Pre-Exposure to Oxidative Stress Decreases the Nuclear Factor-κB-Dependent Transcription in T Lymphocytes

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Reactive oxygen species (ROS) are used as signaling molecules in T cell activation. One of the main targets of ROS is the transcription factor nuclear factor-κB (NF-κB). NF-κB-dependent transcription is inhibited by antioxidants, and the activation is induced or potentiated by ROS. However, chronic oxidative stress is known to reduce the activation of T cells and NF-κB. To analyze these phenomena in more detail, we have exposed Jurkat T cells in vitro to oxidative stress (H₂O₂) at various times before or simultaneously with signals known to activate NF-κB (phorbol dibutyrate (PDBu) and TNF). Simultaneously applied H₂O₂ strongly potentiated the PDBu- or TNF-induced transcriptional activity of NF-κB. In contrast to this, H₂O₂ given 3 to 20 h before the activating signal reduced NF-κB-dependent transcriptional activity. This was not due to the oxidation-induced modification of NF-κB; cytoplasmic NF-κB was able to bind to DNA after dissociation from 1κBα by detergent treatment. H₂O₂ pre-exposure effectively inhibited the PDBu- or TNF-induced phosphorylation and degradation of 1κBα, but H₂O₂ given simultaneously with PDBu or TNF enhanced the degradation. Oxidative stress was also followed by a strongly decreased ability to form intracellular ROS. Taken together, these data indicate that 1κBα phosphorylation is the target of action of ROS, and as the ROS-forming capacity is weaker after chronic oxidative stress, 1κBα is not effectively phosphorylated and degraded, thus leading to decreased NF-κB-dependent transcription. The Journal of Immunology, 1998, 160: 1354–1358.

Lymphocytes are exposed to reactive oxygen species (ROS) derived from activated macrophages and neutrophils during an inflammatory response. In addition to these exogenously derived ROS, it is now becoming more and more evident that ROS are produced within lymphocytes and are used as signaling molecules. Triggering of T cells via the TCR increases intracellular ROS levels, and antioxidants effectively down-regulate the early activation events in these cells (1–3). ROS activate several signaling systems within T lymphocytes, e.g., elevation of intracellular calcium, activation of protein kinase C, increased tyrosine phosphorylation, and activation of the Ras pathway (4–8). One of the more downstream targets of action of ROS is clearly the NF-κB transcription factor, which is involved in the transcriptional regulation of several genes activated during immune and inflammatory responses (1, 2, 9). NF-κB consists of Rel family proteins. These proteins are kept in cytoplasm in an inactive form by the IκB family inhibitors. Cellular activation leads to phosphorylation, ubiquitination, and subsequent proteolytic degradation of IκB, thus allowing the NF-κB proteins to migrate to the nucleus where they bind to DNA, usually as dimers. The ability to control transcription depends on the composition of the NF-κB complex as well as on the phosphorylation status of the NF-κB proteins. In only a few cell types does ROS induce a sufficient signal to activate NF-κB, but NF-κB activation induced by several activators is uniformly inhibited by various antioxidants (10, 11). The exact localization of this redox-controlled step is not known.

During chronic infections or autoimmune diseases, lymphocytes are exposed to long-standing oxidative stress, and this is often associated with decreased T lymphocyte functions. Flescher et al. (12) have shown that normal peripheral blood T cells exposed to oxidative stress for 2 days in vitro have a decreased capacity to activate NF-κB after TCR-mediated stimulation. To further analyze the difference between acute and chronic oxidative stress on NF-κB activation, we now have pre-exposed Jurkat T lymphoma cells to hydrogen peroxide (which is an insufficient signal to activate NF-κB) and analyzed its effect on the inducibility of NF-κB-dependent transcription.

Materials and Methods

Cell cultures

Jurkat T lymphoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Life Technologies, Paisley, Scotland) containing 10% FCS (Life Technologies), 10 mM HEPES buffer, 2 mM L-glutamine, and antibiotics. During exponential growth, the cells were stimulated, at 10⁶cells/ml, with either 100 ng/ml of phorbol dibutyrate (PDBu; Sigma Chemical Co., St. Louis, MO) or 20 ng/ml of TNF (recombinant human TNF, Genzyme Corp., Cambridge, MA) in the presence or the absence of H₂O₂ (0.1 mM) as described in Results.

