ROI production following IL-1 β stimulation. We previously reported that IL-1 β stimulation led to the generation of ROIs in lymphoid cells but not in epithelial transformed cells (6). In order to determine whether ROI production was due to 5-LOX activity, U937, Raji, MCF7 A/Z, and HCT-116 cells were stimulated with IL-1 β (50 U/ml) for 15 min and analyzed with a DFCH probe for ROI detection (Fig. 2). Under these conditions, IL-1 β treatment of Raji cells led to the production of ROIs (Fig. 2A; compare columns 2 and 3). As expected, preincubation of Raji cells with the antioxidant NAC or PDTC abolished ROI accumulation (columns 4 to 7). Interestingly, preincubation of Raji cells prior to IL-1 β stimulation with MK886, a specific inhibitor of FLAP (40) (columns 8 and 9), or with the 5-LOX inhibitor ETYA (columns 12 and 13) completely abolished or markedly decreased ROI production, confirming that 5-LOX activity was required for IL-1 β -induced intracellular oxidative stress in lymphoid cells. As a positive control, we measured ROI production in H₂O₂-treated cells (250 μ M) (columns 10 and 14); MK886 did not influence the ROI production after H₂O₂ treatment, indicating that this inhibitor exerts a specific action (data not shown).

Stimulation of U937 or THP-1 monocytic cells with IL-1 β also generated ROIs (Fig. 2B and C; compare columns 2 and 3). Again, the production of ROIs was completely or partially abolished by preincubation of these cells with the antioxidant NAC or PDTC (columns 4 to 7). Similar data were obtained with HL60 cells (data not shown). However, contrasting with the observation made with Raji cells, MK886 and ETYA failed to block ROI induction in monocytic cells (columns 8, 9, 12, and 13), demonstrating that 5-LOX activity was not responsible for this production.

Stimulation of HCT116 or MCF7 A/Z cells with IL-1 β did not generate any significant ROI production (Fig. 2D and E; compare columns 2, without IL-1 β , and columns 3, in the presence of IL-1 β). The last observation confirmed our previously reported data obtained with ovarian carcinoma OVCAR-3 cells (6), indicating that IL-1 β did not generate any oxidative stress in epithelial transformed cells.

Induction of NF- κ B following IL-1 β stimulation. Several reports stated that NF- κ B induction following a number of stimuli depended on ROI production (50–52). However, we reported that IL-1 β induced NF- κ B in adenocarcinoma cells independently of any cellular oxidative stress (6), an observation confirmed by the experiments described in Fig. 2. We therefore further evaluated the role of 5-LOX activity in NF- κ B activation after IL-1 β stimulation of lymphoid or monocytic cells. Stimulation of Raji, U937, or THP-1 cells with IL-1 β induced nuclear NF- κ B DNA-binding activity as dem-

