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Original Contribution

IMPAIRMENT OF THE MITOCHONDRIAL ELECTRON CHAIN TRANSPORT PREVENTS NF-κB ACTIVATION BY HYDROGEN PEROXIDE

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Abstract—A large body of work has been devoted to mechanisms leading to the activation of the transcription factor NF-κB in various cell types. Several studies have indicated that NF-κB activation by numerous stimuli depends on the intracellular generation of reactive oxygen species (ROS). In this report, we first demonstrated that inhibition of the electron transport chain by either rotenone or antimycine A gave rise to dose-dependent inhibition of NF-κB translocation induced by 150 μM of hydrogen peroxide (H_2O_2). Conversely, the impairment of the mitochondrial respiratory chain did not affect T lymphocyte treatment by TNF-α (tumor necrosis factor α) or pre-B lymphocyte treatment with LPS (lipopolysaccharide). We also showed that oligomycine which inhibits ATP synthase and FCCP, which uncouples respiration also led to dose-dependent inhibition of NF-κB activation by H_2O_2. All these inhibitors were also shown to inhibit mitochondrial respiration in lymphocytes assessed by oxygen comsuption. Although only a transient drop in ATP concentration was observed when lymphocytes were treated by H_2O_2, this effect was remarkably reinforced in the presence of oligomycine demonstrating the crucial role of ATP in the signal transduction pathway induced by H_2O_2. © 1998 Elsevier Science Inc.

Keywords—Hydrogen peroxide, NF-κB, Lymphocytes, Signal transduction, Free radical

INTRODUCTION

NF-κB (Nuclear Factor-κB) is a rapidly inducible transcription factor involved in the response of various cell types to a number of external or internal stimuli. Following its intracellular activation, NF-κB regulates the expression of many genes that code for cytokines, growth factors, acute phase response proteins, or cellular receptors, and thus modulates the cellular response to the applied stimulus.[1–3] Several viruses such as the Human Immunodeficiency Virus type-1 (HIV-1) or the cytomegalovirus also depend on NF-κB for their intracellular replication.[1–3]

NF-κB complexes bind DNA as dimers constituted from a family of proteins designated as the Rel/NF-κB family. In mammals, this family contains the proteins p50, p52, p65 (RelA), RelB, and c-Rel (Rel).[1–3] These five proteins harbor a related, but nonidentical 300 amino acid-long Rel homology domain (RelHD) that is responsible for dimerization, nuclear translocation, and specific DNA-binding. In addition, RelA, RelB, and c-Rel, but not p50 or p52, contain one or two transactivating domains. p50 and p52 derive from cytoplasmic precursors named p105 and p100, respectively. NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family.[4–8] The members of the IκB family are IκB-α, IκB-β, IκB-ε, p100, and p105.[4–8]

Following various stimuli, including the interaction of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) with their receptors, IκB-α is first phosphorylated on serines 32 and 36, then ubiquitinated at lysines 21 and 22, and rapidly degraded by the proteasome, allowing NF-κB nuclear translocation and gene activation.[9–13] In the case of these two types of cytokines, the signal transduction pathways leading to the phosphorylation and degradation of IκB proteins has been recently clarified in HeLa and in L293 cells.[14–18] It is included into a 700–900 kDa complex called signalosome, whose important partners are proteins associated to the TNF-α or IL-1 receptors, NIK and IKK-1 and −2.[14–18] Proinflammatory cytokines such as TNF-α or IL-1β or the bacte-

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rial outer membrane component (LPS) are potent activators of NF-κB, which mediates several of their biological activities such as stimulation of the transcription in lymphocytes through the intracellular generation of an oxidative stress.[19–21] However, the assumption that a similar mechanism is effective in other cell lines such as lymphocytes has not yet been demonstrated. Up to now, the mechanism by which ROS activate NF-κB translocation or how ROS feed in the signalosome pathway is still unknown.

In this work, we have demonstrated that NF-κB activation in human transformed T lymphocytes (Jurkat JR cells) stimulated with 150 μM of H_2O_2 can be strongly inhibited when the mitochondrial respiration is impaired. NF-κB activation in similar conditions by other stimuli such as TNF-α or LPS was not affected by these respiration inhibitors. These data demonstrate that in T lymphocytes, NF-κB translocation mediated by H_2O_2 strongly relies on ATP generated by mitochondria and that H_2O_2 could promote NF-κB translocation by a mechanism involving other components than those present in the signalosome.

MATERIALS AND METHODS

Cell culture and biological reagents

The human T lymphoid cell line Jurkat JR and the murine pre-B 70Z/3 cells were grown in RPMI 1640 medium + ultraglutamine I + 25 mM Hepes (Bio-Whittaker, Belgium), 10% fetal bovine serum (Gibco BRL) and 50 μM β-mercaptoethanol.

NF-κB inducers were usually added in the following final concentrations: H_2O_2 (Merck, Germany) at 150 μM in RPMI medium, LPS (Sigma, St. Louis, Mo) from *E. coli*, serotype 0111:B4 at 5 mg/ml in sterile water and TNF-α (Boeringher Mannheim, Germany) at 100 U/ml in RPMI medium. Before any stimulation, cells were grown in a medium without β-mercaptoethanol for 24 h, and adjusted at a concentration of 10^6 cells/ml.

