JNK (c-Jun NH2-terminal Kinase) Is a Target for Antioxidants in T Lymphocytes*

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AP-1 has been shown to behave as a redox-sensitive transcription factor that can be activated by both oxidant and antioxidant stimuli. However, the mechanisms involved in the activation of AP-1 by antioxidants are largely unknown. In this study we show that the structurally unrelated antioxidant agents pyrrolidine dithiocarbamate (PDTC), butylated hydroxyanisole, and N-acetylcysteine activated JNK (c-Jun NH2-terminal kinase) in Jurkat T cells. This activation differed substantially from that mediated by phorbol 12-myristate 13-acetate (PMA) and Ca²⁺ ionophore or produced by costimulation with antibodies against the T cell receptor-CD3 complex and to CD28. The activation of JNK by classical T cell stimuli was transient, whereas that mediated by PDTC and butylated hydroxyanisole (but not N-acetylcysteine) was sustained. The kinetics of JNK activation correlated with the expression of c-jun which was transient after stimulation with PMA plus ionophore and prolonged in response to PDTC, which also transiently induced c-fos. In addition, JNK activation by PMA plus ionophore was sensitive to inhibitors of signaling pathways involving Ca²⁺, protein kinase C, and tyrosine phosphorylation, which failed to inhibit the activation mediated by PDTC. Transfection of trans-dominant negative expression vectors of ras and raf, together with AP-1-dependent reporter constructs, as well as Western blot analysis using anti-ERK (extracellular signal-regulated kinase) antibodies, indicated that the Ras/Raf/ERK pathway did not appear to mediate the effect of the antioxidant. However, the combined treatment with PDTC and PMA, two agents that synergize on AP-1 activation, resulted in the persistent phosphorylation of ERK-2. In conclusion, our results identify JNK as a target of antioxidant agents which can be regulated differentially under oxidant and antioxidant conditions.

The transcription factor AP-1 plays a central role by integrating the signals elicited by a large number of extracellular stimuli including growth factors, phorbol esters, UV light, and cytokines (1). Homodimers of members of the Jun family of transcription factors and heterodimers formed with these members and those of the Fos family comprise the AP-1 transcription factor, which binds to the TRE[1] site to regulate gene transcription (1, 2).

The capacity of AP-1 to respond to a wide array of extracellular signals is largely mediated by the transcription factors that regulate the c-*fos* and c-*jun* promoters whose binding and activity are in turn regulated through different signaling pathways (2–4). Thus, the major regulatory element within the c-*fos* promoter, the serum response element (SRE), is recognized by a complex that contains the ternary complex factor (TCF)/Elk-1 and the serum response factor (3). TCF/Elk-1 is phosphorylated rapidly in response to a variety of stimuli by the mitogen-activated protein kinases ERK-1 and ERK-2, which are activated by the Ras/Raf/ERK kinase cascade (4, 5). The sequential activation and phosphorylation of ERK and TCF/Elk-1 facilitate the SRE ternary complex formation and the subsequent transcriptional induction of c-*fos* (6–8). Transcription of c-*jun* is activated by binding of preformed homodimers of c-Jun or heterodimers of c-Jun and ATF-2 to the TRE of the c-*jun* promoter. Both transcription factors c-*jun* and ATF-2 are phosphorylated by another group of mitogen-activated protein kinases denominated JNKs/SAPKs (c-Jun NH2-terminal kinases/stress-activated protein kinases) (9–11), which are activated early by upstream signal transduction pathways involving kinase cascades different from those regulating the ERKs (12, 13).

Although several growth factors stimulate the ERK pathway and induce c-*fos* involving the phosphorylation of TCF/Elk-1, exposure of cells to stress induced by UV radiation (14) or by protein synthesis inhibitors (15), which occurred with c-*fos* gene expression, did not involve activation of the ERK pathway. Recently, studies on the mechanisms involved in TCF/Elk-1 phosphorylation indicated that this factor is a point of convergence of mitogen-activated protein kinases/ERKs and JNK/SAPK signaling pathways, since JNKs/SAPKs can also phosphorylate TCF/Elk-1 in response to a variety of stimuli (13, 16–19). Therefore, JNK activation itself could account for AP-1 activation, since TCF/Elk-1, c-Jun, and ATF-2, factors critically involved in the transcription of c-*fos* and c-*jun*, are targets that can be phosphorylated by this kinase.