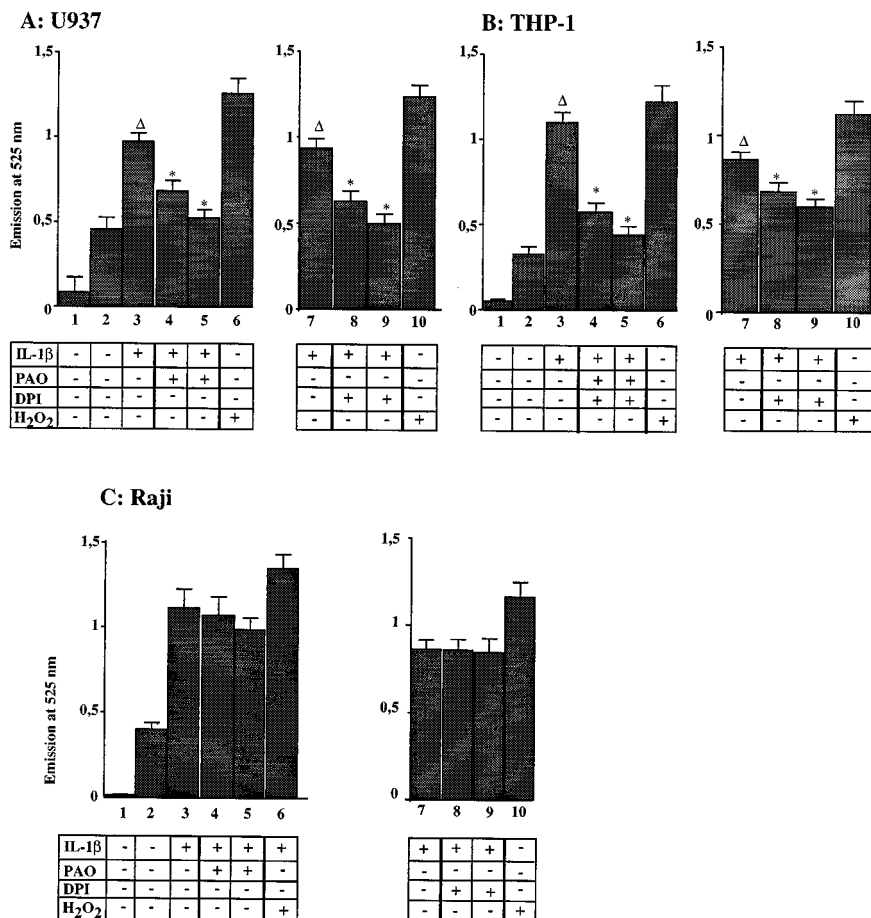


FIG. 6. NADPH oxidase blocked ROI production induced by IL-1β stimulation in monocytic cells. Formation of ROIs was measured by using a DFCH probe in U937 (A), THP-1 (B), and Raji (C) cells after IL-1β stimulation, with and without preincubation in the presence of the NADPH oxidase inhibitors DPI and PAO. Columns 1, background (no DFCH probe); columns 2, unstimulated cells; columns 3, stimulation with IL-1β (50 U/ml); columns 4, as in columns 3 plus PAO (30 μg/ml); columns 5, as in columns 3 plus PAO (60 μg/ml); columns 7, stimulation with IL-1β (50 U/ml); columns 8, as in columns 7 plus DPI (10 μM); columns 9, as in columns 7 plus DPI (30 μM); columns 6 and 10, stimulation with H₂O₂ (250 μM). Asterisks indicate that values are statistically different from the reference values (Δ).

onstrated by EMSA (Fig. 3A, B, and C). Pretreatment with antioxidant NAC or PDTC completely abolished NF-κB activation in the three cell lines. However, pretreatment with the FLAP inhibitor MK-886 or the 5-LOX inhibitor ETYA inhibited IL-1β-dependent NF-κB activation in Raji cells (Fig. 3A) while it did not affect this induction in U937 or THP-1 cells (Fig. 3B and C). Similarly, antioxidants, MK-866, or ETYA failed to abolish NF-κB activation by IL-1β in MCF7 A/Z and HCT-116 cells (Fig. 3D and E), confirming our previous observation in OVCAR-3 and SKOV-3 epithelial cells (6, 7). These data thus indicate that 5-LOX activity plays an important role in ROI production and NF-κB activation in lymphoid cells but not in monocytic or epithelial cells.

Reconstitution of the 5-LOX/FLAP pathway in adenocarcinoma cells. In order to confirm the data obtained with EMSAs, MCF7 A/Z cells were transfected with a reporter plasmid containing a CAT gene driven by the two κB sites from the HIV LTR (HIV-κB-CAT) and then stimulated with IL-1β. Such a treatment induced CAT activity three- to fourfold over the basal activity observed in untreated control cells (Fig. 4A). Cotransfection of the FLAP expression vector did not allow any further induction of CAT activity following IL-1β stimulation (columns 3 and 4). Moreover, the 5-LOX inhibitors

MK-886 and ETYA failed to block IL-1β induction of NF-κB-dependent transcription in either FLAP-expressing (Fig. 4A, columns 5, 6, 8, and 9) or nonexpressing cells (data not shown).