The complexe I inhibitor rotenone (Sigma) was diluted in acetone; the complexe III inhibitors antimycine A (from *Streptomyces*, Sigma) and myxothiazol (from *Myxobacterium myxoccocuc fulvus*, Sigma) were diluted in ethanol; the mitochondrial uncoupler FCCP (Sigma) was diluted in ethanol; the ATPase inhibitor oligomycine (from *S. diastatochromogenes*, Sigma) was diluted in ethanol and the glycolysis inhibitor iodoacetic acid (IAA) (Sigma) was diluted in sterile water.

Nuclear protein extraction

Cells (5×10^6) were harverested and washed in 1-ml cold PBS (137 mM NaCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 2.7 mM KCl; pH 7.4). Cells were centrifuged at 15,000 × *g* for 15 s and resuspended in 400 μl of cold hypotonic buffer (10 mM HEPES–KOH; 2 mM $MgCl_2$; 0.1 mM EDTA; 10 mM KCl; 1 mM DTT; 0.1 mM PMSF; pH 7.9). Cells were allowed to swell on ice for 10 min and then vortexed for 2 or 3 s. Suspension was then centrifuged at 15,000 × *g* for 30 s and the pellets of nuclei were resuspended in 25 μl of cold hypertonic buffer (50 mM HEPES–KOH; 2 mM $MgCl_2$; 0.1 mM EDTA; 300 mM NaCl; 1 mM DTT; 0.1 mM PMSF; pH 7.9) and left on ice for 20 min. After centrifugation (15,000 × *g* for 5 min at 4°C), aliquots of supernatant, containing the nuclear proteins, were stored at −80°C.

Cytoplasmic protein extraction

Cytoplasmic extracts from 10^7 cells were prepared at various time after H_2O_2 treatment. Cells were harverested and washed in 1 ml cold PBS (137 mM NaCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 2.7 mM KCl; pH 7.4). Cells were centrifuged at 15,000 × *g* for 15 s and resuspended in 1 ml of cold washing solution (10 mM HEPES–KOH; 2 mM $MgCl_2$; 0.1 mM EDTA; 20 mM KCl; 1 mM DTT; 1 mM PMSF; pH 7.9). Cells were centrifuged at 15,000 × *g* for 15 s and resuspended in 400 μl of cold hypotonic buffer (10 mM HEPES–KOH; 2 mM $MgCl_2$; 0.1 mM EDTA; 10 mM KCl; 1 mM DTT; 0.1 mM PMSF; igepal 0.2% (v/v), pH 7.9) supplemented with antiproteases (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). Cells were allowed to swell on ice for 30 s. Cellular debris were removed by centrifugation (5 min at 6,000 × *g*) and the supernatant fraction was ultracentrifuged for 1 h with 11% of extraction buffer (0.3 M HEPES; 1.4 M KCl; 30 mM $MgCl_2$; pH 7.9). The supernatant containing cytoplasmic proteins was stored at −80°C. Protein concentrations were measured by the Bradford method with the reagent supplied by Biorad (Belgium).

Electrophoretic mobility shift assays

Electrophoretic Mobility Shift Assays (EMSA) and supershifting analysis were performed as described.[22] Briefly, 5 μg of nuclear proteins were incubated for 30 min at room temperature with 0.2 nM of ^{32}P-labeled oligonucleotidic probe, 2 μg of BSA, and 1.25 μg of poly (dI-dC) · poly (dI-dC) (Pharmacia Biotech, The Netherlands) and binding buffer (20 mM HEPES–KOH, 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$; 1 mM DTT, pH 7.9) in a final volume of 10 μl. DNA–protein complexes were then resolved on a nondenaturing 6% polyacrilamide gel run for 4 h at 180 V in TBE. The gel was then dried and autoradiographied on a

Fuji X-ray film (General Electrics, Belgium). The gels were also analyzed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

The probe (Eurogenetec, Belgium) contains the sequence of NF-κB from the LTR of HIV-1: 5′-GGTTACAAGGGACTTTCCGCTG-3′. The oligonucleotide probe was labeled by filling in with the Klenow DNA polymerase (Boehringer Manheim, Germany). Probe (100 ng) was labeled with 3 μCi of (α-^{32}P)-dATP and (α-^{32}P)-dCTP (3000 Ci/mmol; Du Pont de Nemours International, Belgium) and unlabelled dTTP and dGTP (Boehringer Mannheim, Germany), then purified on a Sephadex G-25 (Pharmacia Biotech, The Netherlands) column and stored at −20°C until use. Specific radioactivity was always $>10^8$ cpm/μg.

Immunoblots

The IκB-α protein were detected by Western blot analysis using specific polyclonal antibody (Upstate Biotechnology, USA). Cytoplasmic protein extracts (10 μg) were added to loading buffer [10 mM Tris/HCl pH 6.8, 1% (w/v) SDS, 25% (v/v) glycerol, 0.1 mM β-mercaptoethanol, 0.03% (w/v) bromophenol blue], boiled, and electrophoresed on a 12% SDS-PAGE gel. After transfer to a nylon membrane (Immobilon-P, Millipore, Bedford, MA) and blocking overnight at 4°C with Phosphate-Buffered Saline–Tween [3.78 mM NaH_2PO_4; 16 mM K_2HPO_4; 0.15 mM NaCl; 0.1% (v/v) Tween 20] plus 5% dry milk, the membranes were incubated for 1 h with the first antibody (1:2000 dilution), washed, and then incubated with the second peroxidase-conjugated antibody. The reaction was revealed with the enhanced chemoluminescence detection method (ECL kit, Amersham, UK).

