Evidence that reactive oxygen species do not mediate NF- κ B activation

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It has been postulated that reactive oxygen species (ROS) may act as second messengers leading to nuclear factor (NF)- κ B activation. This hypothesis is mainly based on the findings that *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), compounds recognized as potential antioxidants, can inhibit NF- κ B activation in a wide variety of cell types. Here we reveal that both NAC and PDTC inhibit NF- κ B activation independently of antioxidative function. NAC selectively blocks tumor necrosis factor (TNF)-induced signaling by lowering the affinity of receptor to TNF. PDTC inhibits the I κ B-ubiquitin ligase activity in the cell-free system where extracellular stimuli-regulated ROS production does not occur. Furthermore, we present evidence that endogenous ROS produced through Rac/NADPH oxidase do not mediate NF- κ B signaling, but instead lower the magnitude of its activation.

Keywords: NAC/NF- κ B/PDTC/reactive oxygen species/redox

Introduction

Recent studies have advanced our understanding of the signal transduction pathways leading to nuclear factor (NF)- κ B activation (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). In non-stimulated cells, most NF- κ B dimers are kept in the cytoplasm through interaction with I κ B inhibitory proteins. Potent NF- κ B activators, such as tumor necrosis factor (TNF) or interleukin (IL)-1, can induce almost complete degradation of I κ Bs within minutes. This process, which is mediated by the 26S proteasome, depends on phosphorylation of two conserved serines in the N-terminal regulatory domain of I κ B. The protein kinase that phosphorylates I κ Bs has been identified as a protein complex composed of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ /NEMO (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). Once phosphorylated, I κ Bs undergo a second post-

translational modification, polyubiquitylation by a specific ubiquitin ligase belonging to the SCF (Skp1/Cul1/F-box) family (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). Upon ubiquitylation, the I κ B proteins are rapidly degraded by the proteasome, thereby freeing NF- κ B, which translocates to the nucleus where it activates transcription.

Beyond the established regulation of NF- κ B described above, reactive oxygen species (ROS) have been proposed to be involved in the activation of NF- κ B (Schreck *et al.*, 1991; Baeuerle and Henkel, 1994; Flohé *et al.*, 1997). This is based on the following observations. (i) Certain types of compounds with antioxidant properties can block NF- κ B activation. (ii) Most, if not all, agents activating NF- κ B are known to trigger formation of ROS. (iii) NF- κ B activation can be induced by treatment with H₂O₂ in certain cell lines. However, the contribution of redox regulation in NF- κ B activation is still subject to intense debate, because of many conflicting reports (Brennan *et al.*, 1995; Korn *et al.*, 2001). In addition, several researchers have reported that H₂O₂-induced NF- κ B activation is highly cell type dependent (Anderson *et al.*, 1994; Bowie *et al.*, 1997; Li and Karin, 1999). Thus, NF- κ B activation does not seem to be a universal response to oxidative stress.

In this study, we examined whether or not the compounds that are thought to act as antioxidants inhibit NF- κ B activation in a redox-dependent manner. The most widely used inhibitors of NF- κ B signaling, *N*-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), are believed to act as antioxidants (Schreck *et al.*, 1991; Baeuerle and Henkel, 1994; Flohé *et al.*, 1997). However, we found that NAC blocks TNF-stimulated NF- κ B activation by inhibiting the binding of TNF to its receptors without affecting IL-1 or TPA-stimulated NF- κ B activation. Furthermore, PDTC inhibits ubiquitin ligase activity towards phosphorylated I κ B α in the cell-free system where extracellular stimuli-dependent ROS production does not occur. These results demonstrate clearly that both NAC and PDTC inhibit NF- κ B activation independently of antioxidative function.

In order to clarify whether or not ROS act as second messengers leading to NF- κ B activation in response to extracellular stimuli, the molecular mechanism of pathway that links cell surface receptors to sites of intracellular ROS generation must be defined. The Rac/NADPH oxidase system is accepted as a candidate responsible for endogenous production of ROS during NF- κ B activation (Sulciner *et al.*, 1996; Bonizzi *et al.*, 1999; Sanlioglu *et al.*, 2001). The small GTP-binding protein Rac, which plays a fundamental role in signaling pathways, controlling actin polymerization and cellular proliferation, is also required for superoxide formation by components of the NADPH oxidase complex (Van Aelst and D'Souza-Schorey, 1997). We established cell lines that inducibly express either a

dominant-negative or a constitutively active form of Rac1. These cell lines enabled us to control the endogenous ROS level by blocking or activating NADPH oxidase in intact cells. Using these cells, we found that endogenously produced ROS do not lead to NF- κ B activation.

Results

Do NAC and PDTC inhibit NF- κ B activation in an antioxidative manner?

A number of reports demonstrate that NAC and PDTC inhibit NF- κ B activation in different types of cells (Allen and Tresini, 2000). The ability of NAC and PDTC to block NF- κ B activation was reproduced in our experiments; both NAC and PDTC inhibited TNF-stimulated I κ B α degradation and NF- κ B DNA binding in Jurkat T cells (Figure 1A). The inhibitory action of NAC and PDTC is widely believed to be due to their antioxidative activities. However, a strong phenolic antioxidant epigallocatechin-gallate (EGCG) and a water-soluble vitamin E analog, Trolox, failed to inhibit TNF-stimulated NF- κ B activation (Figure 1B). To prove that the reagents we used were active in terms of hydrogen donation, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities of NAC, PDTC, Trolox and EGCG were examined. When expressed as the amount of DPPH lost (mol) by 1 mol of the antioxidants, the values were 1.22 (NAC), 2.01 (PDTC), 2.64 (Trolox) and 17.42 (EGCG), respectively, indicating that Trolox and EGCG are more potent hydrogen donors towards DPPH than NAC and PDTC. These results raise the question of whether NAC and PDTC act simply as antioxidants to inhibit NF- κ B activation.