[1] The abbreviations used are: TRE, TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element; SRE, serum response element; TCF, ternary complex factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; SAPK, stress-activated protein kinase; NFκB, nuclear factor κB; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; BHA, butylated hydroxyanisole; kb, kilobase; IL, interleukin; EMSA, electrophoretic mobility shift assay; TBS-T, Tris-buffered saline-Tween; GST, glutathione *S*-transferase.

The activity of the AP-1 and NFκB transcription factors is regulated differentially in T lymphocytes in response to changes in the cell redox state (20). The use of antioxidants with radical scavenger properties has evidenced a role for reactive oxygen intermediates, generated under prooxidant conditions, on the activation of NFκB. Thus, the activation of NFκB in response to oxidative stress is inhibited by several structurally unrelated antioxidants including dithiocarbamates (21–24), the antioxidant enzyme thioredoxin (23–25), and the glutathione precursor *N*-acetylcysteine (21, 26, 27). AP-1 is also activated by a number of stimuli that generate oxidative stress (23, 28–31). However, these antioxidants that block NFκB activation efficiently paradoxically stimulate the DNA binding and transcriptional activities of AP-1 involving induction of the c-*fos* and c-*jun* mRNAs (23, 24, 32) and the transcriptional activation of the SRE (23). Therefore, signals triggered under both prooxidant and antioxidant conditions can lead to the activation of AP-1.

To understand the mechanisms by which antioxidants activate AP-1, we analyzed their effects on different signal transduction pathways involved in T cell activation. We found that different antioxidant agents stimulate JNK activity. Furthermore, we studied the antioxidant-induced signaling in T lymphocytes using pyrrolidine dithiocarbamate (PDTC), a potent antioxidant agent that activates AP-1. Our results are discussed in the context of recent works that implicate JNK as a mediator in the activation of both c-*fos* and c-*jun* transcriptional expression.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Monoclonal Antibodies—The human Jurkat T cell line was grown in RPMI 1640 with GLUTAMAX-I (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Phorbol 12-myristate 13-acetate (PMA), PDTC, butylated hydroxyanisole (BHA), *N*-acetylcysteine, and the calcium ionophore A23187 were purchased from Sigma. Cyclosporin A was from Sandoz. Bisindolylmaleimide and herbimycin A were from Calbiochem. The purified activating anti-CD28.2 monoclonal antibody was provided by Dr. D. Olive, and the anti-CD3 SPV-T3 has been described previously (33).

RNA Analysis—Jurkat cells were incubated with PDTC, PMA plus ionophore, or pretreated with PDTC and then exposed to PMA plus ionophore. After various time points, cells were harvested, and total RNA was isolated using the Ultraspec system (Biotox Laboratories, Inc.). For dot-blot analysis, RNA from each sample (10 μg) was denatured and blotted onto nitrocellulose membranes in a Bio-Dot SF microfiltration apparatus (Bio-Rad). For Northern blot analysis denatured RNA (20 μg) was electrophoresed on a 1% agarose gel and blotted onto a nitrocellulose membrane.

After UV cross-linking, the filters were hybridized with the corresponding specific probes: a 0.8-kb *Bgl*II-*Nco*I fragment of c-*fos* cDNA, a 0.8-kb *Hin*dIII-*Pst*I fragment of c-*jun* cDNA, a 1.8-kb *Eco*RI fragment of human *PAC-1* cDNA (34), and a 0.6-kb *Hin*dIII-*Bam*HI fragment of β-*actin* cDNA.