Oxygen consumption

Jurkat JR cells were harverested and resuspended in PBS (137 mM NaCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 2.7 mM KCl; pH 7.4) at 10^6 cells/ml. PBS was equilibrated at 37°C with atmospheric oxygen for 1 night. Rotenone, antimycine A, oligomycine or FCCP were added in the sample chamber and oxygen consumption was measured with a Clark electrode fitted in a 2-ml thermojacketed sample chamber (37°C) under constant stirring. Mitochondrial inhibitors were added at the following final concentration: rotenone at 1, 20, or 140 nM; antimycine A at 0.625, 2.5, or 5 nM; oligomycine at 1.5, 4.5, or 9 nM; FCCP at 10, 600, or 3000 nM.

ATP measurements

Cells (10^7) were harverested after incubation with H_2O_2 and either oligomycine or IAA and washed in 1 ml PBS (137 mM NaCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 2.7 mM KCl; pH 7.4). Cells were centrifuged and resuspended in 10 μl of PBS, which were rapidly injected in 120 μl boiling TE (500 mM Tris, 20 mM EDTA, pH 7.6) to lyse cells and denature the enzymes that could consume ATP. Cells were boiled for 3 min, and debris were removed by centrifugation (3 min at 15,000 × *g*). ATP content was measured by the luciferine–luciferase method as following: 100 μl of luciferine–luciferase (Sigma) diluted in TE at 5 mg/ml were injected with 120 μl of the ATP containing extracts. The measure of light emitted was done in a luminometer (Lumat LB 9501, Bertold, Germany) for 10 s.

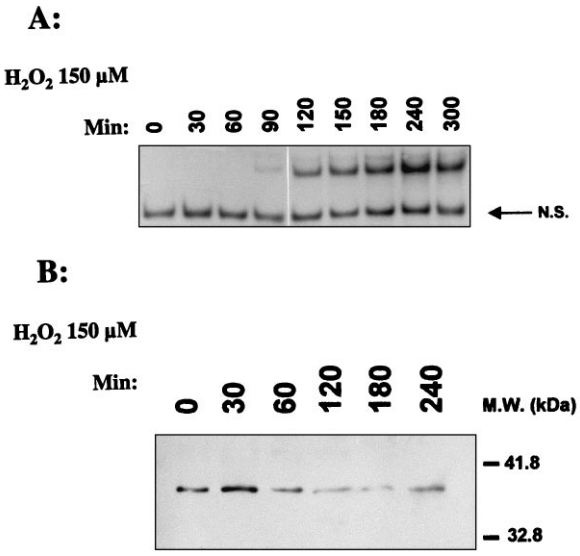

Fig. 1. (A) NF-κB activation in Jurkat JR cells after stimulation with H_2O_2. Jurkat JR cells were stimulated with 150 μM H_2O_2 for the indicated times. Nuclear extracts from these cells were analyzed by EMSA with a labeled probe corresponding to a synthetic κB site. The arrow indicates unspecific NF-κB complexes. (B) Degradation of IκB-α in Jurkat JR cells treated by 150 μM H_2O_2. Immunoblots were performed with cytoplasmic extracts from Jurkat JR cells unstimulated (0) or treated with 150 μM H_2O_2 for the indicated times and revealed with a polyclonal anti IκB-α antibody.

RESULTS

NF-κB activation by H_2O_2 and TNF-α in Jurkat JR cells

Electrophoretic Mobility Shift Assays (EMSA) showed that stimulation of Jurkat JR cells with H_2O_2 at 150 μM induced a strong NF-κB DNA-binding activity, which reached its maximum between 150 and 240 min of treatment (Fig. 1A) but is sustained up to 300 min. Under our experimental conditions, one main band was visible on the autoradiographies. Supershifting experiments performed with antibodies directed toward NF-κB proteins

demonstrated that the shifted band, which was displaced by both p50 and RelA antibodies, was formed mainly of p50/RelA heterodimers (data not shown). Moreover, the addition of cycloheximide at 50 μg/ml did not modify the band intensity demonstrating that, even at long incubation times, NF-κB is activated by a posttranscriptional mechanism, which does not require de novo protein synthesis (data not shown). Similarly, we could also induce NF-κB by TNF-α in the same cell line; the induction being much more transient and rapid (between 10 and 60 min) (data not shown).

To further investigate potential distinct pathways for NF-κB activation by H_2O_2 or TNF-α in Jurkat JR cells, we studied the IκB-α protein by Western blot. We demonstrated that H_2O_2 led to a degradation of the IκB-α protein as observed on immunoblots performed with cytoplasmic extracts (Fig. 1B). The kinetics of IκB-α degradation paralleled NF-κB activation and was thus slower and more partial after H_2O_2 stimulation than after TNF-α induction. Taken together, our data suggest that H_2O_2 and TNF-α induce NF-κB activity in Jurkat JR cells through distinct signal transduction pathways.