NAC inhibits TNF-stimulated signal transduction by lowering TNF receptor affinity

Since an unusually high concentration of NAC (>10 mM) is frequently required to inhibit NF- κ B activation, it is possible that NAC directly affects signaling events independently of antioxidative function. As shown in Figure 2A, NAC inhibited TNF-stimulated I κ B α degradation in HeLa cells. Interestingly, NAC also significantly blocked TNF-induced activation of three MAP kinases, JNK, p38 and ERK (Figure 2A, left panel). In contrast, TPA-stimulated I κ B α degradation, and JNK, p38 and ERK activation were not affected at all by the same treatment with NAC (Figure 2A, right panel). Similarly, the responses of these signaling molecules to IL-1 were not significantly affected by NAC treatment (Figure 2B). Since TNF and IL-1 receptor recruit similar signal transducing proteins, TRAF2 and TRAF6, and elicit similar biological effects (Baud *et al.*, 1999), it is unlikely that only TNF-stimulated signaling pathways depend on NAC-sensitive ROS production while IL-1 signaling is independent of ROS production. The selective inhibition of TNF signaling by NAC was also observed in different types of cells, such as L929 cells, a murine tumorigenic fibroblast line (see Supplementary figure 1 available at *The EMBO Journal* Online), Jurkat T cells, a human T-cell line, and HT-29 cells, a human colonic adenocarcinoma cell line (data not shown). The biological effects of TNF are transduced through two TNF receptors: TNF receptor 1 (TNF R1; 55 kDa) and TNF receptor 2 (TNF R2; 75 kDa),

Fig. 1. Effect of compounds with antioxidant properties on TNF-induced NF- κ B activation in Jurkat T cells. (A) Jurkat T cells were pretreated with either 30 mM NAC or 200 μ M PDTC for 1 h, and then stimulated with 3 ng/ml TNF for the indicated times, and nuclear and cytoplasmic extracts were prepared. Nuclear extracts were used to detect NF- κ B DNA binding by EMSA. Using the same nuclear extracts, EMSA for constitutively DNA-binding protein Oct-1 was carried out as a loading control. The cytoplasmic extracts were analyzed by immunoblotting to detect I κ B α levels. (B) Jurkat T cells were pretreated with the indicated concentrations of either EGCG or Trolox for 1 h, and then stimulated or left untreated with 3 ng/ml TNF for 20 min. EMSA and immunoblotting were carried out as described in (A).

respectively (Vandenabeele *et al.*, 1995). Since almost all TNF activities can be mediated through TNF R1 and only TNF R1 can bind human TNF in murine system (Tartaglia *et al.*, 1991), we examined the effect of NAC on the recruitment of TRAF2 and RIP to TNF R1 using murine L929 cells. TNF R1 immunoprecipitates from TNF-stimulated cells, but not from untreated cells, contained TRAF2. Similarly, recruitment of RIP was observed in TNF-stimulated cells. Interestingly, anti-RIP antibody recognized a high molecular weight diffuse band in TNF-R1 immunoprecipitates from TNF-stimulated cells. No such protein was detected in immunoprecipitates from untreated cells (Figure 3A). NAC treatment strongly inhibited the recruitment of these proteins to TNF R1, while PDTC treatment did not, indicating that NAC, but not PDTC, affects signaling upstream of the recruitment of RIP and TRAF2 to TNF R1. Therefore, we next examined the effect of NAC on binding of TNF to its cell surface receptors. Figure 3B shows the saturation binding curves of TNF in the presence or absence of NAC. Scatchard analyses showed that NAC treatment resulted in a significant increase in the K_d value (Figure 3B), indicating that the affinity of cell surface receptor for TNF was

Fig. 2. NAC selectively inhibits TNF-activated signaling pathways. (A) HeLa cells were pretreated with 30 mM NAC for 1 h, followed by stimulation with either 3 ng/ml TNF or 50 ng/ml TPA for the indicated times. Immunoblotting analyses were performed to detect IκBα, phospho-p38 and phospho-p44/42 ERKs, respectively. c-Jun N-terminal kinase activity was measured using GST-c-Jun (1-79). (B) HeLa cells were pretreated with 30 mM NAC for 1 h, followed by stimulation with either 1 ng/ml IL-1 or 3 ng/ml TNF for the indicated times. Immunoblotting analyses were performed to detect IκBα, phospho-JNKs, phospho-p38 and phospho-p44/42 ERKs. N.S., non-specific.

reduced. These results strongly suggest that NAC inhibits TNF-induced NF-κB activation by interfering TNF binding to its receptor rather than by scavenging intracellular ROS produced in response to TNF.

PDTC inhibits the IκB-ubiquitin ligase activity in a cell-free system

Next we examined the mechanism of action of PDTC, another widely used inhibitor of NF-κB signaling. When L929 cells were treated with increasing concentrations of PDTC, TNF-stimulated IκBα degradation was significantly inhibited in a dose-dependent manner (Figure 4A). PDTC also inhibited JNK activation by TNF, whereas p38 and ERK activation were augmented (Figure 4A). Similarly, IL-1-stimulated IκBα degradation and JNK activation were inhibited, while activation of p38 and ERK was augmented by PDTC in L929 cells (Figure 4B). Similar results were obtained in HT-29 cells. PDTC inhibited IκBα degradation and JNK activation by TNF, IL-1 or TPA, whereas it augmented the activation of p38 and ERK in TNF- or IL-1-stimulated HT-29 cells (Figure 4C). In contrast to the case of TNF or IL-1, TPA-stimulated p38 activation was inhibited by PDTC (Figure 4C). If the molecular action of PDTC is limited to the inhibition of ROS production, it is difficult to explain its opposite effect on p38 signaling. Most likely there are several distinct PDTC-sensitive targets within cells.

