

Involvement of intracellular Ca^{2+} in oxidant-induced NF- κ B activation

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Abstract In human Jurkat T cells and its subclone Wurzburg cells oxidant challenge elevated $[\text{Ca}^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores. In Jurkat cells this effect was rapid and transient, but in Wurzburg cells the response was slow and sustained. H_2O_2 -induced NF- κ B activation in Wurzburg cells was not influenced by the presence of extracellular EGTA but was totally inhibited in cells that were loaded with esterified EGTA. In Jurkat cells that are not sensitive to H_2O_2 -induced NF- κ B activation, H_2O_2 potentiated NF- κ B activation in the presence of sustained high $[\text{Ca}^{2+}]_i$ following thapsigargin treatment. NF- κ B regulatory effect of α -lipoate and *N*-acetylcysteine appeared to be, at least in part, due to their ability to stabilize elevation of $[\text{Ca}^{2+}]_i$ following oxidant challenge. Results of this study indicate that a sustained elevated $[\text{Ca}^{2+}]_i$ is a significant factor in oxidant-induced NF- κ B activation.

Key words: Antioxidant; Redox; Transcription; NF- κ B; Calcium; Thiol

1. Introduction

NF- κ B activity is inducible and regulates a wide variety of immunoregulatory and viral genes, notably IL-2 receptor α chain, IL-2 and human immunodeficiency virus [1]. Because of the close association of NF- κ B function with several human diseases, strategies to modulate the activation of this cytosolic transcription factor are clinically significant. NF- κ B activation is responsive to a wide range of stimuli, and interestingly the response to almost all stimuli is antioxidant inhibitable [2,3]. Such observations have led to the hypothesis that ROS act as a common intracellular messenger that causes NF- κ B activation [2]. The exact mode of ROS-induced activation of NF- κ B and of antioxidant regulation of the NF- κ B activation process, however, is still unclear.

Cytosolic NF- κ B activation can be brought about by a variety of pathogenic or pathogen-elicited stimuli including cytokines, mitogens, bacteria and related products, virus and viral products, physical stress such as UV and ionizing radiation, oxidants such as H_2O_2 and a variety of chemical agents such as phorbol esters, and certain phosphatase inhibitors.

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Abbreviations: NF- κ B, nuclear factor κ B; IL-2, interleukin-2; ROS, reactive oxygen species; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; indo-1, 1-[2-amino-5-(6-carboxylindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane *N,N,N',N'*-tetraacetic acid; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; EGTA, ethyleneglycoltetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NAC, *N*-acetyl-L-cysteine; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; EGTA-AM, acetomethoxyl ester of ethyleneglycoltetraacetic acid.

From our current understanding of the mechanisms involved in NF- κ B activation we know that these stimuli trigger phosphorylation, ubiquitination and subsequent proteolytic degradation of the bound I κ B inhibitory protein [1,4,5]. Loss of I κ B bound to the Rel protein complex p50–p65 is followed by a rapid translocation of the p50–p65 heterodimer to the nucleus. The I κ B protein family is characterized by the presence of (i) a partially conserved domain that docks five to seven ankyrin repeat motifs 33 amino acids long that allow these proteins to interact with the Rel proteins, and (ii) a C-terminal protein destabilizing sequence rich in the amino acids proline, glutamate/aspartate and serine/threonine also known as the PEST sequence. The presence of this PEST sequence facilitates proteolytic degradation of I κ B proteins [4].