Inhibitors of the mitochondrial respiration prevent NF-κB activation by H_2O_2

To further investigate the mechanisms leading to NF-κB activation by H_2O_2 and TNF-α, we have pretreated Jurkat JR cells with several inhibitors of the mitochondrial respiratory chain such as (a) rotenone, which inhibits complex I; (b) TTFA, which inhibits complex II; (c) antimycine A and myxothiazol, which are complex III inhibitors; (d) oligomycine, an inhibitor of proton translocation by ATP synthase and (v) FCCP, which uncouples respiration. In this experimental setting, Jurkat JR cells, cultivated for 24 h in the absence of β-mercaptoethanol, were incubated during 30 min with the inhibitors before being stimulated with 150 μM H_2O_2 and replaced in culture for 180 min. As shown in Fig. 2A, nanomolar concentrations of rotenone strongly inhibited NF-κB translocation induced by H_2O_2. A 50% inhibition of NF-κB translocation was obtained for 20 nM rotenone. Strikingly, rotenone was without any effect when NF-κB translocation was induced by 100 U/ml of TNF-α. It should also be noted that TTFA, which inhibits complex II, had no effect on either H_2O_2 or TNF-α–mediated NF-κB activation (data not shown). On the same manner, nanomolar concentrations of antimycine A also suppressed NF-κB translocation mediated by H_2O_2 (Fig. 2B). This induction is particularly sensitive to antimycine A because the IC50 value was estimated around 2.2 nM. It should however be noted that antimycine A concentrations below 1 nM somewhat stimulated NF-κB in the presence or in absence of H_2O_2. Although this effect is weak, it is clearly significant, and could be interpreted as being due to an increase of electron leakage at the ubiquinone cycle level that gives rise to the generation of superoxide anion.[23,24] This effect com-

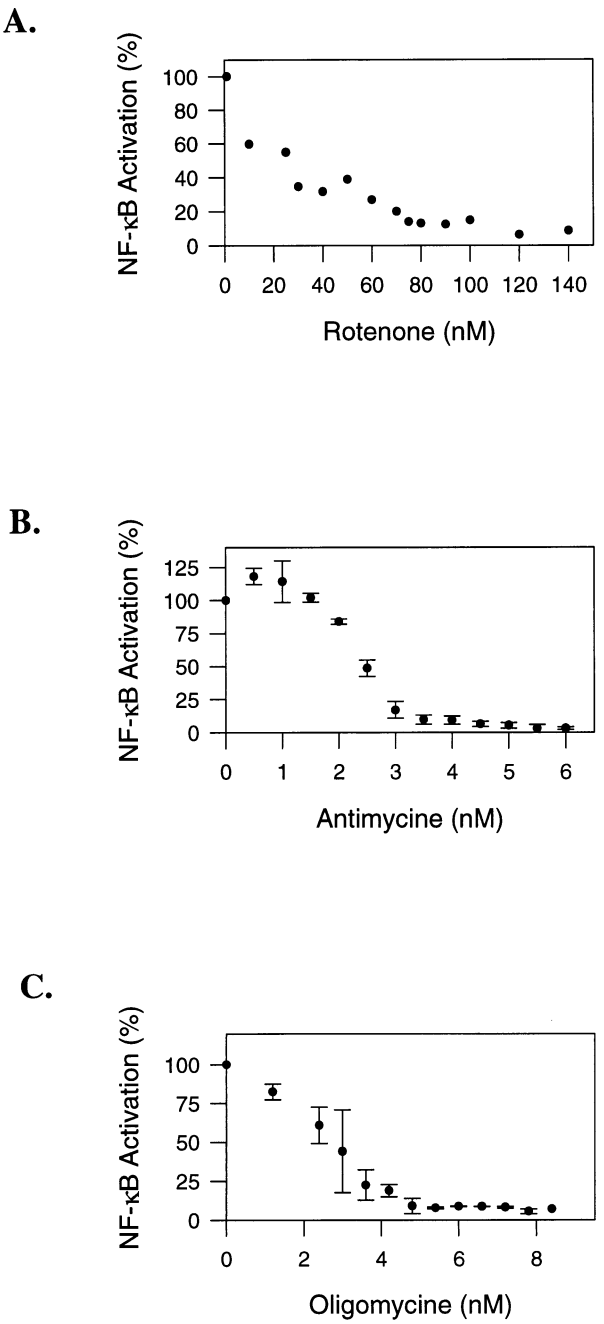

Fig. 2. The electron transport chain inhibitors [Rotenone (A) and Antimycine A (B)] and ATP synthase inhibitor [oligomycine (C)] block H_2O_2-induced NF-κB activation in Jurkat JR cells. Cells were preincubated for 30 min with increasing concentrations of inhibitors prior addition of H_2O_2. Nuclear extracts were prepared after 180 min and analyzed by EMSA with a specific κB probe. The amount of DNA binding assessed by phosphorimaging. The percentage of NF-κB activation is plotted vs. the inhibitor concentrations (in nM).