The same experiment was then performed in MCF7 A/Z cells stably transfected with a 5-LOX expression vector (MCF7 A/Z-LOX). In these cells, IL-1β induced NF-κB transcriptional activity and this activity was not modified in the presence of MK886 (Fig. 4B and data not shown). Transient transfection of MCF7 A/Z-LOX cells with the FLAP expression vector led to a small increase of the IL-1β-induced CAT activity (Fig. 4B, column 4). In the presence of FLAP, treatment of MCF7 A/Z-LOX cells with MK-886 or ETYA completely abolished IL-1β-dependent transcriptional activity (columns 5, 6, 8, and 9).

Consistently with these CAT assays, transient transfections of MCF7 A/Z cells with the FLAP expression vector did not allow any generation of ROIs, even after IL-1β stimulation (Fig. 4C; compare columns 2 and 3). However, transient transfection of the FLAP expression vector in MCF7 A/Z-LOX cells led to a marked increase in ROI production after IL-1β treatment (Fig. 4D; compare columns 4 and 5) while stable expression of 5-LOX alone was not sufficient for such an effect (columns 2 and 3). As expected, NAC, PDTC, MK886, and

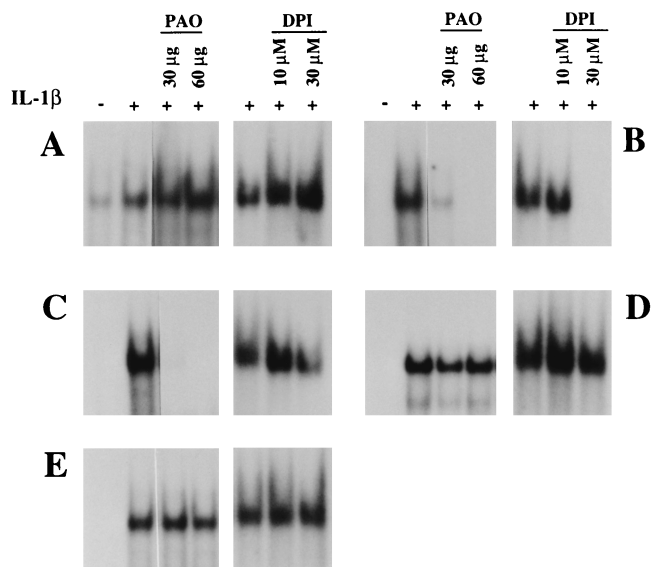


FIG. 7. NADPH oxidase inhibitors blocked NF- κ B activation by IL-1 β in monocytic cells. Nuclear extracts were prepared from Raji (A), U937 (B), THP-1 (C), HCT116 (D), or MCF7 A/Z (E) cells, either untreated or stimulated with IL-1 β (50 U/ml). The same cells were preincubated for 1 h prior to IL-1 β stimulation with increasing concentrations of DPI or PAO, as indicated in the figure. These extracts were analyzed by EMSA for binding to a specific κ B probe.

ETYA reduced ROI production in MCF7 A/Z cells transfected stably with the 5-LOX expression vector and transiently with the FLAP expression vector (columns 6 to 11, 14, and 15).

In HCT-116 cells, which express the 5-LOX enzyme but not the FLAP protein, IL-1 β stimulated threefold the NF- κ B-dependent CAT activity (Fig. 5A) and this stimulation was not blocked by MK886 (data not shown). Again, after cotransfection of the FLAP expression vector, we observed a small increase in the transcription induced by IL-1 β (column 4), an effect which was inhibited by preincubation of the cells with MK886 (columns 5 and 6) or ETYA (columns 8 and 9). Similarly, transient transfection of the FLAP expression vector in HCT-116 cells was sufficient to allow the production of ROIs after IL-1 β stimulation (Fig. 5B), a production which was inhibited by preincubation with NAC, PDTC, MK886, or ETYA.

We can thus conclude that coexpression of 5-LOX and FLAP is required to restore the 5-LOX-dependent pathway leading to production of ROIs and consequent NF- κ B activation.