Plasmid Constructs—The plasmids used for the expression of activated v-Ha-*ras*, dominant negative *ras*, and dominant negative *raf* were provided by Dr. D. Cantrell. These plasmids were generated by inserting a 0.7-kb *Bam*HI fragment of v-Ha-*ras* (activated *ras*), a 4.4-kb *Xho*I-*Bam*HI fragment of c-Ha-*ras* N17 (*ras* dominant negative), and a 0.8-kb polymerase chain reaction fragment containing a carboxyl-terminal truncated form of *raf* (dominant negative) into the pEFBOS expression vector (35). The IL-2Luc reporter construct containing the -326 to $+45$ human IL-2 promoter/enhancer region fused to the luciferase gene (36) was provided by Dr. G. Crabtree. The 73colLuc and 60colLuc plasmids including the AP-1-responsive ($-73/+63$ base pairs) and the AP-1-unresponsive ($-60/+63$ base pairs) regions of the human collagenase promoter fused to the luciferase gene have been described previously (37) and were provided by Dr. M. Karin.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Small scale nuclear extracts were obtained from 10^7 Jurkat cells following a procedure described previously (38) but included in the extracting buffers 10 mM sodium orthomolybdate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride. EMSAs were performed as described (32) except for the bind-

ing reactions that were performed using 5 μg of nuclear proteins and 1 μg of poly(dI·dC) DNA carrier. The double-stranded oligonucleotide used as a probe in EMSAs, 5′-GCCCCCTCTGACTCATGCTGACA-3′, included the -68 to -46 base pairs of the *CD11c* promoter (32) containing an AP-1 consensus site.

Transfection Experiments—Jurkat cells (10^7) were transfected with Lipofectin (Life Technologies, Inc.) as recommended by the manufacturer. Briefly, exponentially growing cells ($3–7 \times 10^5$/ml) were washed twice with phosphate-buffered saline, resuspended in Opti-MEM (Life Technologies, Inc.), and incubated for 12 h at 37 °C with a mixture of 5 μg/ml plasmid DNA and 10 μg/ml Lipofectin in 1 ml of Opti-MEM. Then, cells were diluted with 14 ml of complete medium (RPMI and 10% fetal bovine serum) and incubated for 36 h. Cells were then treated with different stimuli for 12 additional h, collected by centrifugation, and luciferase activity was determined following instructions described in a luciferase assay kit (Promega, Madison, WI). In cotransfection experiments with expression vectors, the same protocol was followed, but the reporter and expression plasmids were added at concentrations of 5 and 10 μg/ml, respectively, and the Lipofectin to 10 μg/ml.

Western Blot Analysis—Western blot was performed as described previously (39) with the following modifications. Cells (10^6) were washed and lysed in 200 μl of buffer A (20 mM Hepes, pH 7.8, 50 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5% Triton X-100, 2 mM MgCl$_2$, 1 mM EGTA, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 3 nM okadaic acid). After vortex, cells were kept on ice for 15 min and microcentrifuged for 15 min. Supernatants were collected, mixed with loading buffer, and separated by 8% SDS-polyacrylamide gel electrophoresis. Gels were transferred to Immobilon-P membranes and then incubated with blocking solution (5% w/v skim milk TBS-T (TBS, 0.05% Tween 20)) for 60 min at 45 °C, washed three times in TBS-T, and incubated with anti-ERK 1, 2 (Zymed, San Francisco) 0.1% v/v in TBS-T, 5% w/v skim milk. Then, membranes were washed six times for 5 min each in 0.5% w/v skim milk in TBS-T, and peroxidase-labeled goat anti-mouse IgG (ICN) (0.1% v/v in TBS-T) was incubated for 60 min at room temperature. After three washes with TBS-T and one with TBS, membrane-bound antibody was visualized by ECL detection reagent (Amersham Corp.).