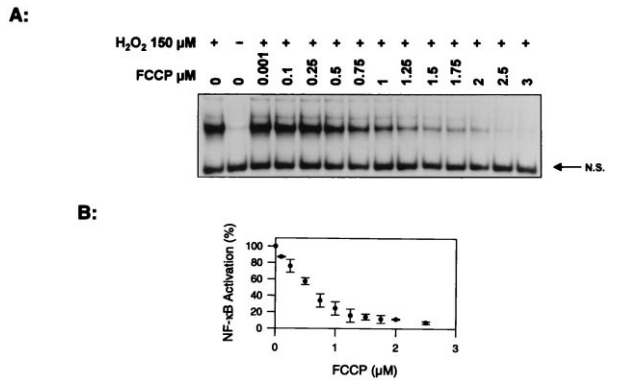

Fig. 3. (A) Inhibition of NF-κB activation in Jurkat JR cells after stimulation with H_2O_2 in the presence of increasing concentrations of FCCP. Jurkat JR cells were preincubated for 30 min, with increasing concentrations of FCCP prior being stimulated with 150 μM H_2O_2 for 180 min. Nuclear extracts from these cells were analyzed by EMSA with a labeled probe corresponding to a synthetic κB site. The arrow indicates unspecific NF-κB complexes. (B) Dose–response curve of the NF-κB inhibition by increasing concentrations of FCCP. The percentage of NF-κB stimulation is plotted vs. the FCCP concentrations (in μM).

pletely disappeared for higher antimycine A concentrations. Again, antimycine A had no effect on NF-κB translocation induced by TNF-α demonstrating that these effects are specifically associated to the stimulation carried out by H_2O_2. Combination between antimycine A and myxothiazol also completely inhibited NF-κB activation by H_2O_2 and had no effect when the inducing agent was TNF-α.

The third inhibitor tested was oligomycine, which inhibits the proton translocation by ATP synthase and then prevents the consumption of the proton gradient generated by the mitochondrial chain on the mitochondrial inner membrane. When Jurkat JR cells are stimulated by H_2O_2 in the presence of oligomycine, NF-κB activation is strongly reduced (Fig. 2C). Above 5 nM oligomycine, NF-κB translocation is totally inhibited and the IC_{50} value was evaluated at 3 nM. As observed with the two other inhibitors, oligomycine did not exert any effect on NF-κB translocation mediated by TNF-α.

When Jurkat JR cells were stimulated by H_2O_2 in the presence of FCCP, which increases the proton permeability of the mitochondrial membrane and dissipates the proton gradient built up by respiration, a strong dose-dependent reduction of NF-κB translocation was also observed (Fig. 3A). In that case, micromolar concentrations were required to observe a complete inhibition of NF-κB translocation; the IC_{50} being evaluated at 0.6 μM. Again, this membrane uncoupler had no effect on NF-κB translocation mediated by TNF-α.

Because the effects of these inhibitors turned out to be totally different on NF-κB induced by either H_2O_2 or TNF-α, we decided to investigate whether the inhibition of the mitochondrial respiration can affect NF-κB translocation by LPS in a pre-B cell line (Jurkat JR cells are not responsive to LPS). 70Z/3 cells were then pretreated by all these inhibitors, within the same concentration range, before being stimulated by LPS. As observed for TNF-α, these inhibitors did not affect NF-κB activation by LPS (data not shown) demonstrating again that the inhibition of mitochondrial respiration specifically inhibits NF-κB activation by H_2O_2.

Inhibitory effects on cellular respiration

To verify that the drugs used above led to a decrease of oxygen consumption by Jurkat JR cells, we have followed the cellular respiration by oxymetry. Jurkat JR cells were treated by the various inhibitors following the same protocol as described above except that they were left unstimulated. As shown in Fig. 4, rotenone, antimycine A, and oligomycine inhibited cellular respiration in the same concentration range than those that inhibited NF-κB translocation by H_2O_2. These data demonstrate that impairment of the cellular respiration by these various drugs can totally prevent NF-κB translocation mediated by H_2O_2. In identical experimental conditions, inhibition of cellular respiration does not influence NF-κB translocation by TNF-α.

ATP measurements

Because the first event in the mechanism leading to NF-κB translocation is the IκB-α degradation triggered by serine phosphorylation, we wanted to determine whether the effects of the inhibitors of the respiratory chain could be explained by a drop in the intracellular ATP concentration. To test this hypothesis, Jurkat JR cells were stimulated by H_2O_2 and ATP was followed by luminometry. As shown in Fig. 5A, H_2O_2 gave rise to a transient drop of ATP. A 70% reduction of the ATP level was recorded as soon as 30 min after stimulation. Two hours after stimulation by H_2O_2, the cells started to recover and the ATP level was back to normal value 24 h after the stress. In similar conditions, oligomycine alone slightly reduced the ATP level (Fig. 5B), while Jurkat JR cells pretreatment during 30 min with oligomycine before H_2O_2 treatment gave rise to an important drop (1% of the initial level) of the ATP level, which remains very low even 120 min after the stimulation (Fig. 5C). These data clearly demonstrated that (a) ATP level is crucial for NF-κB translocation by H_2O_2, (b) mitochondria were not the major source for ATP production in Jurkat JR cells, and (c) Jurkat JR cells can easily switch from mitochondrial respiration to glycolysis. Indeed, it should be noted that the ATP concentration was not importantly modified

