Summary and Conclusions

It is becoming increasingly apparent that NF-κB plays a critical role in regulating the inflammatory response. Data obtained from studies in our laboratories demonstrate that the proteasome plays an important role in the inflammatory cascade by regulating the activation of NF-κB. Indeed, the availability of selective and orally active proteasome inhibitors should prove useful in delineating the roles of the proteasome and NF-κB in other pathophysiological conditions such as cancer and heart disease.

[36] Nuclear Factor κB Activity in Response to Oxidants and Antioxidants

By YVONNE M. W. JANSSEN and CHANDAN K. SEN

Introduction

Oxidation–reduction (redox)-based regulation of gene expression appears to represent a fundamental regulatory mechanism in cell biology.[1–4] The transcription factor, nuclear factor κB (NF-κB), is of considerable interest to the field of free radical biology because it has been shown to be activated by oxidants and its induced activity is inhibited by antioxidants.[1–7] The exact oxidant sensitive sites, the localization of antioxidant action, and signaling events that are responsible for NF-κB activation remain elusive. The discoveries of the TRAF adaptor proteins, NF-κB inducing kinase (NIK), and IκB kinases (IκK) required for the activation of NF-κB by tumor necrosis factor (TNF) α may provide insight into these redox-sensitive sites.[8–10] Because free radicals are involved in numerous

[1] C. K. Sen and L. Packer, *FASEB J.* **10,** 709 (1996).
[2] J. M. Muller, M. R. A. Rupec, and P. A. Baeuerle, *Methods* **11,** 301 (1997).
[3] C. K. Sen, *Biochem. Pharmacol.* **55,** 1747 (1998).
[4] C. K. Sen, *Curr. Top. Cell Regul.* **36,** in press (1998).
[5] C. K. Sen, S. Khanna, A. Z. Reznick, S. Roy, and L. Packer, *Biochem. Biophys. Res. Commun.* **237,** 6645 (1997).
[6] R. Schreck, P. Rieber, and P. A. Baeuerle, *EMBO J.* **10,** 2247 (1991).
[7] R. Schreck, B. Meier, D. N. Mannel, W. Droege, and P. A. Baeuerle, *J. Exp. Med.* **175,** 1181 (1992).
[8] Z. Liu, H. Hsu, D. V. Goeddel, and M. Karin, *Cell* **87,** 565 (1996).
[9] N. L. Malinin, M. P. Boldin, A. V. Kovalenko, and D. Wallach, *Nature (London)* **385,** 540 (1997).
[10] T. Maniatis, *Science* **278,** 818 (1997).

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pathological conditions, the ability to measure NF-κB activation in these circumstances should provide important insight to elucidate the etiology of these disorders.

NF-κB activation leads to the transcriptional activation of genes, many of which are involved in the orchestration of an inflammatory response and immune modulatory events, as well as other processes.[11-13] These observations point to an important role of NF-κB in normal cellular function and disease.

In its latent form, NF-κB is complexed in the cytoplasm to IκB inhibitor proteins. Exposure to an NF-κB activating agent leads to dissociation and, in some cases, degradation of the inhibitory complex via a proteasome pathway.[13,14] This enables the NF-κB protein to translocate into the nucleus under the guidance of a nuclear localization signal, bind to κB sites in promoter regions of target genes, and activate transcription. Five members of the NF-κB/Rel family and seven members of IκB related proteins have been identified in mammalian cells, and new family members continue to be discovered.[15] It is thus evident that the regulation of NF-κB activation by various NF-κB/IκB proteins may be quite complex. Most of the research has focused on classical p65–p50 heterodimers (NF-κB) and its control via release of IκBα or IκBβ. Based on findings from these studies, a number of protocols have been established to measure the activity of p65–p50 and degradation of IκBα/β and are discussed in the following sections. Knowledge of the mechanisms involved in the activation of NF-κB and its target genes in response to different stress situations will be enhanced once the functions of other NF-κB/IκB family member are established.