To identify the PDTC-sensitive point in the NF-κB signaling pathway, we examined whether or not the following signaling events were affected by PDTC: (i)

Fig. 3. NAC attenuates TNF receptor signaling by lowering receptor affinity. (A) L929 cells cultured in 10-cm dishes were pretreated either 30 mM NAC or 200 μM PDTC for 1 h, followed by incubation with 20 ng/ml TNF for the indicated times. Cell extracts were precleared with preimmune serum/protein G-Sepharose and then immunoprecipitated with anti-mouse TNF R1 coupled to protein G-Sepharose, followed by immunoblotting analysis to detect TRAF2. The PVDF membrane was stripped and reprobed to detect RIP. As controls, the anti-mouse TNFR1-coupled beads and the whole-cell extract from non-stimulated L929 cells were directly resolved by SDS-PAGE followed by immunoblotting analyses. (B) L929 cells were incubated with increasing amounts of [^{125}I]TNF in the presence (open circles) or absence (filled circles) of 30 mM NAC at 4°C for 2 h. Specific binding was calculated by subtraction of non-specific binding (in the presence of a 100-fold excess of unlabeled TNF) from total binding. Each point is the mean of duplicate samples (left panel). Scatchard analyses (right panel) yielded the following data: control cells, 2490 sites/cell, $K_d = 0.81$ nM; NAC-treated cells, 2170 sites/cell, $K_d = 4.99$ nM. B/F, bound/free.

phosphorylation of IκBα; (ii) ubiquitylation of IκBα; (iii) degradation of IκBα; and (iv) NF-κB DNA binding. As shown in the top panels of Figure 5A, TNF-stimulated IKK activation was not significantly inhibited by PDTC in either HT-29 or L929 cells. Indeed, the amounts of endogenous IκBα phosphorylated in these cells were not decreased, but rather slightly increased irrespective of PDTC treatment (Figure 5A, second panels). In contrast, the degradation of IκBα and NF-κB DNA binding were significantly inhibited by PDTC treatment (Figure 5A, third and bottom panels, respectively), suggesting that PDTC blocks the process downstream of IκBα phosphorylation and upstream of IκB degradation. Therefore, the effect of PDTC on ubiquitylation of IκBα was examined.

In the presence of proteasome inhibitor MG132, significant accumulation of ubiquitylated IκBα was observed in TNF-stimulated L929 cells transfected with HA-ubiquitin cDNA (Figure 5B). However, when cells were pretreated with MG132 plus PDTC, the amount of ubiquitylated IκBα was remarkably reduced (Figure 5B). The IκB-ubiquitin ligase is classified into SCF type

Fig. 4. Divergent modulation of TNF-, IL-1- and TPA-activated signaling pathways by PDTC. **(A)** L929 cells were pretreated with the indicated concentrations of PDTC for 1 h, followed by the stimulation with 20 ng/ml TNF for 10 min. The cells were lysed and immunoblotting analyses were performed to detect I κ B α , phospho-JNKs, phospho-p38 and phospho-p44/42 ERKs. N.S., non-specific. **(B)** L929 cells were pretreated 200 μ M PDTC for 1 h, followed by the stimulation with 1 ng/ml IL-1 for the indicated times. Immunoblotting analyses were performed as described in (A). **(C)** HT-29 cells were pretreated with 200 μ M PDTC for 1 h, followed by stimulation with 3 ng/ml TNF, 10 ng/ml IL-1 or 100 ng/ml TPA for the indicated times. Immunoblotting analyses were performed as described in (A).

ligases composed of three common (Skp1, Cull1 and ROC1) and single-variable (F-box protein) subunits (Karin and Ben-Neriah, 2000). The actual recognition of phosphorylated I κ B is carried out by FWD1/ β -TrCP (Hatakeyama *et al.*, 1999; Maniatis, 1999). Interestingly, FWD1/ β -TrCP is also known to mediate the ubiquitin-dependent destruction of β -catenin, which plays an essential role in the Wntless/Wnt signaling pathway (Kitagawa *et al.*, 1999; Maniatis, 1999). As shown in the top panel of Figure 5C, anti-HA antibody recognized polyubiquitylated band in the β -catenin immunoprecipitate derived from MG132-treated L929 cells transfected with HA-ubiquitin cDNA. When the PVDF membrane was reprobbed with anti- β -catenin antibody, MG132-induced accumulation of mono- or di-ubiquitylated β -catenin was observed (Figure 5C, bottom panel). PDTC significantly inhibited MG132-induced accumulation of ubiquitylated β -catenin (Figure 5C). Similar results were obtained using anti-ubiquitin antibody to detect the endogenous polyubiquitylation of I κ B α and β -catenin (Figure 5D and E). These results strongly suggest that PDTC inhibits the SCF type ubiquitin ligase towards phosphorylated I κ B α and β -catenin.

To examine the effect of PDTC on the I κ B-ubiquitin ligase activity, L929 cells expressing Flag-tagged Cull1,

HA-tagged FWD1/ β -TrCP, Skp1 and ROC1 were lysed in an extraction/washing buffer containing various concentrations of PDTC. The relevant SCF complexes were then immunoprecipitated with anti-Flag antibody, and *in vitro* I κ B α ubiquitylation assay was carried out using [^{32} P]GST-I κ B α (1–32) as a substrate, keeping the PDTC concentration used in the extraction/washing buffer.