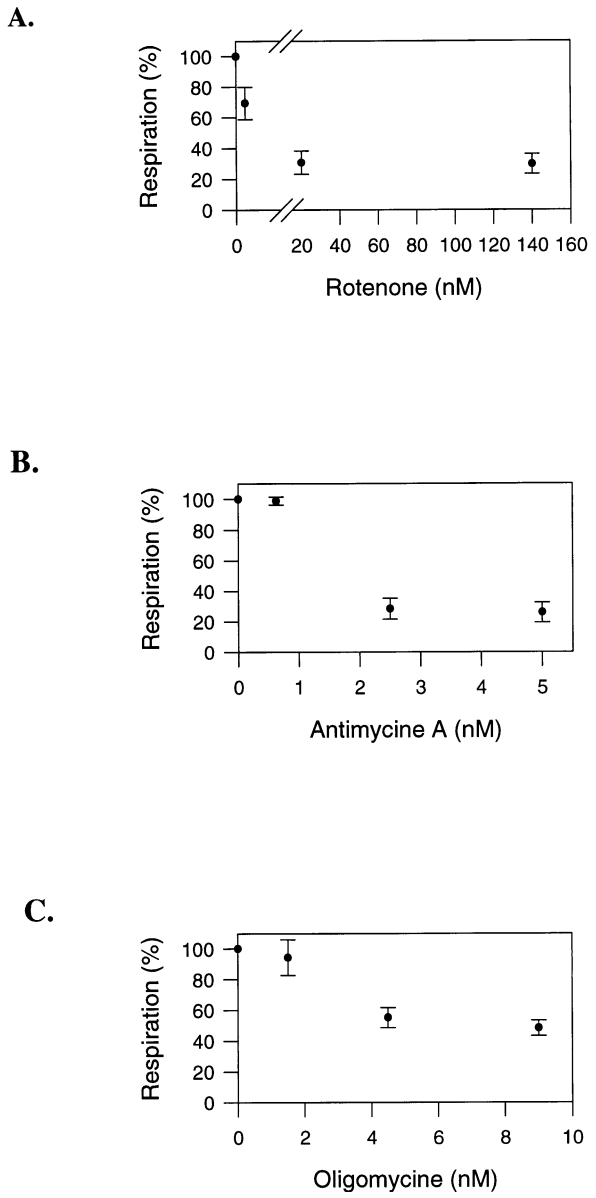

Fig. 4. Jurkat JR cells respiration measured by oxymetry after treatment in increasing concentrations of rotenone (A), antimycine A (B), and oligomycine (C). Respiration in untreated Jurkat JR cells is taken as being 100%.

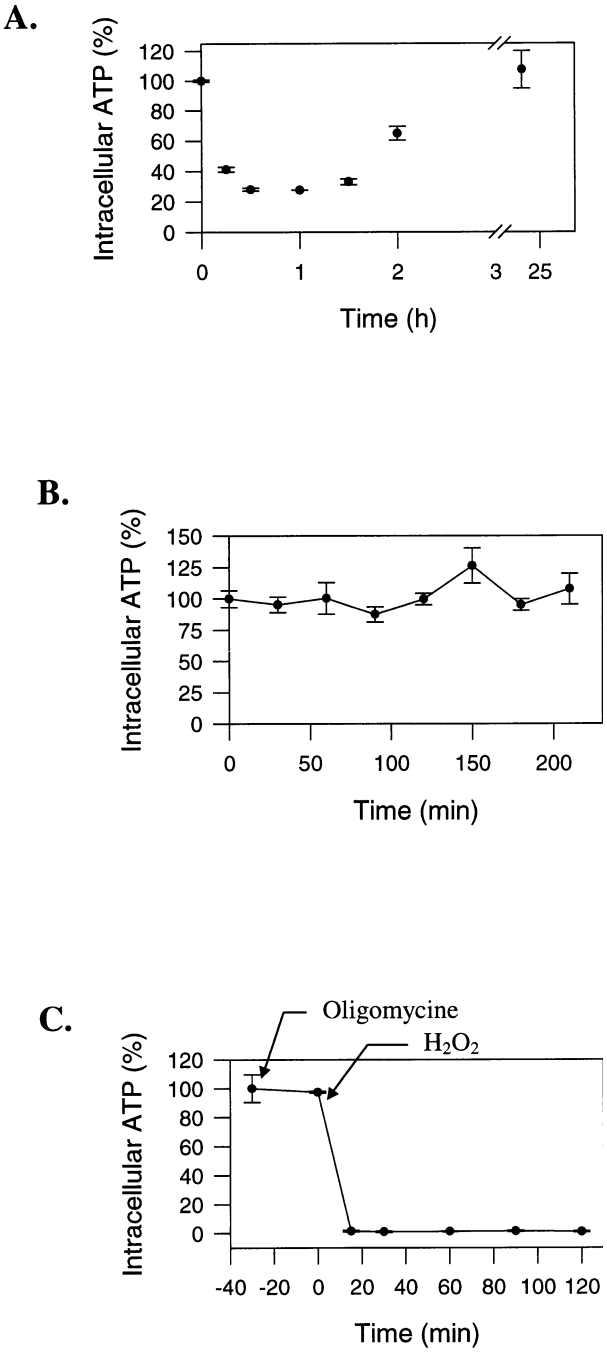

Fig. 5. ATP intracellular concentrations determined by luminometry in Jurkat JR cells treated with 150 μM H_2O_2 (A), with 9 nM oligomycine (B), or during 30 min with 9 nM oligomycine prior being treated with 150 μM H_2O_2 during various period of times (C). ATP intracellular concentrations (in percent) were plotted vs. the incubation times.