Intracellular calcium storage organelles play a key role in calcium signalling. These organelles sequester calcium ions from the cytoplasm through the action of the sarco-endoplasmic reticulum-type calcium transport ATPases and release calcium into the cytoplasm via receptor operated calcium channels [6]. It is well established from several in vitro studies that oxidant challenge is followed by a rapid perturbation of intracellular calcium homeostasis resulting in elevated $[\text{Ca}^{2+}]_i$ [7,8]. Because proteins containing PEST regions are known to be susceptible to degradation by calcium-dependent intracellular proteases, and because I κ B is known to be one such protein we tested the hypothesis that oxidant induced increase in $[\text{Ca}^{2+}]_i$ is implicated in NF- κ B activation. Human Jurkat T-cells are not highly sensitive to oxidant (H_2O_2) induced NF- κ B activation [9], however, a Wurzburg subclone of Jurkat T-cells is highly sensitive to such activation. Using these two cell types we sought to show if an oxidant induced sustained increase in $[\text{Ca}^{2+}]_i$ is a crucial factor in the NF- κ B activation process. α -Lipoate and *N*-acetylcysteine, two thiol antioxidants capable of regulating NF- κ B activation in response to a variety of stimuli, were used to determine if the NF- κ B regulatory effect of these antioxidants was, at least in part, due to their ability to stabilize elevation of $[\text{Ca}^{2+}]_i$ following oxidant challenge.

2. Materials and methods

2.1. Cell culture

Human lymphoma Jurkat T-cells were obtained from American Type Culture Collection (ATCC, Bethesda, MD). Wurzburg cells developed by Dr. Patrick A. Baeuerle (Freiburg, Germany) were a kind gift of Dr. Leonard Herzenberg of Stanford University, CA. Both cells were grown in RPMI 1640 medium supplemented with 10% FCS, 1% (w/v) penicillin-streptomycin, 110 mg/l sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco, CA) in humidified air containing 5% CO_2 .

2.2. Nuclear extraction

Cultured cells were harvested, centrifuged ($125\times g$ for 10 min) and synchronized by resuspending in 1% FCS growth medium either with

or without 1 mM α -lipoate (as indicated in the figure legends) at a density of $\sim 1.25\text{--}1.5 \times 10^6$ cells per ml. Cells were seeded in a 24-well plate with 1.5 ml cell suspension per well. After 18 h of incubation, cells were challenged with 0.25 mM H_2O_2 (Sigma).

Nuclear extracts were prepared from $\sim 2 \times 10^6$ cells as previously described [10,11] with slight modifications [12].

EMSA were performed essentially as described earlier [12,13]. Binding reaction mixtures (20 μl) containing 10 μg protein of nuclear extract, 1 mg poly(dI-dC) (Pharmacia, Sweden), ^{32}P -labeled probe (NF- κB consensus oligonucleotide), 50 mM NaCl, 0.2 mM Na_2EDTA , 0.5 mM DTT, 2% (v/v) glycerol and 10 mM Tris-HCl (pH 7.5) were incubated for 20 min at 37°C. Proteins were separated by electrophoresis in a native 6% polyacrylamide gel using a Tris-borate-EDTA running buffer (12.5 mM Tris-borate containing 0.25 mM Na_2EDTA , pH 8.0), followed by autoradiography. The NF- κB probe (Promega) was labeled with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ (ICN Biomedicals) using T4 polynucleotide kinase (Promega). The labelled probe was purified using a NAP-5 column (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA and stored in aliquots.

2.3. Determination of changes in intracellular Ca^{2+}

Cells grown in 10% FCS containing medium either with or without 1 mM α -lipoate were pelleted ($125 \times g$) and resuspended in Isocove's modified Dulbecco medium (Gibco BRL). For NAC treatment, cells grown in 10% FCS medium for 18 h were pelleted and resuspended in Isocove's modified Dulbecco medium containing 20 mM NAC, pH 7.5 for 2 h before oxidant treatment. Calcium flux was measured in indo-1 (Molecular Probes) loaded cells. Real time changes in intracellular Ca^{2+} were followed using a FACS (EPICS ELITE XL, Coulter) as indicated in the legend of Fig. 1. Unless otherwise indicated, experiments were carried out in the presence of 1 mM of the extracellular calcium chelator EGTA (Sigma) to follow oxidant induced mobilization of intracellular calcium reserves. EGTA was added to the indo-1 loaded cell suspension 10 min before the commencement of flow cytometric analysis.