NADPH oxidase is required for ROI production in IL-1 β -treated monocytic cells. Monocytic cells such as U937 or THP-1 cells produce ROIs in response to IL-1 β stimulation but do not express 5-LOX. They must therefore rely on another source for ROI production, and this source was deemed most likely to be NADPH oxidase.

We first investigated whether NADPH oxidase inhibitors could block ROI production in IL-1 β -stimulated monocytic cells. As already shown in Fig. 2, IL-1 β treatment induced ROIs in THP-1 and U937 cells (Fig. 6A and B; compare columns 2 and 3). Preincubation of these cells with DPI or PAO inhibited in a dose-dependent manner IL-1 β -induced ROI production (Fig. 6A and B, columns 4, 5, 8, and 9). Interestingly, preincubation of Raji cells with the same NADPH oxidase inhibitors did not block ROI production (Fig. 6C).

To investigate whether NADPH oxidase was involved in

NF- κ B activation by IL-1 β , nuclear extracts were prepared from cells preincubated with the same inhibitor, DPI or PAO, prior to IL-1 β stimulation. These inhibitors did not modify NF- κ B activation induced by IL-1 β in Raji, MCF7 A/Z, or HCT-116 cells, as demonstrated on EMSAs (Fig. 7A, D, and E). However, they abolished in a dose-dependent manner the induction of NF- κ B in U937 and THP-1 monocytic cells (Fig. 7B and C), indicating that NADPH oxidase activity was required for the IL-1 β -dependent signaling pathway leading to I κ B α degradation in these cells.

Rac and Cdc42 are required for ROI production and NF- κ B activation in IL-1 β -treated monocytic cells. Rho, Rac1, and Cdc42, all members of the Ras superfamily of GTPases, have been noted to play a role in several transduction processes, including the JNK/SAPK and MAPK pathways (60). More recently, it has been reported that Rho, Rac1, and Cdc42 are involved in NF- κ B induction by TNF- α or bradykinin (45, 46). The NADPH oxidase component p67^{phox} associates with Cdc42/Rac, and Rac1 is required for the activation of NADPH oxidase (1, 5, 21).

Therefore, we investigated whether these GTPases are involved in IL-1 β -induced NF- κ B activation in adenocarcinoma or monocytic cells. MCF7 A/Z cells were cotransfected with the HIV- κ B-CAT reporter plasmid together with expression vectors coding for wild-type or dominant-negative RhoA, Rac1, or Cdc42 (Fig. 8A). Again, IL-1 β stimulation induced three- to four-fold the basal CAT activity. This transcriptional activation was not modified in the presence of wild-type or mutant Rho, Rac1, or Cdc42 proteins. Similarly, treatment of MCF7 A/Z cells with TNF- α or arachidonic acid also activated NF- κ B-dependent transcription of the CAT reporter gene. Again, the expression of wild-type or mutant GTPases did not modify this induction (data not shown). We thus concluded that RhoA, Rac1, and Cdc42 are not required for NF- κ B activation by IL-1 β , TNF- α , or arachidonic acid in MCF7 A/Z cells.

In monocytic cells (THP-1 and U937), however, while transfection of expression vectors for wild-type RhoA, Rac1, or Cdc42 did not modify transcription of an NF- κ B-dependent reporter plasmid, the expression of the Rac1 and Cdc42 dominant-negative mutants almost completely inhibited IL-1 β -induced NF- κ B-dependent gene transcription (Fig. 8B and C).

In order to demonstrate that Cdc42 and Rac1 dominant-negative mutants could block ROI production in monocytic cells, U937 cells were transiently transfected with expression vectors coding for these mutants or the wild-type enzymes. Since the transfection efficiency was very low and did not allow the detection of any difference in ROI production, these cells were then cotransfected with the mentioned plasmids together with an expression vector coding for the CD20 antigen. Transfected cells were purified through two rounds of positive selection with an anti-CD20 monoclonal antibody, first by MACS and then by flow cytometry. Purified transfected cells (1.2×10^6 cells) were then treated with IL-1 β , and ROI production was measured as described above. Under these conditions, IL-1 β stimulation generated ROIs (Fig. 9). This production of ROIs was not modified by the expression of wild-type Cdc42 or Rac1 enzyme, while it was significantly reduced by the two dominant-negative mutants, confirming their implied roles in induced ROI production in monocytic cells.