In-gel Kinase Assays—In-gel kinase assays were performed as described previously (40) with some modifications. Lysates from 10^6 cells were diluted in Laemmli sample buffer and separated by electrophoresis in a 10% SDS-polyacrylamide gel polymerized in the presence of GST-c-Jun$_{1-79}$ fusion protein. GST-c-Jun protein was isolated from 250 ml of bacterial cells expressing pGEX-c-Jun$_{1-79}$ plasmid (provided by Dr. R. Davis) using GSH-Shepharose 4B beads (Pharmacia Biotech Inc.). After electrophoresis, the gel was washed twice for 15 min in 100 ml of 20% v/v 2-propanol, 50 mM Hepes, pH 7.6, and then equilibrated with two 15-min changes of 100 ml of buffer A (50 mM Hepes, pH 7.6, 5 mM β-mercaptoethanol). Proteins were denatured by incubating the gel twice for 15 min in 100 ml of 6 M urea in buffer A at room temperature. This was followed by four sequential renaturation steps (15 min each) by discarding half of the volume (50 ml) and replacing it with ice-cold renaturation buffer (0.05% v/v Tween 20 in buffer A) at 4 °C. After washing three or four times with 150 ml of renaturation buffer at 4 °C, the gel was immersed in 25 ml of kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl$_2$, 5 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 mM dithiothreitol) for 30 min at 4 °C and incubated further in 15 ml of kinase buffer containing 20 μM ATP and 100 μCi of [γ-^{32}P]ATP at 30 °C for 1–2 h. Finally, the gel was washed four times with 150–200 ml of 5% v/v trichloroacetic acid and 1% v/v sodium pyrophosphate at room temperature, dried, and exposed for 1–2 days.

RESULTS

Changes in c-fos and c-jun mRNA Levels in Jurkat Cells Induced by PDTC—Since different antioxidant agents, including PDTC, activate AP-1, we analyzed further the effect of PDTC on c-*fos* and c-*jun* mRNA steady state. Kinetic experiments using total RNA from Jurkat cells treated with PDTC and from cells treated with the combination of the phorbol ester PMA and the Ca^{2+} ionophore A23187, either pretreated or not with PDTC, were carried out (Fig. 1*A*). Incubation with PMA plus A23187 resulted in transient induction of both c-*fos* and c-*jun* mRNA levels, having maximal values at 30–60 min, whereas exposure to PDTC caused a prolonged expression of c-*jun* mRNA reaching maximal levels between 1 and 4 h and remained high for at least 10 h. PDTC induced a weaker
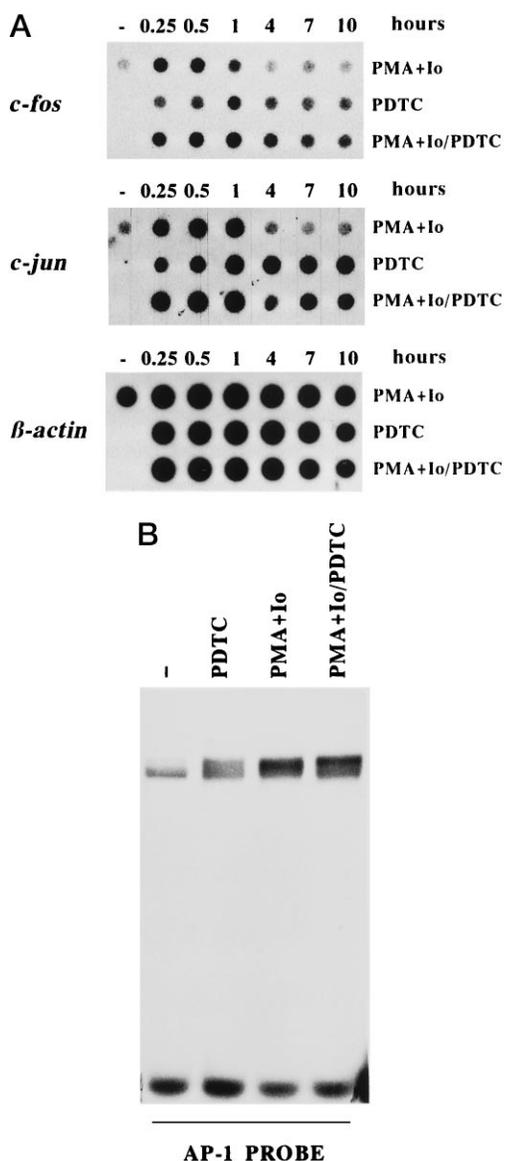
FIG. 1. **Effects of PDTC and PMA plus ionophore on RNA levels of c-*fos* and c-*jun* and on AP-1 binding activity.** *Panel A*, dot-blot analysis using total RNA from Jurkat T cells treated with 50 μM PDTC, 20 ng/ml PMA plus 1 μM A23187 (*Io*) or pretreated with 50 μM PDTC for 2 h and then stimulated with 20 ng/ml PMA plus 1 μM A23187 for the times indicated. RNA was hybridized with probes of c-*fos*, c-*jun*, and β-*actin*. *Panel B*, nuclear extracts from Jurkat cells treated with 50 μM PDTC, 20 ng/ml PMA plus 1 μM A23187 for 4 h, and nuclear extracts pretreated for 2 h with PDTC and then stimulated with PMA plus A23187 (at the same doses as those used in separate treatments) for an additional 4 h were incubated with a probe containing the TRE consensus sequence of the *CD11c* promoter, and complexes were resolved in an EMSA.