3. Results

3.1. Jurkat cell $[\text{Ca}^{2+}]_i$

Real time flow-cytometric monitoring of oxidant induced changes in $[\text{Ca}^{2+}]_i$ in a gated morphometrically homogeneous Jurkat T-cell population revealed that within 60–90 s of 0.25 mM H_2O_2 challenge $[\text{Ca}^{2+}]_i$ was remarkably elevated (Fig. 1a). This phase of a rapid Ca^{2+} spike was followed by a gradual decrease of the intracellular Ca^{2+} signal. Typically, in less than 15–20 min after oxidant challenge $[\text{Ca}^{2+}]_i$ in most of the cells returned to baseline levels (Fig. 1d). To evaluate whether the Ca^{2+} in the oxidant-treated cells originated from the extracellular medium or intracellular stores, the same experiment (Fig. 1a) was carried out in the presence of extracellular calcium chelator EGTA. The presence of extracellular EGTA had minimal effect on oxidant induced elevation of $[\text{Ca}^{2+}]_i$ (Fig. 1b). In Jurkat cells loaded with EGTA-AM, the baseline $[\text{Ca}^{2+}]_i$ level was lower and oxidant challenge had no effect on $[\text{Ca}^{2+}]_i$ increase (Fig. 1c). In cells that were pretreated with thapsigargin, baseline $[\text{Ca}^{2+}]_i$ level was high over a period of more than 2 h from the addition of the sarco-endoplasmic reticulum membrane pump inhibitor. In these cells oxidant treatment resulted in only a transient further elevation of $[\text{Ca}^{2+}]_i$ (Fig. 2). Pretreatment of the Jurkat cells with the thiol antioxidants α -lipoate and NAC partially stabilized oxidant induced perturbation of intracellular calcium homeostasis (Fig. 1d,e). With higher concentrations (2–5 mM) of lipoate pretreatment, oxidant induced elevation of $[\text{Ca}^{2+}]_i$ was remarkably suppressed such that the oxidant treatment had only a minimal effect on $[\text{Ca}^{2+}]_i$ [3]. The active