To clarify the role of NF-κB in various aspects of cell function, several approaches to measure NF-κB activation in cell culture as well as intact tissue models have been used. For example, NF-κB activity can be monitored by electrophoretic mobility shift assays (EMSA), nuclear translocation, and Western blotting, as well as transactivation assays and the measurement of NF-κB regulated protein expression. The goal of this chapter is to review these approaches and discuss their strengths and weaknesses.

I. Electrophoretic Mobility Shift Assay

Translocation of NF-κB proteins from the cytosol to the nucleus leads to increased concentration of NF-κB proteins in the nucleus. Incubation

[11] P. A. Baeuerle and V. R. Baichwal, *Adv. Immunol.* **65,** 111 (1997).
[12] P. A. Baeuerle and T. Henkel, *Ann. Rev. Immunol.* **12,** 141 (1994).
[13] T. S. Finco and A. S. Baldwin, *Immunity* **3,** 263 (1995).
[14] V. Imbert, R. A. Rupec, A. Livolsi, H. L. Pahl, E. B. Traenckner, C. Mueller-Dieckmann, D. Farahifar, B. Rossi, P. Auberger, P. A. Baeuerle, and J.-F. Peyron, *Cell* **86,** 787 (1996).
[15] P. A. Baeuerle and D. Baltimore, *Cell* **87,** 13 (1996).

of a ^{32}P-labeled oligonucleotide containing an NF-κB consensus sequence with nuclear extracts enriched in NF-κB proteins results in enhanced binding of NF-κB proteins to the oligonucleotide. Such binding results in retardation ("shift") of the electrophoretic mobility of the oligonucleotide on a nondenaturing polyacrylamide gel. These "shifts" can be visualized by autoradiography and can be used as a marker of the presence of NF-κB proteins in the nucleus. Thus, EMSA is a measure of the translocation of activated NF-κB proteins to the nucleus, and this assay does not provide any information regarding the transcription regulatory effect of NF-κB.

A. Preparation of Nuclear Extracts

Cells ($2–10 \times 10^6$), adherent or grown in suspension, are harvested and centrifuged at 750*g* for 5 min at 4°. The cell pellet is gently resuspended in ice-cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged at 750*g* for 5 min at 4°. The cell pellet thus obtained is resuspended in 400 μl of ice-cold hypotonic buffer containing 10 m*M* HEPES (pH 7.8), 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* ethylenediaminetetraacetic acid (EDTA, sodium salt), 0.2 m*M* NaF, 0.2 m*M* Na_3VO_4, 0.4 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.3 mg/ml leupeptin, and 1 m*M* dithiothreitol (DTT). When available, the use of molecular biology grade reagent is recommended for all procedures described in this work. Stock solutions of PMSF (10 m*M*) in isopropanol and leupeptin (10 mg/ml) in water may be stored as single-use aliquots at −20°. PMSF, leupeptin, and DTT should be added fresh. The cell suspension is kept on ice for 15 min, following which 25 μl of 10% Nonidet P-40 is added. The suspension is vortexed vigorously for 15 sec and then centrifuged at 16,000*g* for 1 min. This results in a pellet of nuclei.

Nuclei obtained as described above are resuspended in 50 μl of ice-cold high-salt extraction buffer [50 m*M* HEPES (pH 7.8), 50 m*M* KCl, 300 m*M* NaCl, 0.1 m*M* EDTA, 0.2 m*M* NaF, 0.2 m*M* Na_3VO_4, 0.4 m*M* PMSF, 0.3 mg/ml leupeptin, 1 m*M* DTT and 10% sterile glycerol]. PMSF, leupeptin, and DTT should be added fresh. The nuclear suspension is placed on a rocking platform for 30 min at 4° to facilitate lysis of the nuclei. The nuclear lysates are then centrifuged at 16,000*g* for 10–20 min at 4°. The supernatant is collected and stored as 15 μl aliquots at −80°. Nuclear extracts should not be thawed more than twice. The protein concentration in the supernatant is determined from a 5 μl aliquot using an assay method (Bradford protein assay reagent, Bio-Rad, Hercules, CA) that does not interfere with DTT present in the nuclear protein extract.