DISCUSSION

The signaling pathways leading to NF- κ B activation following cellular stimulation with proinflammatory cytokines have been the subject of many investigations but remain a matter of

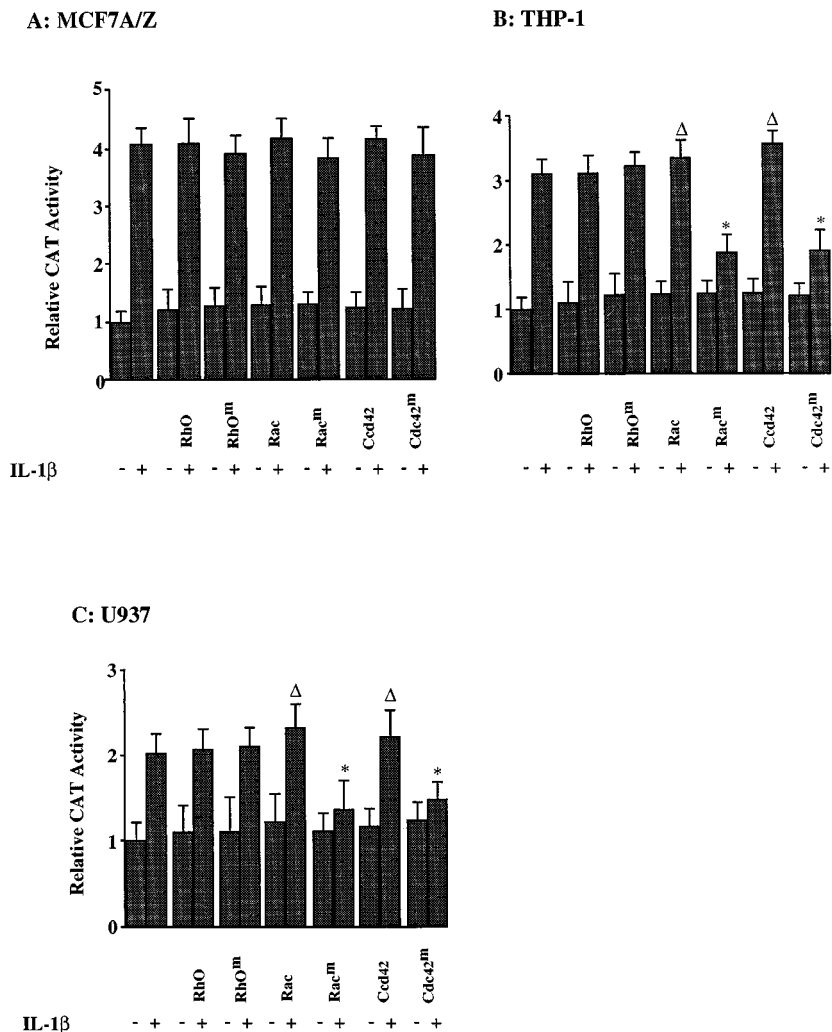


FIG. 8. GTPases are required for NF-κB transcriptional activity in monocytic cells. MCF7 A/Z (A), THP-1 (B), or U937 (C) cells were transfected with the HIV-κB-CAT reporter plasmid alone or together with expression vectors for wild-type or dominant-negative RhoA, Rac1, and Cdc42. Cells were either untreated (–) or stimulated with IL-1β (+) for 6 h, and CAT activities were measured. Each column represents the mean of three independent experiments (± SD). Asterisks indicate that values are statistically different from the reference values (Δ).

discussion. The recent discovery of IκBα kinases (IKK-α, IKK-β, and IKK-γ) and their activation by the NIK kinase led to the hypothesis that TNF-α or IL-1β receptors are connected to IκBα through TRAF adapter proteins, NIK and IKKs (20, 33, 39, 42, 56, 66). However, such a model did not incorporate the results of previous studies indicating the role of other signaling intermediates such as ROIs, ceramide, or Rho proteins and left unidentified other members of the large signaling complex. These discrepancies could be explained by cell type specificities. Indeed, most of the studies reported so far investigated a single cell line, and the models derived from such experiments might not be confirmed in different settings.