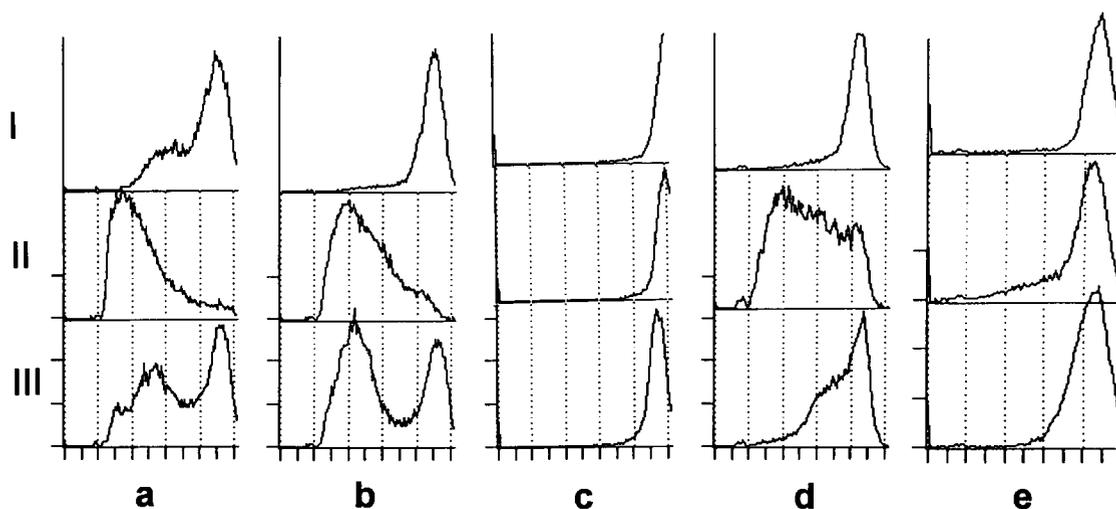


Fig. 1. Changes in intracellular Ca^{2+} in Jurkat T-cells challenged with H_2O_2 . Data are presented as cell population-fluorescence distribution plots. The x-axis represents the unbound/bound indo-1 ratio. A decrease in this ratio, i.e. shift towards the origin, represents an increase in cytosolic Ca^{2+} . The ordinate represents relative cell count. I (top) represents the mean of a 45 s baseline measurement of resting cells before H_2O_2 treatment; II (middle) represents the mean of a 1 min measurement of cells 1 min after 0.25 mM H_2O_2 treatment; III (bottom) represents the mean of a 1 min measurement of cells during the last min of the 10 min measurement. Cells loaded with indo-1 were not treated with any calcium chelator (a), treated with 1 mM EGTA 10 min before H_2O_2 treatment (b), or loaded with 0.5 mM of lipophilic EGTA-AM (Molecular Probes Inc.) and treated with 1 mM EGTA 15 and 10 min before H_2O_2 treatment (c). To observe the effects of NF- κB regulatory thiol antioxidants α -lipoate and NAC on oxidant induced perturbation of intracellular Ca^{2+} homeostasis, cells were treated with either 1 mM α -lipoate for 18 h (d) or 20 mM NAC for 2 h (e) before being challenged with H_2O_2 . Intracellular Ca^{2+} was followed using a FACS (EPICS ELITE XL, Coulter). The ratio of fluorescence intensities at 510 nm and 405 nm in response to a 350 nm UV laser excitation was continuously followed for 10 min at a flow rate 250–300 cells/s. After 45 s of baseline measurement, cells were challenged with 0.25 mM H_2O_2 . Experiments b–e were carried out in the presence of 1 mM of the extracellular calcium chelator EGTA to follow oxidant induced mobilization of intracellular calcium reserves.

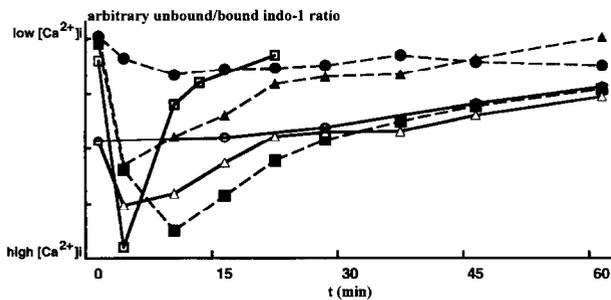


Fig. 2. Changes in intracellular Ca^{2+} in Jurkat and Wurzberg T-cells challenged with H_2O_2 . All experiments were carried out in the presence of 1 mM of the extracellular calcium chelator EGTA to follow oxidant induced mobilization of intracellular calcium reserves. Solid and hatched lines represent Jurkat and Wurzberg cell results, respectively. Intracellular Ca^{2+} was measured from 15 000 cells at the given time points after 0.25 mM H_2O_2 challenge. The zero time data represent baseline intracellular Ca^{2+} levels before H_2O_2 challenge. Wurzberg cells were either not pretreated with any antioxidant (\blacksquare), treated with 1 mM α -lipoate for 18 h (\blacktriangle), or treated with 20 mM NAC for 2 h (\bullet) before H_2O_2 challenge. Jurkat cells were either not treated with the calcium pump inhibitor (\square) or treated with 1 mM thapsigargin for 1 h (\triangle) before H_2O_2 challenge. To monitor the changes in intracellular Ca^{2+} as result of thapsigargin treatment alone, one set of cells were treated with thapsigargin (as in \triangle) but not treated with H_2O_2 (\circ).

antioxidant form of α -lipoate is dihydrolipoate. Dihydrolipoate as such is highly unstable in its reduced form. We have observed that Jurkat and Wurzberg cells rapidly reduce lipoate to dihydrolipoate and release the latter to the cell culture medium [14]. In cells that were incubated for 18 h in 1 mM α -lipoate and washed in lipoate-free medium, lipoate and dihydrolipoate contents per 10^6 cells were 13.18 and 6.51 pmol, respectively (result not shown).

3.2. Wurzberg cell [Ca^{2+}]_i

The kinetics of oxidant dependent changes in [Ca^{2+}]_i in Wurzberg cells was quite different compared to the parental Jurkat cell line (Fig. 2). In Wurzberg T-cells, the 0.25 mM H_2O_2 induced peak [Ca^{2+}]_i was observed after about 10 min of the challenge. Even after 60 min of oxidant treatment, [Ca^{2+}]_i level was higher than the baseline. Pretreatment of Wurzberg cells with α -lipoate or NAC resulted in lower post-oxidant [Ca^{2+}]_i mobilization.