B. Labeling of NF-κB Consensus Oligonucleotide Sequence and EMSA

NF-κB oligonucleotide (8.75 pmol; Promega, Madison, WI) is incubated with 50 μCi [γ-^{32}P]ATP in the presence of 10 U T4 polynucleotide kinase

and 1× kinase buffer supplied with the kinase (Boehringer, Indianapolis, IN) at 37° for 30 min. The reaction is stopped by addition of 1 volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1). After gentle mixing by pipette, the mixture is centrifuged at 16,000*g* for 2 min. The upper aqueous phase is collected and subjected to column filtration. Nonincorporated ^{32}P is removed by centrifugation through a Sephadex G-50 column (Boehringer) equilibrated with saline–Tris–EDTA buffer (10 m*M* Tris, 100 m*M* NaCl, and 10 m*M* EDTA, pH adjusted to 7.5 using HCl). Labeled oligonucleotide is precipitated at −20° until use.

C. DNA Binding Reaction and Electrophoresis

The nuclear protein extract (5 μg) obtained as described in Section A in a total of 4 μl of high-salt extraction buffer is incubated in the presence of 0.1 pmol ^{32}P-end-labeled NF-κB oligonucleotide, 40 m*M* HEPES (pH 7.8), 10% (v/v) glycerol (or 4% Ficoll 400), 1 m*M* $MgCl_2$, 0.1 m*M* DTT, and 200 ng poly(dI-dC) · poly(dI-dC) (Pharmacia, Piscataway, NJ) in a final volume of 20 μl for 20 min at room temperature. Following incubation, the sample is loaded on a 0.8 mm 6% nondenaturing polyacrylamide gel in 0.5× Tris/boric acid/EDTA (TBE) buffer [10× stock solution is available from Bio-Rad (Hercules, CA)]. The gel is prepared using a 40% acrylamide/bis acrylamide solution 29 : 1 (3.3% C) in 1× TBE. The mixture is polymerized using 0.1% (w/v) ammonium persulfate and 0.1% TEMED (Bio-Rad). Samples are electrophoresed in 0.5× TBE at 120 V for 2 hr. Gels are dried under heat and vacuum and band shifts examined by autoradiography using a ^{32}P-sensitive film (Hyperfilm, Amersham Life Science Inc., Arlington Heights, IL).

For the DNA binding reaction it is critical that the volume of high-salt extraction buffer present in each binding assay be identical. This can be achieved by diluting the more concentrated nuclear protein extracts in the same extraction buffer. The salts present in the extraction buffer determine the binding kinetics of the protein to the oligonucleotide and should therefore be strictly controlled for relative concentration in different samples.

D. Competition and Supershift Assays

To ascertain the specificity of NF-κB DNA binding, reactions are performed in the presence of a 10-fold molar excess of unlabeled NF-κB oligonucleotide, which will result in the disappearance of all NF-κB complexes. Incubation with an unrelated oligonucleotide or a mutated NF-κB oligonucleotide (one base pair altered, Santa Cruz Biotechnology Inc., Santa Cruz, CA) should not compete for binding (Fig. 1).