Kim *et al.* reported that the inhibition of NF- κ B activity by PDTC depended on zinc ions and free EDTA but not zinc-saturated EDTA (EDTA-Zn) interfered with the inhibitory action of PDTC (Kim *et al.*, 1999). Therefore, we prepared extraction/washing buffer containing either free EDTA or EDTA-Zn and examined their effect. As shown in the top panel of Figure 6A, significant polyubiquitylation of [^{32} P]GST-I κ B α (1–32) was observed in the absence of PDTC, regardless whether SCF complexes were immunopurified in the presence of free EDTA (lane 2) or EDTA-Zn (lane 7). The complete absence of chelator (EDTA or EDTA-Zn) during the isolation of SCF complex resulted in a remarkable decrease in ubiquitin ligase activity (data not shown). The amount of high molecular weight multiple conjugates observed as the bands in the top of the gel (indicated by an arrowhead) was reduced by 250 μ M PDTC treatment. By increasing the concentration of PDTC to 2000 μ M, the

Fig. 5. PDTC inhibits ubiquitylation of IκBα and β-catenin. (A) L929 and HT-29 cells were pretreated with 200 μM PDTC for 1 h, followed by the stimulation with TNF for the indicated times. Then cytoplasmic and nuclear extracts were prepared as described in Materials and methods. The cytoplasmic extracts were used for IKK assay and immunoblotting analyses to detect phospho-IκBα and IκB. Nuclear extracts were used to measure NF-κB and Oct-1 DNA binding activities by EMSA. (B) L929 cell transiently overexpressing HA-tagged ubiquitin were pretreated with 10 μM MG132 in the presence or absence of 200 μM PDTC for 1 h, followed by stimulation with 3 ng/ml TNF for the indicated times. Cell extracts were immunoprecipitated with anti-IκBα antibodies followed by immunoblotting to detect HA epitope. Then PVDF membrane was stripped and reprobed with anti-IκBα antibody. Ig, immunoglobulin heavy chain. (C) L929 cells transiently overexpressing HA-tagged ubiquitin were pretreated with 10 μM MG132 in the presence or absence of 200 μM PDTC for 2 h. Cell extracts were immunoprecipitated with anti-β-catenin antibodies followed by immunoblotting to detect HA epitope. Then PVDF membrane was stripped and reprobed with anti-β-catenin antibody. (D) HT-29 cell extracts were pretreated as described in (A) and immunoprecipitated with anti-IκBα antibodies followed by immunoblotting to detect endogenous ubiquitin. (E) L929 and HT-29 cell extracts were prepared as described in (B) and immunoprecipitated with anti-β-catenin followed by immunoblotting to detect endogenous ubiquitin.

levels of polyubiquitylated ladder observed just above the band of [³²P]GST-IκBα declined to the background level (Figure 6A, top panel). As shown in the middle and the

bottom panels in Figure 6A, each lane contained equal amounts of Flag-tagged Cul1 and HA-tagged FWD1/β-TrCP irrespective of PDTC addition. It should be noted

that 2000 μ M PDTC was required to inhibit I κ B degradation in L929 cells overexpressing SCF complex (Figure 6B), although 200 μ M PDTC was sufficient in parental L929 cells (Figure 4A). PDTC also inhibited the I κ B-ubiquitin ligase activity of recombinant SCF complex reconstituted *in vitro* (Figure 6C; Supplementary figure 2). These results demonstrate that PDTC inhibits the I κ B-ubiquitin ligase activity independently of antioxidative function.

Endogenously produced ROS do not mediate NF- κ B activation

To further examine the involvement of ROS in NF- κ B signaling, we focused on the NADPH oxidase system, since this enzyme complex is regulated by the small GTP-binding protein, Rac, which is known to participate in diverse extracellular stimuli-evoked signaling pathways (Van Aelst and D'Souza-Schorey, 1997). We established stable cell lines in which expression of either a dominant-negative form of Rac1 or a constitutively active form of this protein is regulated by a tetracycline derivative, doxycycline (Dox). This Tet-On system, by allowing comparison between the 'on' and 'off' states of the transfected cDNA, enables us to assess the direct consequences of expression of a single gene in an internally controlled system. As shown in Figure 7A, Dox treatment led to the significant induction of dominant-negative N17Rac1 in the cell line derived from HeLa Tet-On cells. We then investigated the effect of N17Rac1 expression on the TNF- or TPA-stimulated NF- κ B activation and ROS production. When control (-Dox) cells were stimulated with TNF or TPA, significant elevation of NF- κ B DNA binding activity was observed (Figure 7B). Expression of N17Rac1 did not affect the induction of NF- κ B DNA binding activity by 3 ng/ml TNF. Even at the lower concentrations (0.03, 0.3 and 1 ng/ml) of TNF, TNF-induced NF- κ B activation was not inhibited by N17Rac1 (data not shown). In contrast, TPA-induced NF- κ B activation was significantly inhibited by N17Rac1 induction (Figure 7B). Similar results were obtained by immunocytochemical analysis (Figure 7C). Cells were treated with Dox for 48 h, and then subjected to immunostaining to detect N17Rac1 and p65 subunit of NF- κ B using anti-Myc antibody and anti-p65 antibody, respectively. As the characteristic morphological feature, cell-cell association seemed to be increased in cells expressing N17Rac1. TNF induced nuclear translocation of p65 in most cells regardless of N17Rac1 expression (Figure 7C, middle panels). In contrast, TPA-induced nuclear translocation of p65 was significantly inhibited by N17Rac1 expression, while p65 nuclear translocation was observed in cells that did not express N17Rac1 (indicated by white arrowheads in Figure 7C, bottom panels). Next, we investigated whether or not dominant-negative N17Rac1 expression inhibited the TNF- or TPA-induced ROS production using DCFH-DA, a membrane-permeable fluorescent dye, which can be oxidized to DCF by intracellular ROS, resulting in increased fluorescence of the dye. As shown in Figure 7D, 30 min TNF treatment, sufficient for the induction of NF- κ B activity, did not elevate ROS production, whereas 2 h incubation with TNF resulted in a significant increase in ROS production in both control (-Dox) and N17Rac1-expressing (+Dox) cells.