3.3. NF- κ B activation

When nuclear protein extracts of H_2O_2 stimulated Wurzberg T-cells were examined by EMSA using the labeled NF- κ B binding oligonucleotide a distinct NF- κ B retardation band was observed (Fig. 3). The specificity of the NF- κ B band was demonstrated by treating the nuclear protein of positive control extracts with an excess of cold competitor probe or cold mutant (one base pair altered, Santa Cruz Biotechnology Inc.) probe for 15 min before the EMSA was performed (Figs. 3 and 4). H_2O_2 induced activation of NF- κ B was not influenced by the presence of extracellular EGTA. In cells that were loaded with EGTA-AM either 15 min before oxidant challenge or even 30 min after the challenge, H_2O_2 failed to induce NF- κ B activation at all. Pretreatment of the Wurzberg cells with the thiol antioxidants α -lipoate or NAC resulted in a marked inhibition of H_2O_2 induced NF- κ B activation in these cells.

In contrast to the Wurzberg cells, Jurkat T-cells are barely

responsive to H_2O_2 with respect to NF- κ B activation (Fig. 4). Treatment of the Jurkat cells with thapsigargin for 3 h weakly induced NF- κ B activation. This effect of thapsigargin was strongly potentiated by H_2O_2 . Pretreatment of the cells with thapsigargin for 1 h before the oxidant challenge markedly enhanced NF- κ B activation compared to thapsigargin alone. Using pretreatment of the nuclear protein with an excess of either cold NF- κ B oligonucleotide or the respective cold mutant probe it was demonstrated that the gel retarded band obtained by H_2O_2 challenge of thapsigargin pretreated cells was that of NF- κ B. NF- κ B activation by H_2O_2 in thapsigargin pretreated cells was not sensitive to the presence of extracellular EGTA. In thapsigargin treated cells preloaded with the EGTA-AM 15 min before H_2O_2 challenge, oxidant treatment failed to activate NF- κ B.

4. Discussion

NF- κ B is a transcription factor whose activity is essential for many immunoregulatory and other genes [1]. Elucidation of the cellular factors that modulate NF- κ B function is a necessary prerequisite to design effective strategies to pharmacologically manipulate NF- κ B activation and thus the pathogenesis of related health disorders [3]. Because antioxidants are capable of inhibiting NF- κ B activation in response to almost all of the diverse range of activating stimuli, a central role of ROS in the activation process has been suggested [2], however, the mechanism of ROS involvement is still unclear. In a number of systems it has been shown that intracellular calcium homeostasis is highly sensitive to ROS [7,8]. Although Ca^{2+} is widely recognized as an intracellular messenger, direct evidence supporting the involvement of intracellular Ca^{2+} in oxidant induced NF- κ B activation has not been reported until now.

In a number of cell types H_2O_2 is known to induce NF- κ B activation. However, human Jurkat T-cells are known to be insensitive to H_2O_2 with respect to NF- κ B activation whereas in Wurzberg T-cells, a daughter clone of Jurkat cells, H_2O_2 does induce NF- κ B activation [9]. We observed a clear difference in intracellular Ca^{2+} response in these two cell types after treatment with H_2O_2 . Although in both types of cells oxidant challenge resulted in a remarkable increase in [Ca^{2+}]_i level followed by a recovery to the baseline, the kinetics of the response was clearly different (Figs. 1 and 2). In Jurkat cells, the oxidant induced elevation of [Ca^{2+}]_i was rapid and transient. In contrast, in Wurzberg cells the peak level of [Ca^{2+}]_i was observed about 10 min after oxidant treatment, and even 1 h after the treatment [Ca^{2+}]_i was higher than baseline values.