by the addition of oligomycine, demonstrating that, in our cell culture conditions and with oligomycine (11 mM glucose), Jurkat JR cells generated an important part of their ATP through the glycolytic pathway. To assess the role of ATP generation by both the glycolytic and mitochondrial routes, we have used IAA, which specifically inhibits glyceraldehyde-3-phosphate-deshydrogenase and indirectly the mitochondrial respiration. As shown in Fig. 6A, the addition of IAA at 500 μM induced an important drop of the ATP level within 30 min. This drop could also be recorded when cells were pretreated first with 9 nM oligomycine before the addition of IAA (Fig. 6A). From these data, we can conclude that Jurkat JR cells mainly produce ATP by switching easily from the mitochondrial to the glycolytic pathways. To determine whether or not ATP generated is important for NF-κB activation by TNF-α, Jurkat JR cells were pretreated by

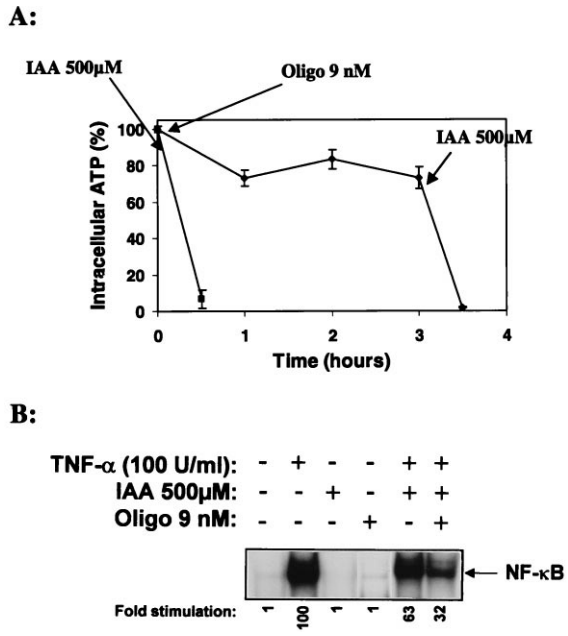

Fig. 6. (A) Intracellular ATP concentrations were measured when Jurkat JR cells were treated with 500 μM IAA or for 180 min with 9 nM oligomycine before being treated with 500 μM IAA. ATP concentrations (in %) were plotted vs. incubation times. (B) NF-κB activation in Jurkat JR cells induced with 100 U/ml TNF-α. Cells were pretreated during 30 min with either 500 μM IAA, or 9 nM oligomycine, or with both inhibitors. Nuclear extracts were prepared 30 min after induction and NF-κB DNA binding was measured by EMSA with a synthetic NF-κB probe. Level of binding was determined by phosphorimaging.

IAA for 30 min before being stimulated by TNF-α for 30 min. EMSA analysis revealed that IAA alone had no effect on NF-κB activation, while it gave rise to a 34% inhibition of NF-κB activation by TNF-α alone or a 63% reduction in combination with oligomycine (Fig. 6B). These data demonstrated for the first time that the intracellular ATP level is crucial for NF-κB activation by two cell inducers, i.e., H_2O_2 and TNF-α.

DISCUSSION

Because NF-κB can be activated by an extraordinarily large number of different signals, ranging from ultraviolet radiation, oxidative stress to cytokines, and T cell activation, the mechanism by which these signals converge on IκB-α is of the wide interest. Recently, many components from certain signaling pathways that lead to NF-κB activation have been described.[14–18] The recent identification of a high molecular weight IκB kinase complex and the identification of two unusual IκB kinases (IKK-1 and -2) provides a framework for resolving the problem of integrating multiple NF-κB signaling pathways.[14–18] In this article, we have shown, as many other authors, that kinetics by which NF-κB translocates into the nucleus can be very different from a stimuli to another, i.e., H_2O_2 and TNF-α.[25–27] Because a crucial event in the activation of NF-κB is the phosphorylation of the IκB on serine residues 32 and 36, we can better understand why these two inducers promote NF-κB with so different kinetics. As ATP is probably used as phosphate donor by the two IKKs to phosphorylate IκB-α, its transient drop during the first 60 min after the oxidative stress can explain why NF-κB translocation by H_2O_2 is delayed in comparison to TNF-α. During this drop phase, ATP concentration could well be limiting for an efficient IκB-α phosphorylation by IKK-1 and -2. With TNF-α as an inducer, no intracellular ATP variation can be recorded and in that case, phosphorylation of IκB-α by IKK-1 and -2 occurs within 10 min.