Transfection, luciferase, and β-galactosidase assays

Jurkat cells were transfected using the DEAE-dextran method. Cells (10 × 10⁶/ml) in RPMI 1640 were suspended with 250 μg of DEAE-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden), 50 mM Tris-HCl (pH 7.5), 3 μg of NF-κB-Luc plasmid p-55IgκLuc, and 3 μg of β-galactosidase control plasmid (13). p-55IgκLuc contains three tandem Igκ NF-κB motifs driving a minimal (~ 55 to +19) human IFN-β promoter (14). These plasmids were provided by Prof. K. Sakseła (Institute of Medical Technology, Tampere, Finland). The amount of transfected DNA was equalized to 20 μg using herring sperm DNA (Sigma Chemical Co.). Samples were incubated at 37°C for 90 min. After incubation the samples were treated for 2 to 3 min with DMSO (final DMSO volume, 10%) and suspended in culture.
medium. Transfected cells were stimulated 24 h after the transfection. Luciferase activity was measured by using a commercial luciferase assay system (Promega, Madison, WI). β-Galactosidase activity was measured in luciferase assay lysates using the following procedure: 50 μl of cell lysate, 5 μl of 10× lucZ buffer (10× lucZ = 500 mM NaCl, 100 mM MgCl2, and 100 mM β-ME), and 50 μl of 10 mM ONPG (Sigma). Samples were incubated at 37°C for 1 h, and the reaction volume was adjusted to 1 ml with H2O. β-Galactosidase activity was measured spectrophotometrarily (OD = 420 nm).

**Measurement of intracellular ROS**

2′,7′-Dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR) is a stable, nonfluorescent, cell-diffusible dye (15). Intracellular esterases cleave the acetyl groups from the molecule to produce nonfluorescent DCFH. This is trapped inside the cell, and in the presence of ROS, DCFH is further modified to fluorescent DCFH, which can be detected by flow cytometry. Cells were preloaded with 5 μM DCFH-DA at 37°C for 15 min and stimulated as indicated. Ten thousand individual data points were collected for each sample point using a Becton Dickinson FACScan flow cytometer (Mountain View, CA). The data are expressed as the mean fluorescence of unstimulated samples was subtracted from that of the stimulated ones at each data point.

**Western blotting**

The cytoplasmic protein fractions were prepared as previously described (16). Proteins (5 μg) were analyzed in 10% SDS-PAGE and transferred to Immobilon-P (PVDF) membranes (Millipore Corp., Bedford, MA). Membranes were incubated overnight at 4°C with anti-IκBα (1/1000) Ab (Santa Cruz Technology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated swine anti-rabbit Ig (1/3000; Dako) for 30 min at room temperature and streptavidin-biotinylated horseradish peroxidase complex (1/1000) Ab (Amersham, Aylesbury, U.K.) for 1 h at room temperature. Signals were visualized using enhanced chemiluminescence system (ECL) according to the manufacturer’s recommendation (Amersham). Results were quantitated using densitometric scanning. The equal loading of proteins was verified by using Coomassie brilliant blue R staining according to the manufacturer of the PVDF membranes.

**Results**

**Hydrogen peroxide pre-exposure decreases the transcriptional activity of NF-κB**

To examine the effect of hydrogen peroxide pre-exposure on the induction of NF-κB-dependent transcription, the cells were transfected with a luciferase reporter plasmid (p-55IgLuc) containing three repeats of NF-κB binding sites in front of a human IFN-β promoter (14). To control the efficiency of transfection and the viability of the transfected cells, a β-galactosidase plasmid was cotransfected together with the p-55IgLuc plasmid. The cells were treated with 0.1 mM H2O2 for either 3 or 20 h, and the cultures were stimulated with either TNF (20 ng/ml) or PDBu (100 ng/ml). Four hours thereafter the cells were lysed, and both luciferase and galactosidase activities were measured as described in Materials and Methods. The data shown in Figure 1 demonstrate that H2O2 pre-exposure clearly reduced both the TNF- and PDBu-induced NF-κB-dependent transcription, while simultaneously applied H2O2 had an increasing effect.

To analyze whether chronic oxidative stress could have an influence on the DNA binding ability of cytoplasmic NF-κB, we first treated cytoplasmic extracts with detergents (deoxycholate and Nonidet P-40) to dissociate the NF-κB/IκBα complex. NF-κB DNA binding was analyzed by electrophoretic mobility shift assay. Pre-exposure of cells to H2O2 for 3 h did not prevent the DNA binding of cytoplasmic NF-κB detergent treatment, indicating that the reduced transcriptional activity after H2O2 pre-exposure was not due to the modified DNA binding ability of NF-κB (data not shown).