expression of c-*fos* mRNA although displaying kinetics different from that exerted by the combination of PMA and ionophore. Strikingly, the incubation of Jurkat cells with PDTC prior to stimulation with PMA plus ionophore led to a prolonged induction not only of the c-*jun* mRNA levels, but also of the c-*fos* levels, which were, on the other hand, higher than those obtained with PDTC and PMA-ionophore separately. As shown previously for other cell types (23, 24, 32), EMSA using nuclear extracts from Jurkat T cells and a consensus AP-1 probe confirmed that PDTC (and PMA-ionophore) induced AP-1 DNA binding activity (Fig. 1*B*) and TRE-dependent transcriptional activation (Fig. 2). Taken together these data indicated that in Jurkat T cells, PDTC is able, by itself, to induce a
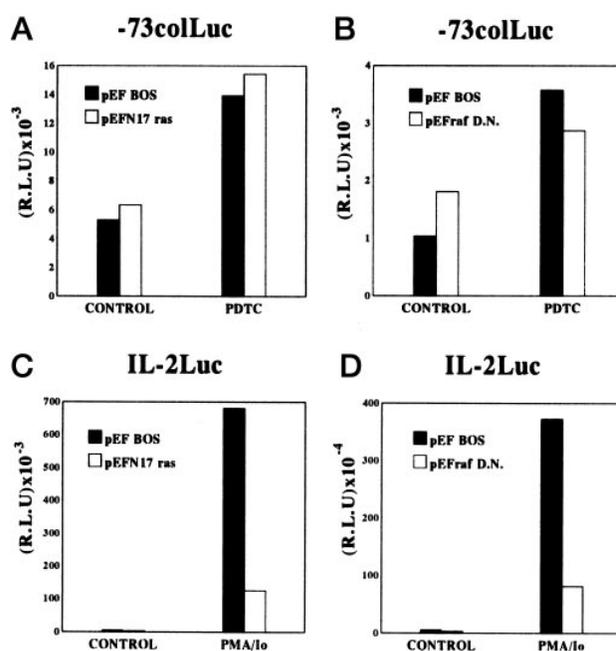

FIG. 2. **Roles of *Ras* and *Raf* on the AP-1 *trans*-activation mediated by PDTC.** Jurkat T cells were cotransfected by lipofection with 5 μg/ml luciferase-based plasmids directed by the AP-1-responsive ($-73/+63$ base pairs) sequence of the collagenase promoter (-73colLuc) (*panels A* and *B*) or by the IL-2 promoter (IL-2Luc) (*panels C* and *D*) together with 10 μg/ml expression vectors of the *trans*-dominant negative forms of *ras* (*panels A* and *C*) and *raf* (*panels B* and *D*) or its parental empty vector, pEFBOS, for 12 h. 36 h post-transfection cells were treated with 50 μM PDTC or 20 ng/ml PMA plus 1 μM A23187 for an additional 12 h. The results are expressed as relative light units (*R.L.U*) measured for 30 s. Results are representative of three independent experiments.

transient expression of c-*fos*, a strong and prolonged expression of c-*jun*, and a durable activation of both c-*fos* and c-*jun* triggered by PMA plus ionophore.