Fig. 6. Direct inhibition of Cul1-ROC1-Skp1-FWD1/ β -TrCP SCF ubiquitin ligase complex by PDTC. (A) L929 cells overexpressing FLAG-tagged Cul1, HA-tagged FWD1/ β -TrCP, Skp1 and ROC1 were harvested in the extraction/washing buffer containing either free EDTA (EDTA) or zinc-saturated EDTA (EDTA-Zn) as described in Materials and methods. Before harvesting the cells, the extraction/washing buffer was supplemented with the indicated concentrations of PDTC. The cell extracts were immunoprecipitated with anti-FLAG antibodies and the resultant immunoprecipitates were analyzed by an *in vitro* ubiquitylation assay in the presence of the indicated concentrations of PDTC. Separately, the cell extracts were subjected to immunoblotting analyses to detect either FLAG-Cul1 or HA-FWD1/ β -TrCP. The bands marked by asterisks are non-specific. (B) L929 cells overexpressing FLAG-tagged Cul1, HA-tagged FWD1/ β -TrCP, Skp1 and ROC1 were pretreated with the indicated concentrations of PDTC, followed by stimulation with 20 ng/ml TNF for 15 min. The cells were lysed and the immunoblotting analysis was performed to detect I κ B α . (C) Recombinant SCF ubiquitin ligase complex was purified and preincubated with the indicated concentrations of PDTC. Then *in vitro* ubiquitylation assay was carried out as described in Materials and methods. E3, recombinant SCF complex bound to the beads.

These results demonstrate clearly that TNF-induced ROS production is not mediated by Rac/NADPH oxidase and occurs as a later event independently of NF- κ B activation. On the other hand, TPA treatment for 30 min elevated the ROS level in control (-Dox) cells and this elevation was significantly blocked by induction of N17Rac1 (Figure 7D).

Since dominant-negative N17Rac1 inhibited TPA-induced ROS production, it is likely that the NADPH oxidase complex is involved in TPA-evoked signaling cascades. However, we cannot conclude that ROS production through NADPH oxidase is required for the

Fig. 7. TNF-induced NF- κ B activation occurs independently of Rac/NADPH oxidase while TPA-induced NF- κ B activation depends on Rac. (A) Hygromycin-resistant clonal cell lines derived from HeLa Tet-On cells transfected with pTRE-Myc-N17Rac1 (HeLa Tet-On-N17Rac1 cells) were treated or left untreated with 2 μ g/ml Dox for 48 h. The cells were lysed and immunoblotting analyses were performed using either anti-Rac1 antibody or anti-Myc antibody. (B) HeLa Tet-On-N17Rac1 cells were treated with 2 μ g/ml Dox for 48 h. The cells were then stimulated with either 3 ng/ml TNF or 3 ng/ml TPA for the indicated times. Nuclear extracts were used to detect NF- κ B and Oct-1 DNA binding by EMSA. (C) HeLa Tet-On-N17Rac1 cells were seeded on glass coverslips and treated or left untreated with 2 μ g/ml Dox for 48 h. The cells were stimulated with either 3 ng/ml TNF or 3 ng/ml TPA followed by the immunostaining analyses to detect N17Rac1 (Myc) and p65 subunit of NF- κ B (p65). (D) HeLa Tet-On-N17Rac1 cells were treated or left untreated with 2 μ g/ml Dox for 48 h. The culture medium was replaced with Hank's balanced salt solution (HBSS) and the cells were stimulated with either 3 ng/ml TNF or 3 ng/ml TPA for the indicated times. ROS production was measured using DCFH-DA as described in Materials and methods. ** P < 0.01; *** P < 0.001.

NF- κ B activation by TPA, because Rac has several different downstream targets other than NADPH oxidase. Therefore, we examined the role of NADPH oxidase in TPA-induced NF- κ B activation using a cell line that inducibly expresses constitutively active form of Rac1 (V12Rac1). As shown in Figure 8A, Dox treatment strongly elevated the expression of V12Rac1 detected by both anti-Rac1 and anti-Myc antibodies, although the low level of the leaky expression was observed in control (-Dox) cells. V12Rac1-expressing cells showed the representative morphological feature, membrane ruffling (Supplementary figure 3). As shown in Figure 8B, significant elevation of ROS production was observed in cells treated with Dox for 48 h. When V12Rac1-expressing (+Dox) cells were treated with diphenyleneiodonium chloride (DPI), a known NADPH oxidase inhibitor (O'Donnell *et al.*, 1993), ROS production was significantly inhibited in both untreated and TPA-treated cells, suggesting that the elevated ROS production observed in

V12Rac1-expressing (+Dox) cells is mediated through NADPH oxidase (Figure 8B). DPI treatment also reduced ROS production in cells not incubated with Dox (data not shown), suggesting that leaky expression of V12Rac1 in control (-Dox) cells would cause constitutive ROS production. We next examined the effect of V12Rac1 expression on NF- κ B activation. Interestingly, V12Rac1 expression resulted in a slight inhibition of TPA-stimulated NF- κ B activation rather than the augmentation of its activation (Figure 8C, lanes 4–6 compared with lanes 1–3). Similar results were obtained by immunocytochemical analysis (Supplementary figure 3). DPI treatment, which prevented NADPH-oxidase-mediated ROS production, significantly augmented the TPA-stimulated NF- κ B activation in both control (-Dox) and V12Rac1-expressing (+Dox) cells (Figure 8C, lanes 7–9 compared with lanes 1–3, and lanes 10–12 compared with lanes 4–6, respectively). These results suggest that ROS produced through NADPH oxidase act negatively to lower the magnitude of

Fig. 8. ROS produced through NADPH oxidase do not mediate TPA-induced NF- κ B activation. (A) Hygromycin-resistant clonal cells derived from HeLa Tet-On cells transfected with pTRE-Myc-V12Rac1 (HeLa Tet-On-V12Rac1) were treated or left untreated with 2 μ g/ml Dox for 48 h. Immunoblotting analyses were performed as described in Figure 6A. (B) HeLa Tet-On-V12Rac1 cells were treated or left untreated with 2 μ g/ml Dox for 48 h. The cells were further cultured in the presence or absence of 20 μ M DPI for 3 h. The culture medium was replaced with HBSS and the cells were stimulated or left untreated with 3 ng/ml TPA for 30 min in the presence or absence of DPI. ROS production was measured using DCFH-DA as described in Materials and methods. * P < 0.05; ** P < 0.01. (C) HeLa Tet-On-V12Rac1 cells were treated or left untreated with 2 μ g/ml Dox for 48 h. The cells were further cultured in the presence or absence of 20 μ M DPI for 3 h. The cells were then stimulated with 3 ng/ml TPA for the indicated times. Nuclear extracts were subjected to EMSA to detect NF- κ B and Oct-1 DNA binding activities.