**Effect of hydrogen peroxide pre-exposure on IκBα**

Nuclear localization of NF-κB proteins is controlled by inhibitor proteins, of which IκBα is the most prominent. To test whether the decreased transcriptional activation capacity of NF-κB after chronic oxidative stress is due to the decreased degradation of IκBα, the cytoplasmic extracts were analyzed in SDS-PAGE followed by immunoblotting with anti-IκBα. As previously shown (17, 18), stimulation of cells with TNF and PDBu induced the degradation and reformation of IκBα (Fig. 2A). Stimulation of cells together with PDBu and H2O2 or with TNF and H2O2 induced a clear degradation that was more long lasting in TNF- plus H2O2-stimulated cells than after TNF stimulation. When the cells were first treated with H2O2 for 3 h and then stimulated with PDBu or TNF, clear degradation was not detected. H2O2 alone did not induce IκBα-degradation (Fig. 2B).

The phosphorylation of IκBα has been shown to precede its degradation (17, 19, 20). To test whether the inhibited degradation of IκBα after H2O2 pre-exposure is due to the impaired phosphorylation, the total cellular extracts were analyzed in SDS-PAGE followed by immunoblotting with anti-IκBα. Stimulation of cells with TNF for 5 min induced the formation of a band with slower mobility (Fig. 2C, lane 2), which has previously been identified as a phosphorylated form of IκBα (21–24). Pre-exposure of cells to H2O2 did not induce the formation of this band (Fig. 2C, lane 4), thus indicating that the reduced degradation of IκBα after H2O2 pretreatment is due to the inhibited phosphorylation of IκBα. Similar results were obtained when cells were stimulated with PDBu, but the phosphorylation was weaker, and it was seen after 10 min of stimulation (data not shown).

**Effect of hydrogen peroxide pre-exposure on the induction of intracellular ROS formation**

As the NF-κB activators are also potent inducers of intracellular ROS formation, we analyzed intracellular ROS levels using the
ROS-reactive fluorochrome DCFH. In normal Jurkat cells H$_2$O$_2$ induced a strong ROS formation that had begun to increase by 5 min after the stimulation (Fig. 3). These levels declined to the baseline after 1 h. PDBu in untreated cells caused a much smaller response (note the different y-axis scale) that peaked 30 to 45 min after the stimulation (Fig. 4). In contrast to this, in the H$_2$O$_2$-preexposed (for 3 h) cells, PDBu had almost no effect. TNF as the inducer behaved in the same way as PDBu, but the kinetics of ROS formation were faster, and the down-regulative effect was not as strong (Fig. 5). Thus, it may be concluded that H$_2$O$_2$ pre-exposure had strongly decreased the ROS forming capacity of PDBu and TNF.

**Discussion**

In this report we have demonstrated that, in contrast to acute oxidative stress, long-lasting oxidative stress down-regulates NF-κB-dependent transcription. This reduction took place at the level of phosphorylation and degradation of the IκBα cytoplasmic inhibitor; H$_2$O$_2$ given simultaneously with the activating signal (TNF or PDBu) enhanced the IκBα degradation, but H$_2$O$_2$ given 3 h earlier effectively inhibited the phosphorylation and degradation of IκBα. Furthermore, we have excluded the possibility that H$_2$O$_2$-induced oxidation would be responsible for the reduced activation of transcription; cytoplasmic NF-κB derived from H$_2$O$_2$-pre-exposed cells and released from IκBα by detergent treatment had a normal DNA binding capacity. When the intracellular ROS levels were measured, it was observed that H$_2$O$_2$ pre-exposure clearly diminished the ROS formation induced by TNF or PDBu. Therefore it seems likely that reduced ROS formation in the pre-exposed cells is responsible for the deficient IκBα degradation, which then leads to weaker activation of the NF-κB-dependent transcription.

Cells use various mechanisms to neutralize the effects of oxidative stress (25). Glutathione (GSH; L-$\gamma$-glutamyl-L-cysteinyl-glycine) is probably the most important intracellular antioxidant. GSH reduces peroxides and is thus converted to the oxidized form, GSH disulfide. Activation of T cells via the TCR is known to

**FIGURE 2.** Effect of H$_2$O$_2$ pre-exposure on IκBα. A, Jurkat cells were stimulated with PDBu (100 ng/ml), TNF (20 ng/ml), or PDBu/TNF plus H$_2$O$_2$ (0.1 mM), or cells were first incubated for 3 h with 0.1 mM H$_2$O$_2$ and then stimulated with PDBu/TNF. B, Jurkat cells were stimulated with 0.1 mM H$_2$O$_2$. Cells were collected at the time points indicated. Cytoplasmic extracts (5 μg) were fractioned with 10% SDS-PAGE and transferred onto PVDF membranes. C, To analyze the phosphorylation of IκBα the cells were stimulated with TNF for 5 min or first incubated for 3 h with 0.1 mM H$_2$O$_2$ and then stimulated with TNF. Total cellular extracts (50 μg) were fractioned with 15% SDS-PAGE and transferred onto PVDF membranes. Immunodetections were performed using anti-IκBα (1/10000) Ab. Arrowheads indicate the position of IκBα, and an arrow in C points the phosphorylated IκBα. The data shown are from one representative experiment of four performed.