Effect of PDTC on the Ras/ERK Pathway—The Ras/ERK pathway is a common route that serves multiple extracellular signals that activate the SRE of the c-*fos* promoter (4, 5). Since the SRE has been shown to act as a primary antioxidant responsive element that can be activated by PDTC (23), we analyzed whether the activation of AP-1 by this antioxidant was mediated through activation of this pathway in Jurkat T cells. As shown in Fig. 2*A*, the activation of an AP-1 reporter construct (-73colLuc) in response to PDTC was not affected by the expression of a dominant negative *ras*, c-Ha-*ras* N17 (Fig. 2*A*). The efficient expression of the *ras* construct was confirmed in parallel cotransfections, where expression of c-Ha-*ras* N17 caused a severe transcriptional inhibition of both a luciferase plasmid directed by the human IL-2 promoter (Fig. 2*C*) and the AP-1 reporter plasmid in response to PMA plus ionophore (data not shown). Similar experiments were performed to analyze whether Raf-1 kinase was required for the activation of AP-1 induced by PDTC. AP-1 *trans*-activation by the antioxidant was not significantly affected by the expression of a *trans*-dominant negative mutant of Raf-1 kinase (Fig. 2*B*) which efficiently inhibited the IL-2Luc *trans*-activation, as well as the AP-1Luc *trans*-activation by PMA plus ionophore (Fig. 2*D* and data not shown).

To explore whether the signals elicited by PDTC affected ERK activity, we performed Western blot analysis using anti-Erk-1, -2 antibodies to detect any possible decrease in the electrophoretic mobility of ERKs which would be indicative of its activation (41). PDTC failed to modify the mobility of ERK-2, which appears to be the predominant ERK isoform
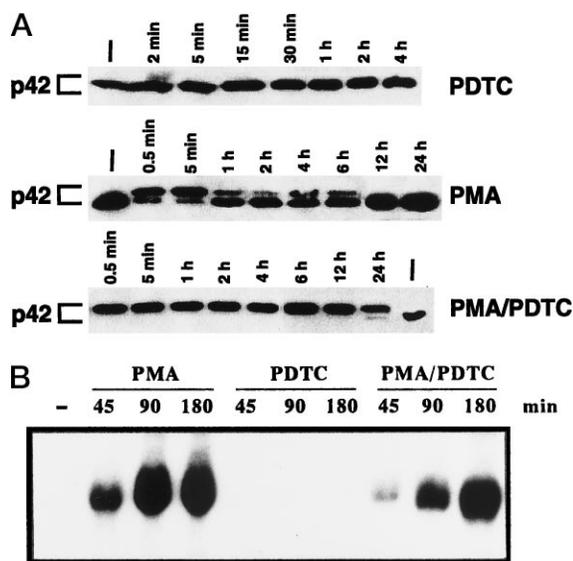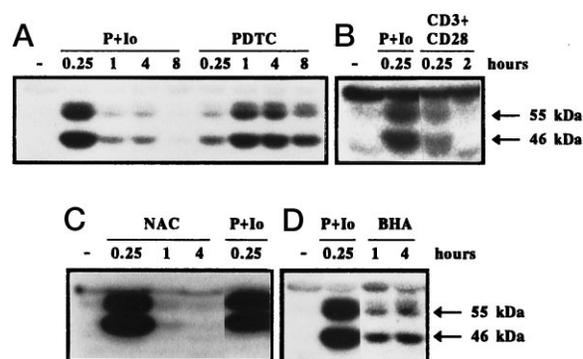
FIG. 3. **Effects of PDTC and PMA on ERK activity and *PAC-1* gene expression.** *Panel A*, Western blot analysis of total lysates from Jurkat cells (10^6) incubated with 50 μM PDTC, 20 ng/ml PMA, or preincubated with 50 μM PDTC for 2 h and then treated with 20 ng/ml PMA for the times indicated. A monoclonal antibody against ERK-1 and ERK-2 was used. The electrophoretic mobility of the *upper* and *lower* *bands* corresponded to the phosphorylated and unphosphorylated bands of ERK-2, respectively. *Panel B*, total cytoplasmic RNAs (10 μg) from Jurkat cells treated as described in *panel A* were prepared at different times of treatment and subjected to Northern blot analysis using a probe specific for *PAC-1*.