This transient drop in ATP concentration observed after treatment with H_2O_2 could be due to a partial inhibition of glyceraldehyde-3-phosphate deshydrogenase[28] or to minor alterations of the mitochondria such as the release of cytochrome *c*[29] or the inactivation of aconitase[30] or to an activation of the poly (ADP-ribose) polymerase, which leads to a drop in NAD and ATP levels.[31] Our data definitively demonstrate the important role of the cytoplasmic ATP in the mechanism leading to IκB-α phosphorylation and to NF-κB translocation. When the mitochondrial functions are uncoupled from ATP synthesis, NF-κB translocation induced by H_2O_2 is completely abolished. Unexpectedly, these inhibitors have no effect when either TNF-α or LPS are used as inducers. The inhibition of the electron chain transport by itself could lead to an accumulation of reducing metabolites such as NADH, UQH_2, and the formation of a so-called reductive stress.[32] This causes an increased production of ROS through a noncatalytic oxidation by O_2 of the reduced metabolites, thus further increasing oxidative damages to macromolecules.[32] When these effects are cumulated with those induced by the extracellular addition of H_2O_2, we can suspect that the normal antioxidant defenses of Jurkat JR cells are overwhelmed, and these cells cannot easily be rapidly adapted to high oxidant conditions by turning on expression of genes encoding proteins important for damage repair.

Addition of an inhibitor of ATP synthase also abolishes NF-κB inducibility by H_2O_2. These effects at first sight could be due to a drop in intracellular ATP level caused by the inhibitor. However, the addition of oligomycine alone, which inhibits ATP synthesis, only slightly reduced the ATP level, allowing suspicion that, in Jurkat JR cells cultivated in the presence of glucose and oligomycine, most of the ATP synthesis does not come from mitochondrial respiration, but from glycolysis. These conclusions are reinforced by the observation that IAA alone can lead to a severe drop in ATP level. The lack of NF-κB inducibility by H_2O_2 in the presence of oligomycine could be explained by the collapse of

ATP level when both oligomycine and H_2O_2 are added together. Indeed, oligomycine could block the ATP synthesis, depending on mitochondria with H_2O_2 partially inhibiting the ATP synthesis, depending on glycolysis. These two agents seem to synergize because the ATP level drops below 10% of the normal value within 20 min. With a so low intracellular ATP level in the presence of both oligomycine and H_2O_2, one can easily suspect that the important phosphorylation events leading to NF-κB activation are inhibited.

From the data described above, we can conclude that addition of 150 μM H_2O_2 on Jurkat JR cells leads to moderate modifications of mitochondrial functions and a transient drop in ATP. Changes in ATP concentration due to mitochondrial alterations are probably not drastic enough to abolish NF-κB inducibility. However, when mitochondrial functions are impaired or when ATP synthesis through glycolysis is inhibited, the addition of H_2O_2 has deleterious biochemical effects. Among them, we can suspect that the inhibition of kinase activities, such as IKK-1 and -2, is responsible for the lack of NF-κB induction in these conditions. In case of TNF-α, we have shown that inhibition of mitochondrial functions does not abolish NF-κB–activation in Jurkat JR cells cultivated in the presence of glucose. Our observations are somewhat in contrast with those of Schulze-Osthoff et al.,[33] who showed that, in L929 cells, the interference with the mitochondrial electron transport chain inhibits NF-κB inducibility by TNF-α. Although we could not observe the same effects in Jurkat JR cells, these discrepancies could be due to differences in glucose concentrations in the cell medium or in the dependence of this particular cell type on mitochondria to synthesize ATP. Indeed, respiration-deficient L929 subclones revealed a strongly reduced NF-κB activation by TNF-α demonstrating the importance of ROS generated by mitochondria in TNF-α–induced activation of NF-κB.[30] This dependence on ROS for NF-κB is cell-type dependent.[34–37] Indeed, we have recently shown that in epithelial cells, activation of NF-κB by either IL-1β or TNF-α, does not require the intracellular generation of ROS, but seems to rely on the activation of an acidic sphingomyelinase activity in the lyzosomal membrane.[35] Thus, depending on the cell type studied, it is plausible that ROS generation by mitochondria during TNF-α stimulation may have various importance in term of NF-κB activation. In epithelial cells, the transduction machinery based on the coordinated activation of various proteins belonging to the signalosome does not seem to require the generation of ROS, while in other cells like lymphocytes, ROS could well be important partners for an efficient activation.

In summary, we have shown that NF-κB induction by H_2O_2 requires a fully active mitochondrial electron transport chain because its inhibition results in an abolition of NF-κB activation due to a severe drop in intracellular ATP. This effect turns out to be specific because inhibition of mitochondrial function does not affect NF-κB inducibility by TNF-α in Jurkat JR cells, and by LPS in 70Z/3 cells.

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ABBREVIATIONS

EMSA—electrophoretic mobility shift assay
FCCP—carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
H_2O_2—hydrogen peroxide
HEPES—4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA—iodoacetic acid
IκB—inhibitor κB
LPS—lipopolysaccharide
NF-κB—nuclear factor-κB
ROS—reactive oxygen species
TNF-α—tumor necrosis factor α
TTFA—thenoyltrifluoroacetone