There are two possible ways to increase [Ca^{2+}]_i in response to cell stimulation: (i) influx of Ca^{2+} from the extracellular medium, and/or (ii) mobilization of intracellular calcium pools particularly stored in the sarco-endoplasmic reticula and mitochondria [6]. Experiments performed in the absence or presence of extracellular EGTA demonstrated that most of the oxidant induced increase in intracellular Ca^{2+} originated from the release of Ca^{2+} from intracellular stores. Treatment of the cells with thapsigargin, a sarco-endoplasmic reticulum membrane calcium pump inhibitor, remarkably elevated [Ca^{2+}]_i. In the sarco-endoplasmic reticulum a large pool of intracellular calcium is maintained against a large concentration gradient by calcium pumps. Clearly, inhibiting calcium

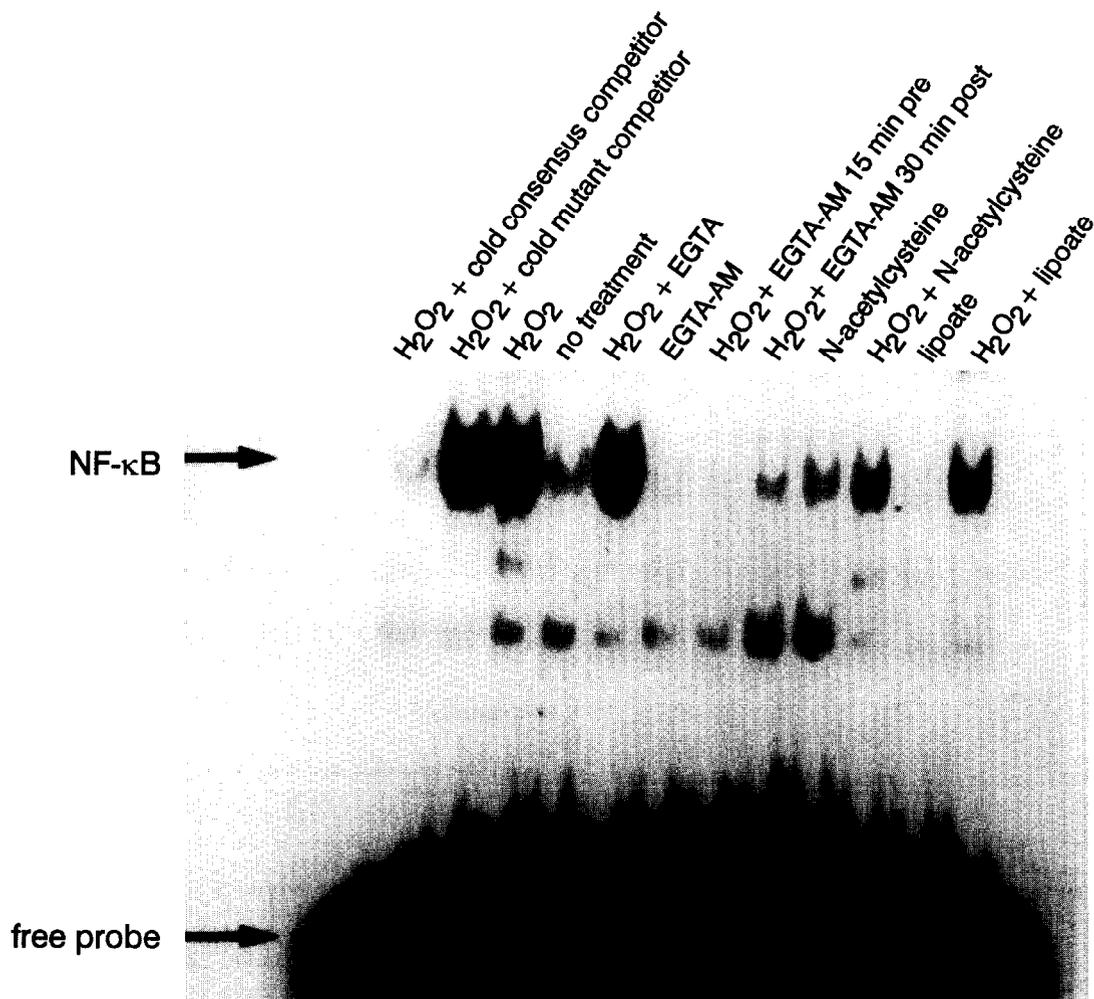


Fig. 3. Regulation of H_2O_2 induced NF- κB activation in Wurzburg cells by calcium chelators and thiol antioxidants. To test the specificity of the NF- κB band, nuclear extracts from activated cells were treated with an excess of unlabelled consensus NF- κB oligonucleotide (lane 1; from left to right) or with an excess of cold mutant NF- κB oligonucleotide (lane 2) before incubation with ^{32}P -labelled consensus NF- κB probe; lanes 3 and 4, cells were either treated or not treated with 0.25 mM H_2O_2 , respectively; lane 5, cells treated with 1 mM EGTA 10 min before H_2O_2 challenge; lane 6, cells treated with 0.5 mM EGTA-AM; lane 7, cells treated with 0.5 mM EGTA-AM 15 min before H_2O_2 challenge; lane 8, cells treated with 0.5 mM EGTA-AM 30 min after H_2O_2 challenge; lane 9, cells treated with 20 mM NAC for 6 h; lane 10, cells pretreated with 20 mM NAC for 2 h before H_2O_2 treatment for 4 h; lane 11, cells treated with 1 mM α -lipoate for 22 h; lane 12, cells pretreated with α -lipoate for 18 h followed by H_2O_2 treatment for 4 h.