We previously reported that ROIs and the PKC λ/ι isoform play a cell-specific role in NF-κB activation by IL-1β or TNF-α (6–8). In this study, we explored the sources of ROIs in different cell types and their roles in NF-κB activation by IL-1β. At least three different pathways that lead to induction of nuclear NF-κB DNA-binding activity and transactivation of a reporter gene after cellular treatment with IL-1β can be identified (Fig. 10). Lymphoid cells, such as Raji or 70Z/3 cells,

express 5-LOX and its activating protein FLAP, and 5-LOX enzymatic activity is required for ROI production and NF-κB activation following stimulation by IL-1β. In these cells, the ROIs are essential for IκBα degradation and NF-κB activation (6) but it is not known whether ROIs can directly or indirectly activate the IKK kinases or whether other IκBα kinases are involved.

Epithelial cells do not express the 5-LOX/FLAP enzymatic complex and do not produce ROIs in response to IL-1β or TNF-α. Reconstitution of the 5-LOX/FLAP system in these cells showed that, despite high catalase activity (7), this enzymatic activity was sufficient to restore ROI production and ROI-dependent NF-κB activation. In other words, once the 5-LOX pathway is functional, it becomes predominant. These data, as well as those obtained with monocytic and lymphoid cells, suggest that the ROIs might activate the signalsome. In the absence of 5-LOX, as we had previously reported, an alternative pathway which involves the activation of acid sphingomyelinase and the production of ceramide could be essential

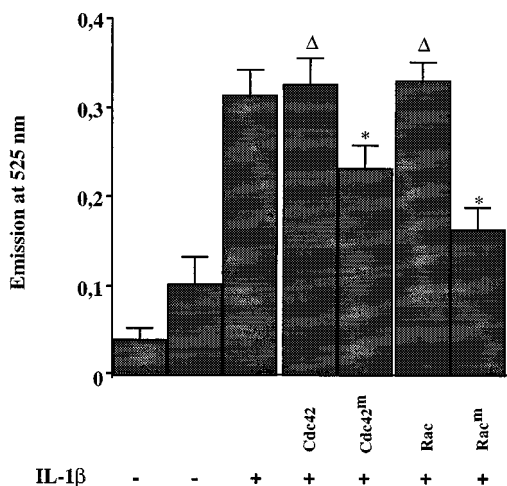


FIG. 9. GTPases are required for ROI production in response to IL-1β in monocytic cells. U937 cells were transfected with the CD20 expression vector and with plasmids coding for wild-type (Cdc42 and Rac) or dominant-negative (Cdc42^m and Rac^m) Rac1 and Cdc42. CD20-expressing transfected cells were positively selected with an anti-CD20 monoclonal antibody through MACS and flow cytometry cell sorting. Selected cells were either untreated (-) or stimulated with IL-1β (+) for 15 min, and formation of ROIs was measured by using a DFCH probe. Asterisks indicate that values are statistically different from the reference values (Δ).

for NF-κB activation by IL-1β in epithelial cells (7), but such an hypothesis remains to be confirmed by genetic studies.

Finally, monocytic cells (THP-1 or U937 cells) do not express the 5-LOX enzyme but produce ROIs in response to IL-1β or TNF-α. In these cells, ROIs are generated by the NADPH oxidase and are required for NF-κB activation by proinflammatory cytokines. Moreover, the induction of NF-κB by IL-1β, TNF-α, or arachidonic acid in monocytic cells also requires the activity of small GTPases. We concluded that Rho and Rac proteins stimulated ROI production through NADPH

oxidase activation in macrophages/monocytes. As for lymphoid cells, we do not know how ROIs are connected to IκBα kinases.