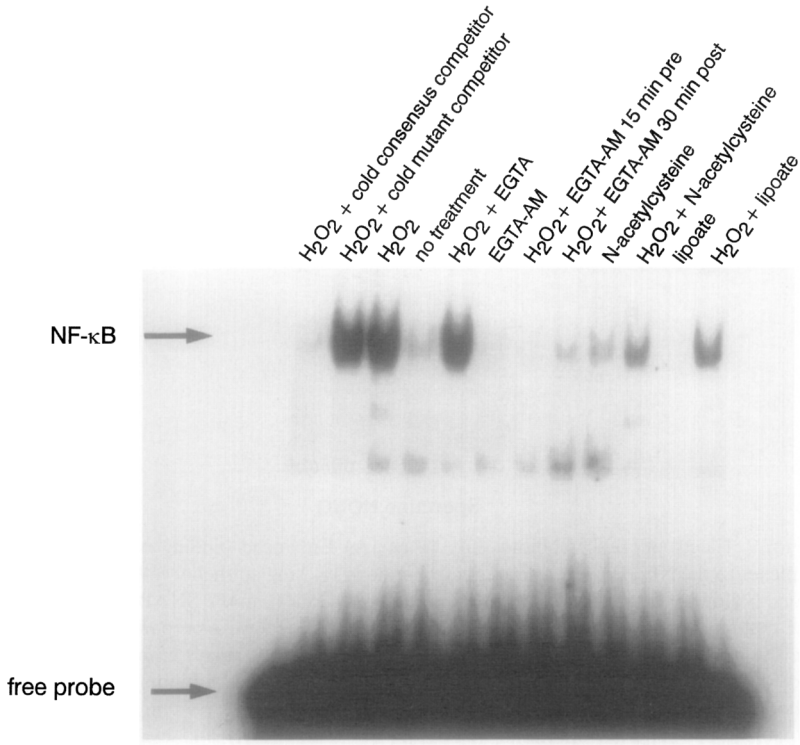

FIG. 1. Inhibition of H_2O_2-induced NF-κB activation in Wurzburg cells by intracellular calcium chelators and thiol antioxidants. To test the specificity of the NF-κB band, nuclear extracts from activated cells were treated with an excess of unlabeled consensus NF-κB oligonucleotide (lane 1; from left to right) or with an excess of cold mutant NF-κB oligonucleotide (lane 2) before incubation with ^{32}P-labeled consensus NF-κB probe. Lanes 3 and 4, cells were either treated or not treated with 0.25 m*M* H_2O_2, respectively; lane 5, cells treated with 1 m*M* EGTA (ethylene glycol tetraacetic acid) 10 min before H_2O_2 challenge; lane 6, cells treated with 0.5 m*M* lipophilic EGTA-AM (acetomethoxyl ester of EGTA); lane 7, cells treated with 0.5 m*M* EGTA-AM 15 min before H_2O_2 challenge; lane 8, cells treated with 0.5 m*M* EGTA-AM 30 min after H_2O_2 challenge; lane 9, cells treated with 20 m*M* *N*-acetyl-L-cysteine (NAC) for 6 hr; lane 10, cells pretreated with 20 m*M* NAC for 2 hr before H_2O_2 treatment for 4 hr; lane 11, cells treated with 1 m*M* α-lipoate for 22 hr; lane 12, cells pretreated with α-lipoate for 18 hr followed by H_2O_2 treatment for 4 hr. Reprinted from Sen *et al.* (1996),[16] with kind permission from Elsevier Science, Amsterdam, The Netherlands.

To assess the subunit composition of DNA binding proteins, specific antibodies are available to detect p65 and p50 proteins (Santa Cruz Biotechnology Inc., Santa Cruz, CA). DNA binding reactions (18 μl) are carried out as described, and are followed by the addition of 2 μl antibody for an additional 30 min prior to electrophoresis. In cases where antibody binding

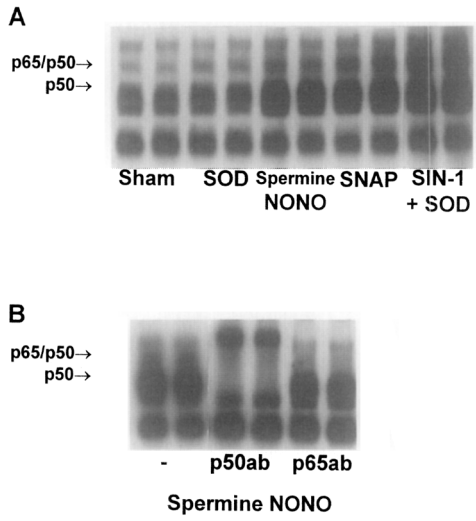

FIG. 2. Electrophoretic mobility shift assay. (A) Enhanced binding of nuclear protein complexes to the NF-κB consensus oligonucleotide in rat lung epithelial cells exposed to NO· donors. Following 6 hr of exposure to spermine NONOate (1 m*M*). SNAP (1 m*M*) plus 100 μ*M* L-cysteine, or SIN-1 in presence of SOD, nuclear extracts were prepared and evaluated. Note the increases compared to sham controls. (B) Supershift assays employing antibodies directed against p65 or p50 subunits of NF-κB. Incubation of DNA binding complexes with antibodies prior to electrophoresis abrogates two specific bands, verifying the presence of p65/p50 and p50 subunits. Spermine NONO, [*N*-2-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine]; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; SNAP, (±)-*S*-nitroso-*N*-acetylpenicillamine.