pump activity seriously compromised the ability of the cells to maintain a low baseline $[\text{Ca}^{2+}]_i$. Loading the cells with EGTA-AM resulted in chelation of intracellular Ca^{2+} as indicated by an elevated baseline unbound/bound indo-1 ratio. Because of an excess of intracellular calcium chelator, oxidant challenge failed to elevate $[\text{Ca}^{2+}]_i$ in EGTA-AM treated cells.

Consistent with previous reports we observed that H_2O_2 treatment resulted in a strong activation of NF- κB in Wurzburg cells [9]. NF- κB activation was not influenced by the lowering of extracellular calcium by EGTA, however, H_2O_2 induced activation was totally inhibited in cells that were loaded with the esterified calcium chelator EGTA-AM. Such an effect of EGTA-AM was observed in cells that were loaded with the ester 15 min before H_2O_2 treatment, and in cells loaded with EGTA-AM even after 30 min of the oxidant challenge indicating that a sustained increase in $[\text{Ca}^{2+}]_i$ is necessary for NF- κB activation. Although the specific site of high $[\text{Ca}^{2+}]_i$ action is not known it may be suggested that activation of several key cellular processes, e.g. cleavage of

PEST containing proteins by calcium activated proteases [15], and/or phosphorylation reactions may have been induced. It is known that the phosphorylation and subsequent degradation of the PEST containing inhibitory protein I κB are prerequisites for NF- κB activation [1,4,5]. Pretreatment of the cells with the NF- κB regulatory antioxidants α -lipoate or NAC dampened the oxidant induced intracellular Ca^{2+} response (Fig. 2). In support of the hypothesis that elevated $[\text{Ca}^{2+}]_i$ is involved in oxidant induced NF- κB activation, α -lipoate or NAC pretreatment of cells inhibited H_2O_2 induced NF- κB activation in Wurzburg cells. In a recent study it has been reported that the calcium antagonist TMB-8 inhibited okadaic acid induced NF- κB activation in HeLa cells [16]. We have observed a similar inhibitory effect of TMB-8 on H_2O_2 induced NF- κB activation in Wurzburg cells (data not shown). Recently it has been also suggested that intracellular calcium is also required for activation of NF- κB by endoplasmic reticulum stress inducing conditions [17].