FIG. 4. **Activation of JNK by antioxidants and T cell stimuli.** In-gel kinase assay performed with whole-cell extracts from Jurkat cells (10^6) treated with 20 ng/ml PMA plus 1 μM A23187 (*P+Io*), or 100 μM PDTC (*panel A*), 10 μg/ml anti-CD3 plus 2 μg/ml anti-CD28 (*panel B*), 50 mM *N*-acetylcysteine (*NAC, panel C*), and 50 μM BHA (*panel D*) for the times indicated. Controls from untreated and PMA plus iono-phore-treated cells are included in each experiment to compare the relative induction obtained with the different antioxidants. Whole-cell extracts were separated by SDS-polyacrylamide gel electrophoresis on a gel polymerized in the presence of GST-c-Jun$_{1-79}$. After a denaturation-renaturation process, the gel was incubated with [γ-^{32}P]ATP, washed extensively, and exposed. The bands corresponding to the 46- and 55-kDa JNK forms are indicated by *arrowheads*.

expressed by Jurkat cells (Fig. 3*A*). As expected, PMA caused a very rapid transient shift in the mobility of ERK-2 which was detected as early as 30 s and declined after 1 h of treatment (Fig. 3*A*). Interestingly, when Jurkat cells were pretreated with PDTC and stimulated further with PMA, ERK-2 persisted in its activated form after 24 h of treatment (Fig. 3*A*).

The persistent phosphorylation of ERK-2 observed might be due to an inhibition of the PAC-1 T cell phosphatase, which has been shown to dephosphorylate ERK-2 *in vivo* (34, 42). However, Northern blot analysis indicated that mRNA steady-state levels of *PAC-1*, induced early by PMA, were not significantly affected by previous treatment with PDTC after 90 and 180 min (Fig. 3*B*) when a complete activation of ERK-2 was found in cotreated cells (Fig. 3*A*). Taken together, our results indicated that the Ras/Raf/ERK pathway did not appear to mediate the effect of the antioxidant PDTC. In addition, the combined treatment of PDTC and PMA, which has been shown to syner-gize on AP-1-dependent *trans*-activation (23, 24) caused a per-sistent phosphorylation of ERK-2 by mechanisms different from the inhibition of the gene expression of *PAC-1*.

Activation of JNK by Antioxidants—Signals leading to T cell activation by costimulation with phorbol ester and Ca^{2+} iono-phore or by agonistic antibodies to the T cell receptor-CD3 complex and to CD28 involve the activation of JNKs (43). Besides the ability to phosphorylate c-Jun (40), JNKs have recently been shown to phosphorylate ATF-2 (11, 44, 45) and TCF-Elk-1 (16–19). Thus, JNKs phosphorylate factors involved in the activation of AP-1 not only by regulating *c-jun* but also by *c-fos* transcription. Therefore, we decided to determine whether PDTC affected the activity of JNKs, a mechanism that could account for the observed c-*fos* and c-*jun* activation in the apparent absence of ERK activation. In-gel kinase assays of GST-c-Jun$_{1-79}$ revealed activation of the 46- and 55-kDa forms of JNK in extracts of Jurkat cells treated with PDTC (Fig. 4*A*). Strikingly, whereas the treatment with either PMA plus iono-phore or anti-CD3 plus anti-CD28 resulted in a transient acti-

vation of JNK (Fig. 4, *A* and *B*), PDTC exerted a prolonged activation of JNK which was maintained for at least 8 h (Fig. 4*A*). To determine whether the activation by PDTC was due to its antioxidant effect, we then analyzed the effects on JNK activity of two structurally unrelated antioxidants, *N*-acetyl-cysteine and BHA, which have been shown to increase AP-1 activity (23, 24). Exposure of Jurkat cells to BHA resulted in a weak although sustained activation of JNK which persisted for more than 4 h (Fig. 4*D*). Treatment with *N*-acetylcysteine led, on the other hand, to a transient activation of JNK, although in this case it was even stronger than that mediated by PMA plus ionophore (Fig. 4*C*). Thus, signals triggered by different anti-oxidant agents that increase or activate AP-1 involve the acti-vation of JNK.