NF- κ B activation rather than transduce NF- κ B activating signal. Thus we conclude that ROS do not act as mediators leading to NF- κ B activation.

Discussion

ROS have been proposed to be involved in the activation of NF- κ B (Schreck *et al.*, 1991; Baeuerle and Henkel, 1994; Flohé *et al.*, 1997). However, how do ROS, as highly reactive but non-specific molecules, mediate well coordinated NF- κ B signaling? It seems difficult to envision any known protein in the signaling pathways that lead to NF- κ B activation as a specific receptor for ROS.

The initial hypothesis that ROS mediate NF- κ B signaling was based on the finding that putative antioxidants can inhibit NF- κ B activation (Schreck *et al.*, 1991; Baeuerle and Henkel, 1994). The most widely used NF- κ B inhibitors, NAC and PDTC, have been thought to act as such antioxidants. However, much stronger phenolic radical scavengers such as EGCG and Trolox failed to inhibit NF- κ B activation (Figure 1B). Therefore, it seemed questionable that NAC and PDTC inhibit NF- κ B activation in a manner dependent on their antioxidant activity.

NAC blocks TNF-evoked signaling by lowering the affinity of TNF R1, while IL-1- or TPA-activated signaling pathways are not affected at all (Figures 2 and 3). So far, two TNF receptors with a molecular weight of 55 kDa (TNF R1) and 75 kDa (TNF R2) have been identified and cloned. The extracellular domain of TNF R1 and R2 is composed of four conserved cysteine-rich repeats. In each repeat, four to six cysteines are involved in disulfide bridge formation (Vandenabeele *et al.*, 1995). Since NAC has a reducing thiol group, it can cause the structural changes in TNF receptors by reducing the disulfide bridges. NAC has been known to inhibit HIV replication (Roederer *et al.*, 1990). On the other hand, TNF is known to play a central role in the progression of AIDS. Indeed, TNF levels are abnormally high in serum samples from AIDS patients (Lahdevirta *et al.*, 1988). It is possible that NAC inhibits TNF-induced TNF production in HIV-infected lymphoid cells by blocking TNF binding to its receptors.

PDTC, another widely used NF- κ B inhibitor, is a stable analog of dithiocarbamates. It has been postulated that dithiocarbamates chelate iron thereby inhibit ROS production through the Fenton reaction (Schreck *et al.*, 1992). However, a ROS-independent mechanism by which dithiocarbamates exert their effects has also been suggested. For example, dithiocarbamates, including PDTC, are known to decompose at cellular pH to compounds such as carbon disulfide and isothiocyanates, both of which can modify proteins (Valentine *et al.*, 1992). Several groups have reported that the metal-transporting activity of PDTC was important for its inhibitory action toward NF- κ B signaling; a zinc-ionophore activity was reported by Kim *et al.* (1999), while a copper-ionophore activity was suggested by Iseki *et al.* (2000).

We found that PDTC divergently modulated TNF-, IL-1- and TPA-activated signaling pathways (Figure 4). These results suggest that PDTC has different targets within cells rather than simply inhibits ROS production. The PDTC-sensitive step in the NF- κ B signaling pathway was found to be at the level of I κ B polyubiquitylation (Figure 5B and D). Interestingly, polyubiquitylation of β -catenin was also blocked by PDTC (Figure 5C and E). Since I κ B and β -catenin have the similar phosphorylation-dependent destruction motifs recognized by the same SCF type ubiquitin ligase (Maniatis, 1999), it is likely that

PDTC directly inhibits the ubiquitin ligase towards phosphorylated I κ B and β -catenin. Indeed, the inhibition of the I κ B-ubiquitin ligase activity by PDTC was demonstrated in a cell-free system (Figure 6A and C), indicating that the NF- κ B inhibition by PDTC is independently of its antioxidative function.

In certain types of cells, such as Wurtzburg Jurkat T cells or human endothelial cell line ECV304, NF- κ B is activated by the treatment of cells with H₂O₂ (Anderson *et al.*, 1994; Bowie *et al.*, 1997). However, H₂O₂ fails to activate NF- κ B in other types of cells (Bowie *et al.*, 1997; Li and Karin, 1999). In order to clarify whether or not ROS can act as second messengers leading to NF- κ B activation, it was necessary to use the physiologically controlled system through which ROS can be produced endogenously in intact cells. For this purpose, we established the HeLa Tet-On cell lines inducibly expressing either a dominant-negative form or a constitutively active form of Rac1, since Rac is known as the regulatory component of ROS-producing NADPH oxidase (Van Aelst and D'Souza-Schorey, 1997).

By operating this Tet-On system, we could control the endogenous ROS production. As shown in Figures 7D and 8B, dominant-negative N17Rac1 expression inhibited TPA-induced ROS production, while constitutively active V12Rac1 induction resulted in significant ROS production. Using these cell lines, we found that TNF-stimulated NF- κ B activation occurred independently of Rac/NADPH oxidase system and TPA-stimulated NF- κ B activation depended on Rac, but not on ROS produced through NADPH oxidase. In the case of TNF stimulation, NF- κ B activation is terminated before remarkable elevation of ROS production is observed, suggesting that TNF-stimulated NF- κ B activation does not require endogenous ROS production. Furthermore, we found that ROS produced through Rac/NADPH oxidase lowered the magnitude of NF- κ B activation (Figure 8C). These data demonstrate clearly that endogenously produced ROS do not mediate NF- κ B activation as second messengers.