Because the long terminal repeat region of the HIV proviral

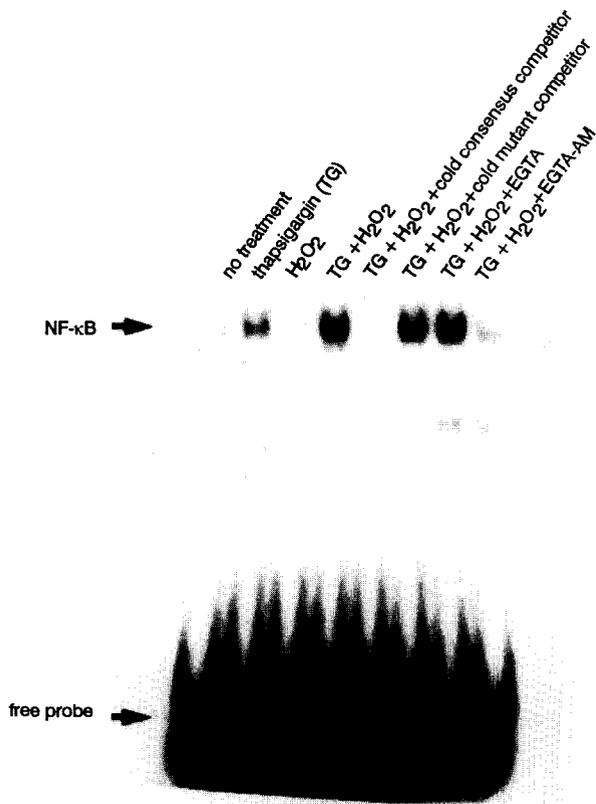


Fig. 4. Activation of NF- κ B in Jurkat cells by thapsigargin and H₂O₂. Lane 1 (left to right), no treatment; lane 2, cells treated with 1 mM thapsigargin for 3 h; lane 3, cells treated with 0.25 mM H₂O₂ for 2 h; lane 4, thapsigargin (1 mM) was added to the cells 1 h before H₂O₂ treatment; lane 5, nuclear extracts as of lane 4 were treated with an excess of unlabelled NF- κ B consensus oligonucleotide before incubation with labelled NF- κ B probe; lane 6, nuclear extracts as of lane 4 were treated with an excess of unlabelled NF- κ B mutant oligonucleotide before incubation with labelled NF- κ B probe; lane 7, thapsigargin and H₂O₂ treatment as in lane 4 was carried out in the presence of 1 mM EGTA added to the cells 10 min before H₂O₂ challenge; lane 8, thapsigargin and H₂O₂ treatment as in lane 4 was carried out in cells that were loaded with 0.5 mM EGTA-AM 15 min before H₂O₂ challenge.

DNA contains two binding sites for the transcriptional activator NF- κ B, activation of NF- κ B is implicated in HIV transcription [1]. Inhibition of the sarco-endoplasmic reticulum membrane calcium pump in T-lymphoid cells has been shown to stimulate HIV expression [18]. Our results suggest that the calcium pump inhibition induced HIV expression is likely mediated by activated NF- κ B.

That only a transient rise in [Ca²⁺]_i is not sufficient to cause NF- κ B activation is evident from our Jurkat cell data. Consistent with observations in other laboratories, H₂O₂ was barely capable of activating NF- κ B in these cells. Because

of their compromised ability to maintain the large sarco-endoplasmic reticulum calcium pool, thapsigargin treated cells exhibited above-baseline [Ca²⁺]_i over an extended time period, an effect which, by itself, was capable of only weakly activating NF- κ B. This effect of thapsigargin was markedly potentiated in cells challenged with H₂O₂. Oxidant induced further elevation of [Ca²⁺]_i in Jurkat cells was only slight and transient. Thus, oxidant induced potentiation of thapsigargin induced NF- κ B activation does not appear to be a calcium-dependent effect. Oxidant treatment triggered reactions which in combination with the presence of a sustained elevated [Ca²⁺]_i were capable of inducing further NF- κ B activation. Thus we are led to conclude that a sustained elevated [Ca²⁺]_i is a significant factor in oxidant induced NF- κ B activation. In support of the hypothesis that H₂O₂ induced activation of NF- κ B in thapsigargin treated Jurkat cells was elevated [Ca²⁺]_i-dependently, such activation was totally inhibited in cells that were preloaded with EGTA-AM. Taken together, our results suggest the involvement of intracellular Ca²⁺ in oxidant induced NF- κ B activation.

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