**FIGURE 3.** The effect of H$_2$O$_2$ on intracellular ROS levels in Jurkat cells. Jurkat cells were preloaded with DCFH-DA and exposed to 0.1 mM H$_2$O$_2$. At different time points thereafter, the intracellular fluorescence was quantitated by flow cytometry. The data shown are expressed as an increase in mean fluorescence intensity (the mean fluorescence of unstimulated samples was subtracted from that of the stimulated samples at each time point). The data shown are from one representative experiment of three performed.

**FIGURE 4.** The effect of H$_2$O$_2$ pre-exposure on the PDBu-induced intracellular ROS levels. Jurkat cells were preloaded with DCFH-DA, exposed to 0.1 mM H$_2$O$_2$ for 3 h, and stimulated with 100 ng/ml of PDBu. At different time points thereafter, the intracellular fluorescence intensity was quantitated by flow cytometry. The data shown are expressed as an increase in mean fluorescence intensity (the mean fluorescence of unstimulated samples was subtracted from that of the stimulated samples at each time point). The data shown are from one representative experiment of three performed.
probable that several T cell functions (both NF-
stress reduced the intracellular ROS levels and inhibited the phos-
dely exposed to oxidative stress (delivered both from the TCR/
As the maintenance of immunologic memory probably requires
inhibit the new ROS formation induced by a second stimulus.
It has previously been shown that cells overexpressing catalase are unable to activate NF-
but, in contrast, overexpression of Cu/Zn-dependent superoxide dismutase, which enhances the production of H2O2 from superoxide, potentiated NF-
It has also been shown that the phosphorylation and degra-
dation of IκBα are inhibited when GSH peroxidase is overex-
press (30). In this report we have shown that chronic oxidative stress reduced the intracellular ROS levels and inhibited the phos-
phorylation and degradation of IκBα. Chronic oxidative stress could enhance the function of detoxifiant enzymes, which could inhibit the new ROS formation induced by a second stimulus.
We have previously reported that naïve T cells (CD45RA+) exposed to H2O2 demonstrate higher NF-κB nuclear translocation than T cells of the activated/memory (CD45RO+) phenotype (31). As the maintenance of immunologic memory probably requires continuous or repeated antigenic contact (reviewed in Refs. 32 and 33), we hypothesized that memory cells are, consequently, repeat-
edly exposed to oxidative stress (delivered both from the TCR/ 
CD28-mediated signals and from extracellular sources), and this would then modify their antioxidative capacity. The data reported here support this hypothesis, but it should be noted that the Jurkat
cells used in the present studies are continuously proliferating, malignant cells, and although they are widely used as a model in T cell signaling studies, they do not necessarily behave in the same way as normal, resting T lymphocytes.

The data shown here might also explain some previously pub-
lished discrepant observations. Shatov et al. (34) observed that HIV gp120, which binds to the CD4 molecule on the T cell surface, is a strong inducer of intracellular ROS and consequently is able to augment the TNF-induced NF-κB activation when given simultaneously with TNF. Jabado et al. (35) have shown that pre-
treatment with gp120 resulted in inhibition of phorbol ester-in-
duced NF-κB activity. Thus, it is likely that ROS induced by the gp120-CD4 interaction have a similar timing-dependent effect on NF-κB activation as the exogenously added H2O2 used in our ex-
periments. Moreover, it could be speculated that T cells activated by gp120 alone would be hyporeactive to a subsequent TCR/CD28 stimulus, thus providing one explanation for the T cell deficiency associated with HIV infection.

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References

\[ \text{\textbf{FIGURE 5.}} \quad \text{The effect of H}_2\text{O}_2 \text{ pre-exposure on TNF-induced intracel-
ular ROS levels. Jurkat cells were preloaded with DCFH, exposed to 0.1 mM H}_2\text{O}_2 \text{, and stimulated with 20 ng/ml of TNF. At different time points thereafter, the fluorescence intensity was quantitated by flow cytometry. The data shown are expressed as an increase in mean fluorescence intensity (the mean fluorescence of unstimulated samples was subtracted from that of stimulated samples at each time point). The data shown are from one representative experiment of three performed.} \]