There have been several papers suggesting the involvement of Rac/NADPH oxidase during NF- κ B activation (Sulciner *et al.*, 1996; Bonizzi *et al.*, 1999; Sanlioglu *et al.*, 2001). The observations conflicting with ours can not be explained as the consequence of difference of cell lines used in the experiments, since Sulciner *et al.* reported that expression of V12Rac1 induced NF- κ B activation by itself and N17Rac1 expression inhibited IL-1-stimulated NF- κ B activity using HeLa, i.e. the parental cell line of HeLa Tet-On used in our experiments (Sulciner *et al.*, 1996). In most cases, studies were carried out using transient expression systems where the gene of interest was expressed in a small percentage of cells. Furthermore, in those experiments, the activation of NF- κ B was assessed by reporter gene assay in which cells were usually treated with activating agents such as TNF or IL-1 for a long period of time (i.e. 6–24 h). The reporter gene assay is a method for determining whether NF- κ B is finally activated as the transcriptionally active form but is not suitable for studying the initial signaling events that lead to NF- κ B activation.

The use of HeLa Tet-On-derived stable cell lines enabled the biochemical analysis of initial signaling events in a large number of genetically altered cells. In

addition, by measuring DCF fluorescence, which reflects the mass of ROS produced, we were able to critically assess the involvement of ROS in signaling. It should also be notified that Jefferies *et al.* found that Rac1 was not involved in IL-1-stimulated signaling pathway leading to I κ B α phosphorylation and degradation by the use of an inducible expression system under the control of an isopropyl- β -D-thiogalactopyranoside-responsive promoter (Jefferies *et al.*, 2000).

NF- κ B is an important molecular target for pharmacological intervention, since it regulates a wide variety of genes controlling immune and inflammatory responses, pathogenesis of AIDS and cancer (Baldwin, 2001). However, the compounds with the property of NF- κ B inhibitor must be characterized extensively on the following viewpoints: (i) what molecule in the multiple steps of NF- κ B signaling pathway is their direct target; and (ii) whether or not their inhibitory action is restricted to NF- κ B signaling. The evidence we present above should be useful in identifying highly specific NF- κ B inhibitors, since one can exclude from such a search antioxidants, which according to our results will not function as specific inhibitors of NF- κ B activation.

Materials and methods

Antibodies

Antibodies (Abs) specific for phospho-I κ B α (Ser32), phospho-p38 (Thr180/Tyr182) and phospho-p44/42 ERK were from Cell Signaling Technology; phospho-JNK (Thr183/Tyr185) was from Promega Corporation; NF- κ B p65 (C20), I κ B α (C21), I κ B α (C15), TRAF2 (C20), IKK α (M-280) and Ub (P4D1) were from Santa Cruz Biotechnology; IKK α (B78-1), RIP (clone 38), β -catenin (clone14) and Rac1 (clone 102) were from BD PharMingen and Transduction Laboratories; mouse TNF R1 was from R&D Systems; the FLAG epitope (M2) was from Sigma-Aldrich; and the HA epitope (12CA5) and the Myc epitope (9E10) were from Roche Applied Science.

Cell treatment

Subconfluent layers of adherent cells (HeLa, HT-29 and L929 cells) or Jurkat T cells at a density of 1×10^6 cells/ml were stimulated with activating agents (TNF, IL-1 or TPA) as indicated in the figure legends. One hour before adding the activating agents to the culture, cells were pretreated with NAC, PDTC or other antioxidants. The solutions of NAC and PDTC were freshly prepared on the day of treatment. The NAC solution was adjusted to pH 7.4 by the addition of 8 M NaOH. Unless otherwise noted, cells were seeded in 35-mm dishes and harvested for the following studies.

Preparation of the nuclear and cytoplasmic extract

Cells treated with various agents were washed with ice-cold phosphate-buffered saline (PBS) and then harvested in buffer A [10 mM HEPES/KOH pH 7.9, 10 mM KCl, 0.1% NP-40, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 3 μ g/ml pepstatin]. After centrifugation for 30 s at 12 000 g at 4°C, the cytoplasmic extracts were immediately adjusted to the condition for immunoprecipitation by adding the immunoprecipitation (IP) buffer (2 $\times$) described below. The nuclei were further washed once with buffer A and extracted with buffer C (20 mM HEPES/KOH pH 7.8, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF and 25% glycerol).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (Schreck *et al.*, 1991). Binding reaction mixtures containing 5 μ g protein of nuclear extract, 2 μ g poly(dI-dC) and ³²P-labeled probe, were incubated for 20 min at room temperature. Samples were analyzed by native 4% polyacrylamide gels. The sequence of NF- κ B probe was 5'-AATTCTCAGAGGGGACTTTCCGAGAGG-3'. The sequence of Oct-1 probe was 5'-CTAGATATGCAAATCATTTG-3'.

Hydrogen donating capabilities of water-soluble antioxidants to DPPH

Donation of hydrogen atom to stable free radical DPPH was conducted in a biphasic system as described previously (Hiramoto *et al.*, 2002). DPPH was dissolved in *n*-hexane to give 5 ml of 80 μ M solution. Each test compounds dissolved in 5 ml PBS were mixed and vigorously shaken for 2.5 h. The decrease in DPPH absorption was measured at 517 nm.