results in an additional electrophoretic retardation, this is referred to as "supershift" (Fig. 2B). Examples of EMSA and supershifts are shown in Figs. 1 and 2. Results in Fig. 2A demonstrate increases in NF-κB DNA binding in response to exposure of rat lung epithelial (RLE) cells to the NO· generating compound spermine NONOate. Several antioxidants have been shown to inhibit inducible NF-κB activation. Figure 1 illustrates the inhibition of hydrogen peroxide induced NF-κB activation in Würzburg T cells by thiol antioxidants and calcium chelators.[16]

II. Nuclear Translocation of p65

Activation of NF-κB is characterized by translocation of NF-κB proteins into the nucleus. This can be detected by immunocytochemical or immuno-

[16] C. K. Sen, S. Roy, and L. Packer, *FEBS Lett.* **385,** 58 (1996).

fluorescence approaches or by performing a Western blot of nuclear extracts.

A. Evaluation of Nuclear p65 by Immunofluorescence

Cells are grown on glass coverslips and treated with NF-κB activating agents. After the appropriate incubation times, cells are washed twice in PBS (4°) and fixed in 100% methanol for 30 min at room temperature, followed by two washes with PBS. To permeabilize cells, sections are treated with 0.1% Triton X-100 in PBS for 20 min. To block nonspecific binding of antibody, sections are incubated in PBS containing 1% bovine serum albumin (BSA) twice for 30 min each at room temperature. Coverslips are then incubated with primary antibody against p65 (2.5 μg/ml, Santa Cruz Biotechnology Inc.) in PBS/BSA by inverting the coverslips onto 50 μl of antibody suspension. After 1 hr of incubation in primary antibody at room temperature, sections are washed in PBS/BSA (3 × 20 min each) and incubated with 10 μg/ml goat anti-rabbit antibody conjugated with the fluorophore Cy3 (Jackson Immunoresearch, West Grove, PA) for 45 min (10 μg/ml in PBS/BSA). To verify staining specificity, control sections are not incubated with primary antibody. Only secondary antibody is applied to these samples. Such treatment should result in a lack of immunostaining. Coverslips are then washed with PBS and incubated with the DNA stain YOYO. The use of a nuclear stain such as YOYO-1 iodide (500 n*M*, Molecular Probes, Eugene, OR) allows colocalization of p65 in the nuclear component.[17] An example of immunofluorescence of p65 in control cells, as well as cells treated with the NF-κB activator lipopolysaccharide (LPS), is shown in Fig. 3.

B. Detection of NF-κB Proteins in Nuclear Extracts by Western Blotting

An alternative approach is to perform Western blot analysis of the nuclear protein extract for the specific detection of NF-κB proteins. Increased presence of these proteins in the nucleus indicates NF-κB activation.

III. Transactivation of NF-κB Directed Gene

Activation of the transcription factor NF-κB should enhance the transcription of NF-κB regulated target genes. Translocation of p65 into the nucleus, evidenced by the techniques described above, does not provide any information regarding the transcriptional activity of NF-κB. The tran-

[17] Y. M. W. Janssen, K. E. Driscoll, B. Howard, T. R. Quinlan, M. Treadwell, A. Barchowsky, and B. T. Mossman, *Am. J. Pathol.* **151,** 389 (1997).

scription regulatory aspect of NF-κB activity may be studied in cells transiently transfected with NF-κB consensus sequence linked to a reporter gene, e.g., luciferase, β-galactosidase, human growth hormone, or green fluorescent protein, that is not expressed endogenously in mammalian cells. Increased reported gene expression in these transiently transfected cells reflects the transcription regulatory activity of NF-κB. Firefly luciferase is a convenient reporter enzyme that gives a linear response over a wide range of activity and has an enhanced sensitivity compared to β-galactosidase, which can occur endogenously in some mammalian cells.[18] A major drawback of this assay is that the transient transfection of NF-κB luciferase plasmid results in an artificially high amount of target sequence that is not physiologically relevant. Thus, this method could result in the overestimation of the transcription regulatory effect of NF-κB.