These data thus disclosed two distinct, cell-specific sources of ROIs, which are each required for NF-κB activation by proinflammatory cytokines, and a third pathway leading to NF-κB activation independently of ROI production (Fig. 10). Such cell specificity might be explained by the association of distinct proteins with the IL-1 receptor. Indeed, IL-1R associates with TRAF6, MyD88, IRAK, and IRAK-2 proteins, which are all required for NF-κB activation (14–16, 31, 44, 61). Moreover, other proteins might also associate with this receptor. It is possible that the expression of each of these receptor-associated adapter proteins or kinases is cell specific and therefore initiates distinct transduction pathways. Indeed, it has been shown that, although TRAF6, MyD88, IRAK, and IRAK-2 mRNAs could be detected in most cell types and organs, their levels of expression vary considerably (15, 16, 28, 44). It would be most interesting to determine whether different proteins are associated with the IL-1 type I receptor in lymphoid, monocytic, or epithelial cells and play specific, cell type-related, signaling roles.

These signaling pathways leading to NF-κB activation might be related to distinct IL-1β biological activities in different cell types. The nature of the activated NF-κB complexes might be different for each pathway and lead to the transcription of distinct target genes. Each pathway is also controlled differently. For instance, antioxidant cellular defenses could regulate pathways involving ROI production while distinct phosphatases could dephosphorylate kinase substrates and specifically regulate signal transduction. Finally, the activation of distinct pathways leads to the concomitant activation of NF-κB and other specific transcription factors. In a given cellular context, the activation of a specific set of transcription factors, including NF-κB, certainly regulates the expression of distinct target genes and thus allows an appropriate cellular response to the applied stimulus. Further experiments are required in order to identify NF-κB target genes specifically induced by IL-1β in lymphoid, monocytic, or epithelial cells and to determine cell-specific mechanisms of regulation for each of them.

It has been shown that IL-1β can exert pro- or anti-apoptotic activities in HeLa and L929 cells (27). Moreover, high IL-1β levels have been measured in the sera of patients with ovarian adenocarcinomas (67). It is therefore crucial to better understand the role of IL-1β in normal and transformed epithelial cells and to identify the biochemical pathways mediating its biological activities.

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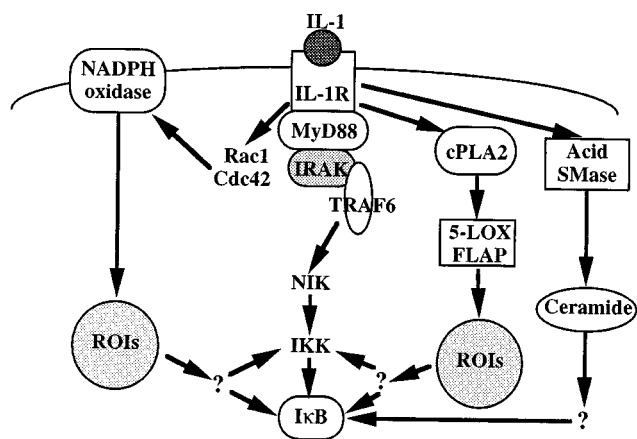


FIG. 10. Signaling pathways for NF-κB activation by IL-1β. Following the interaction of IL-1 with the type I IL-1 receptor, the NIK-IKK pathway can be activated through the MyD88, IRAK, and TRAF6 signaling proteins. Alternatively, Rac1 and Cdc42 can activate the NADPH oxidase complex and induce NF-κB activity through the production of ROIs. Other pathways involve, in lymphoid cells, the cytoplasmic phospholipase A2, the 5-LOX, and FLAP complex and the production of ROIs or, in epithelial cells, the acid sphingomyelinase (SMase) and the production of ceramide. CPLA2, cytosolic phospholipase A2.

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