Immunoblotting, IP and immunostaining

Cells were washed with PBS and cell extracts were prepared using either SDS-PAGE sample buffer or IP buffer described below. After normalization of protein content according to the protein assay, samples were resolved by SDS-PAGE and subjected to immunoblotting analyses. The immunocomplexes on the PVDF membranes were visualized using enhanced chemiluminescence detection (Amersham Bioscience). For immunoprecipitation analyses, cells were lysed in IP buffer (DiDonato *et al.*, 1997) with a slight modification (NP-40 concentration was increased to 1.0%). To detect polyubiquitylated I κ B α and β -catenin, cells were lysed in RIPA buffer (IP buffer supplemented with 0.5% deoxycholate and 0.1% SDS). The cell extracts were incubated with the relevant antibodies and protein G-Sepharose beads and the immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting. Double-immunofluorescence staining for the Myc epitope and NF- κ B p65 was carried out as follows. Cells cultured on glass coverslips were fixed with 4% paraformaldehyde, then permeabilized with methanol/acetone (1:1), and blocked with 10% goat serum in PBS. The cells were incubated with a mixture of mouse anti-Myc Ab and rabbit anti-p65 Ab for 2 h, followed by the incubation with a mixture of FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h. These samples were analyzed by a Leica DM IRB microscope.

Kinase assay

Cell extracts were prepared using IP buffer (see above). Solid phase JNK assay was performed as described previously, using GST-c-Jun (1–79) as the substrate (Hibi *et al.*, 1993). Endogenous IKK was immunoprecipitated with anti-IKK α antibody (B78-1 for human IKK and M-280 for mouse IKK) and *in vitro* kinase assay was performed as described previously, using GST-I κ B α (1–54) as the substrate (DiDonato *et al.*, 1997).

IP *in vitro* ubiquitylation assay

L929 cells transiently overexpressing FLAG-tagged Cul1, HA-tagged FWD1/ β -TrCP, Skp1 and ROC1 were lysed in the extraction/washing buffer (25 mM HEPES/NaOH pH 7.4, 0.15 M NaCl, 10% glycerol, 0.5% NP-40, 20 mM β -glycerophosphate, 10 mM *p*-nitrophenyl phosphate, 10 mM NaF, 0.3 mM Na-*O*-vanadate, 100 nM okadaic acid, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 3 μ g/ml pepstatin, and various concentrations of PDTC) containing either 0.4 mM EDTA or 0.4 mM EDTA-Zn. FLAG-immunoprecipitates were washed four times with the extraction/washing buffer, followed by one washing with ubiquitylation reaction buffer (50 mM HEPES/NaOH pH 7.4, 0.1% NP-40, 5 mM MgCl₂, 2 mM NaF, 10 mM okadaic acid). To obtain recombinant IKK β , Sf9 cells expressing GST-IKK β was lysed in the IP buffer and subjected to glutathione-Sepharose 4B purification. GST-IKK β eluted from the beads was used to phosphorylate GST-I κ B α (1–54) in the presence of [γ -³²P]ATP. The SCF complex bound to the beads was incubated in the reaction mixture (25 μ l) containing [³²P]GST-I κ B α (1–54), E1 (100 ng), UbcH5c as E2 (1 μ g), 2 mM ATP, 30 mM creatine phosphate, 0.2 mg/ml creatine kinase, 200 μ g/ml ubiquitin, 0.1 mM DTT and various concentrations of PDTC for 1 h at 37°C. The samples were resolved by 7.5% SDS-PAGE followed by autoradiography.

Recombinant SCF *in vitro* ubiquitylation assay

Sf9 cells expressing either GST-Cul1/His-ROC1 or GST-FWD1/Skp1 (Morimoto *et al.*, 2003) were lysed in the extraction/washing buffer containing 0.4 mM EDTA as described above. The resultant extracts were combined and subjected to glutathione-Sepharose 4B purification. Equal amounts of gel slurry binding recombinant SCF complex were preincubated in the ubiquitylation reaction buffer containing various concentrations of PDTC for 10 min at 37°C. The ubiquitylation reaction was started by adding phosphorylated GST-I κ B α (1–54) and incubated for 1 h at 37°C. The resultant samples were resolved by 7.5% SDS-PAGE followed by immunoblotting using anti-I κ B α antibody (C15) that recognizes the N-terminal domain of I κ B α .

Transfection and establishment of stable cell lines

Transfection was carried out using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. To establish the stable cell lines that inducibly express either a dominant-negative or a constitutively active form of Rac1, HeLa Tet-On cells (LOT 710056; Clontech) were cotransfected either with pTRE-Myc-N17Rac1 and pTK-Hyg or with pTRE-Myc-V12Rac1 and pTK-Hyg. The hygromycin-resistant clonal cell lines were tested for Dox-regulated Myc-tagged mutant Rac1 expression.

Measurement of ROS production

HeLa Tet-On-derived sublines were treated or left untreated with 2 μ g/ml Dox for 48 h. The culture medium was replaced with Hank's balanced salt solution (HBSS) supplemented with 0.1% BSA. The cells were stimulated with either TNF or TPA as indicated in the figure legends. During the last 15 min of the incubation for stimulation, 10 μ M DCFH-DA (Molecular Probes) was loaded. After washing the excess amount of unincorporated DCFH-DA, cells were fixed and permeabilized with PBS containing 20% ethanol and 0.1% Tween-20. The cell extract was centrifuged and resultant supernatants were collected. DCF fluorescence was measured using a spectrofluorometer (excitation, 492 nm; emission, 526 nm). To assess the total amount of fluorescence probe incorporated into the cells, cells left untreated with Dox were incubated with 10 μ M DCFH-DA for 15 min, then washed with PBS, followed by the treatment with 400 μ M H₂O₂ for 4 h to oxidize incorporated DCFH to DCF. Then ethanol and 10% Tween-20 were added to give the final concentrations of 20% and 0.1%, respectively. Fluorescence intensity of the supernatant was calibrated to 100%. Assays were performed in triplicate. Data were analyzed by the Student's *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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