A. Transient Transfection

Cells are transiently transfected using optimal protocols, e.g., calcium phosphate coprecipitation, DEAE dextran, lipofectamine, or electroporation. Detailed descriptions of these basic laboratory protocols are available in the literature.[19] In our hands, cells difficult to transfect have responded well to electroporation (Bio-Rad, Genepulser electroporator) using complete medium with 10% serum and 0.8–1 × 10^6 cells at 240 V. Cells are transfected with an NF-κB–luciferase reporter vector in presence of the control vector PSV β-Gal (Promega), which serves as a vector control and allows to correct for variabilities in transfection efficiencies. Cells are transfected using 5–10 μg of DNA, plated in 35 mm dishes, incubated for 4–6 hr, and allowed to recover overnight in complete culture medium. After this, the cells are stimulated with appropriate agents and harvested at selected time points. In general, cells may be harvested after 48 hr of transfection or at time points during which expression of the transfected reported gene is optimal.

B. Reporter Activity Assay

To harvest cells for assessment of luciferase and β-galactosidase activities, cells in 35 mm dishes are washed twice in ice-cold PBS and lysed in 200 μl reporter lysis buffer (Promega). Dishes are scraped with a rubber

[18] J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987).

[19] "Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology" (F. Ausubel, R. Brent, R. E. Kingston, *et al.*, Eds.), 3rd ed. Wiley, New York, 1995.

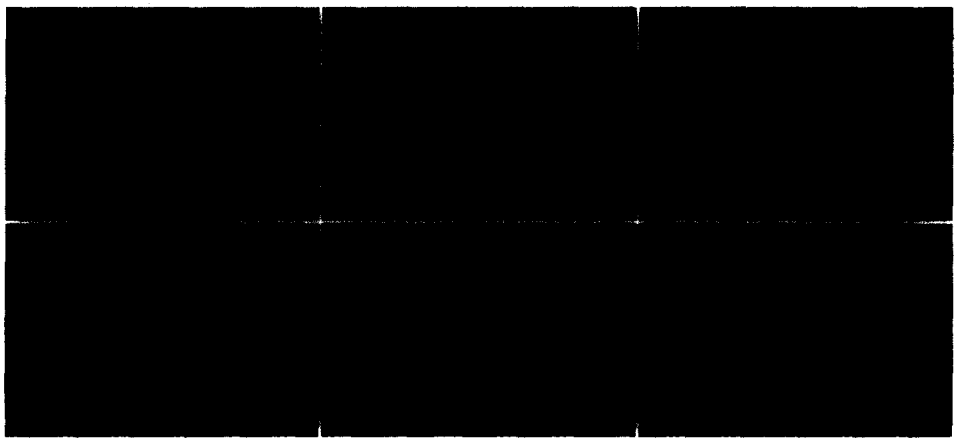

FIG. 3. Confocal microscopy illustrating the translocation of p65 into the nucleus of rat pleural mesothelial cells exposed to lipopolysaccharide (LPS) for 2 hr. Cells were incubated with p65 antibody, followed by incubation with a secondary antibody coupled to the fluorochrome Cy3™, resulting in a red staining to illustrate the subcellular localization of p65 (Panels B and E). Cells were simultaneously stained with YOYO, to stain the DNA green (Panels A and D). Overlap of green and red colors results in an orange/yellow color formation and verifies the presence of p65 in the nucleus (Panels C and F). Panels A–C are sham controls, panels D–F are LPS-exposed rat pleural mesothelial cells (magnification 1080×).

TABLE I
TIME COURSE OF ACTIVATION OF NF-κB IN RAT LUNG EPITHELIAL CELLS EXPOSED TO TNFα[a]

Step	Luc	β-Gal	Luc/β-Gal
Sham	15,863	43,296	366
	11,472	46,887	245
TNFα, 1 hr	13,214	44,553	297
	9,238	45,126	205
TNFα, 2 hr	41,872	29,004	1,444
TNFα, 8 hr	82,508	29,548	2,792
	116,372	28,407	4,097

[a] Luc, Luciferase activity; β-Gal, β-galactosidase activity; Luc/β-Gal, Luc activities normalized to β-Gal. The two rows of data for each treatment group indicate replicate values from two different experiments. Data are arbitrary luminescence values.

policeman and lysates are stored at −80° until analysis. Lysates are centrifuged at 16,000*g* for 2 min at 4°. The supernatant is used for the measurement of reporter gene activities. The amount of extract required for the assay may be optimized. For this, 100 μl of luciferase assay reagent containing luciferin and ATP (Promega) is added to the extract and the generated luminescence is detected using a standard luminometer.

β-Galactosidase activity may be measured similarly using a Galactolight assay kit (Tropix, Bedford, MA) and a luminometer. Instrument settings should be optimized according to the detected activities in the sample. Typically, luciferase activities are measured in 10 sec, and β-galactosidase activities in 5 sec. Increased NF-κB activity is manifested as increased luciferase activity. β-Galactosidase is constitutively expressed in cells and its activity is used to normalize luciferase activity results. This controls for variations in transfection efficiencies that may occur in these transient assays. As a sham control, cells can be transfected with a luciferase vector not regulated by NF-κB or a vector where NF-κB sites are mutated. Luciferase activity detected from such samples should not respond to the treatment of cells with NF-κB activating agents. An example of NF-κB regulated luciferase activities and β-galactosidase activities in control RLE cells as well as after various times of exposure to TNF (tumor necrosis factor) α is illustrated in Table I. We have also demonstrated increases in NF-κB-luciferase activities in RLE cells exposed to a range of reactive oxygen or

TABLE II
ACTIVATION OF NF-κB IN RAT LUNG EPITHELIAL CELLS[a]

Step	Luciferase/β-Gal	
	I	II
Sham	488	648
300 U/ml SOD	631	
300 μ*M* SIN-1	1337	1127
SIN-1 + SOD	1514	1020
300 μ*M* ONOO-	1151	1895
300 μ*M* H_2O_2	9410	10,741

[a] Exposed to reactive oxygen or reactive nitrogen species for 8 hr. β-Gal, β-galactosidase activity; luciferase/β-Gal, luciferase activities normalized for β-Gal. The two columns of data (I, II) for each treatment group indicate replicate values obtained from two different experiments. Data are arbitrary luminescence values.

nitrogen species for 8 hr as is shown in Table II. More striking increases in NF-κB luciferase activities are apparent after 16 hr of activation (unpublished data).

IV. NF-κB Regulated Protein Expression

The transient transfection of NF-κB luciferase plasmid results in an artificially high amount of target sequence that is not physiologically relevant. Thus, a more reasonable approach to assess NF-κB activation is to evaluate the expression of an NF-κB target gene that (1) contains NF-κB sequences in its promoter and (2) requires NF-κB activity for transcription. The latter can be confirmed by promoter deletion assays where loss of NF-κB sequences results in a loss of gene transcription. The power of this approach is that it can be performed in both *in vitro* and *in vivo* situations. The process requires minimal manipulation of the cell, and this is clearly an advantage compared to transfection requiring methods where cells must be subjected to considerable chemical or physical treatments for transfection (see Section III).

A number of target genes have been identified to be reasonable end points that reflect NF-κB activation in certain species. These genes and their products include macrophage inflammatory protein-2, interleukin-1, interleukin-2 receptor, interleukin-6, interleukin-8, intercellular adhesion

molecule (ICAM)-1, and inducible nitric oxide synthase. In this context it should be noted that the expression of a gene is mostly, if not always, regulated by multiple transcription factors and that enhanced expression of a particular gene may be the result of cooperation between multiple transcription factors that include NF-κB. Additionally, the involvement of NF-κB in the activation of these genes appears to be species specific.

A. Determination of ICAM-1 Expression

Cells (1×10^6/ml) may be either pretreated or not with an antioxidant for an optimized duration necessary for the antioxidant to function best. Following the pretreatment, cells are either activated or not by appropriate agents, e.g., 100 μM H_2O_2 for 18 hr. To study ICAM-1 expression, cells (0.5×10^6/ml) are incubated with 20 μl of ICAM-1 monoclonal antibody coupled with the fluorochrome fluorescein isothiocyanate (FITC) (Immunotech, Coulter Corporation, Miami, FL) for 30 min at 4°. After the incubation, cells are washed twice in ice-cold Dulbecco's PBS, pH 7.4 (D-PBS), and finally resuspended in fresh D-PBS. Expression of ICAM-1 may be detected by Western blotting or using a flow cytometer as described below.

The fluorescence and light scattering properties (forward scatter, FS; side scatter, SS) of cells may be determined by using a standard EPICS-XL (Coulter, Miami, FL) flow cytometer. In each sample, at least 10,000 gated viable cells are examined. Cells stained with FITC-conjugated antibodies are studied at a rate of 300–500 events per second. FITC fluorescence is detected using a 488 nm argon ion laser, and the emission is recorded

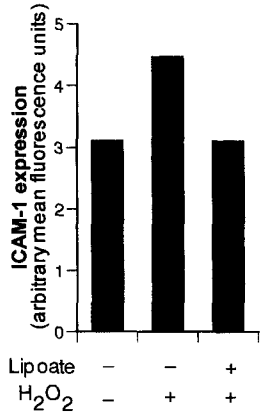

FIG. 4. Pretreatment of Wurzburg T cells with α-lipoate inhibits H_2O_2-induced expression of ICAM-1. α-Lipoate, 250 μM, 24 hr; H_2O_2, 100 μM, 18 hr. ICAM-1 expression was measured using a flow cytometer as described in the text.

at 525 nm. Autofluorescence of cells is recorded and subtracted from total mean fluorescence detected from FITC stained cells to obtain ICAM-1 specific fluorescence data. Pretreatment of cells with the antioxidant α-lipoate inhibited H_2O_2 induced expression of ICAM-1 (Fig. 4) in cells where H_2O_2 is known to result in NF-κB activation as shown by EMSA (Fig. 1).

V. Summary

The goal of this chapter was to review the current protocols that are available to measure the activation of NF-κB. The methods discussed all have their pitfalls when used in isolation. To obtain meaningful information. nuclear translocation and transcriptional activation should be studied in conjunction. Study of NF-κB regulated protein expression is the most physiologically relevant approach to monitoring the transcription regulatory effect of NF-κB. Because of the limitations of transcriptional analysis in primary cell cultures or tissues, incorporation of multiple approaches is recommended when the involvement of NF-κB in a disease process is evaluated.

[37] Assessing Induction of IκB by Nitric Oxide

By MARTIN SPIECKER and JAMES K. LIAO

Introduction

The activation of nuclear factor-κB (NF-κB) is required for the transcriptional induction of many proinflammatory mediators involved in vascular inflammation and atherogenesis such as cellular adhesion molecules, cytokines, and growth factors.[1,2] Factors which modulate the activity of NF-κB activation, therefore, could potentially regulate inflammatory processes and atherogenesis.

NF-κB is a family of homo- or heterodimeric cytosolic proteins, which can be further divided into two groups based upon their structure and function. The first group consists of p65 (Rel A), c-Rel, and Rel B, which contain transcriptional activation domains necessary for gene induction.[3] The second group consists of p105 and p100, which on proteolytic processing

[1] T. Collins, *Lab. Invest.* **68,** 499 (1993).
[2] H. B. Peng, T. B. Rajavashisth, P. Libby, and J. K. Liao, *J. Biol. Chem.* **270,** 17050 (1995).
[3] D. W. Ballard, E. P. Dixon, N. J. Peffer, H. Bogerd, S. Doerre, B. Stein, and W. C. Greene, *Proc. Natl. Acad. Sci. USA* **89,** 1875 (1992).

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