Inhibitors of T Cell Activation Discriminate the Signaling Pathways of PDTC versus PMA plus Ionophore—To search for mechanisms involved in the activation of JNK by antioxidant agents, a panel of inhibitors that interfere with upstream sig-nals involved in T cell activation was used. Thus, inhibition of protein tyrosine kinases with herbimycin A resulted in signif-icant inhibition of the JNK activation mediated by PMA plus ionophore (Fig. 5*A*) and by anti-CD3 plus anti-CD28 antibodies (data not shown), but it did not interfere with that mediated by PDTC. In addition, blockade of the Ca^{2+}/calcineurin signaling pathway with cyclosporin A, which has been shown to attenu-ate JNK activation in response to either PMA plus ionophore or anti-CD3 plus anti-CD28 (43), had no effect on PDTC-mediated JNK activation at the doses analyzed (Fig. 5, *A* and *B*, and data not shown). Moreover, inhibition of PKC with bisindolylmale-imide (46), which completely abrogated the activation of JNK induced by PMA plus ionophore (Fig. 5*A*), had the opposite effect on the PDTC-mediated activation, resulting in increased JNK activity when bisindolylmaleimide was added (Fig. 5*B*). This effect, which was not observed either with extracts from cells treated with PMA plus ionophore, with anti-CD3 plus anti-CD28 cells, or with control cells pretreated with the PKC inhibitor (Fig. 5*A* and data not shown), requires further study. Nevertheless, the effect of bisindolylmaleimide clearly discrim-inates the signaling pathways induced by PDTC *versus* PMA plus ionophore. Thus, the signals that converge in JNK activa-tion mediated by PDTC appear to be distinct from those elicited
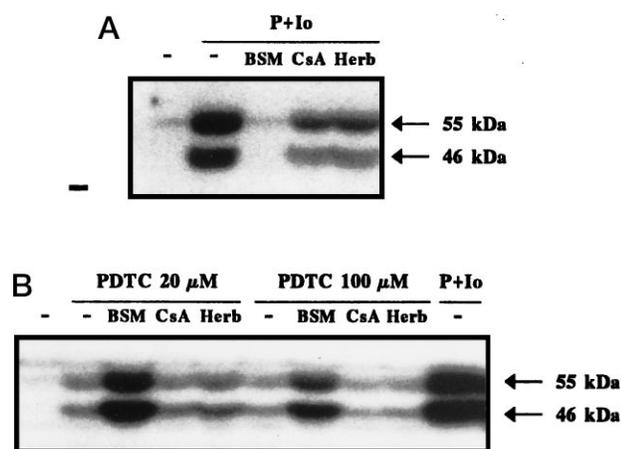
Fig. 5. **Effects of T cell activation inhibitors on the JNK activity induced by PDTC and T cell stimuli.** In-gel kinase assays were performed with whole-cell extracts from Jurkat cells preincubated or not with 10 μM bisindolylmaleimide (*BSM*), 100 ng/ml cyclosporin A (*CsA*), or 800 nM herbimycin A (*Herb*) for 4, 1, and 12 h, respectively. After preincubation, cells were treated with 20 ng/ml PMA plus 1 μM A23187 (*P+Io*) for 15 min (*panel A*) or with 20 and 100 μM PDTC for 2 h (*panel B*). Whole-cell extracts were then obtained, and an in-gel kinase assay of GST-c-Jun$_{1-79}$ was performed. The bands corresponding to the 55- and 46-kDa forms of JNK are indicated by *arrowheads*.

through stimulation of T cells with PMA plus ionophore or anti-CD3 plus CD28.

DISCUSSION

The physiological and pharmacological modifications of the cellular redox state provoke drastic changes in the activities of the transcription factors AP-1 and NFκB which play a central role in the regulation of the immune response. NFκB and AP-1 are affected differentially by signals generated by prooxidant and antioxidant agents (20); whereas NFκB is induced by a large number of T cell stimuli that cause reactive oxygen intermediate generation (20, 47), its activation is inhibited by different antioxidant agents with radical scavenger properties. However, AP-1 is not only induced by a wide array of stimuli that generate oxidative stress (23, 28–31), but also by a number of antioxidant agents (23–25, 32). In this study, we compared the signaling pathways through which antioxidants and T cell stimuli lead to the activation of AP-1 and identify JNK as a target activated by different antioxidant agents.

Although treatment of Jurkat T cells with either PMA plus ionophore or anti TCR/CD3 plus CD28 on one hand and with antioxidants on the other increases the activity of JNK, our data clearly indicate that the signal transduction mechanisms involved in both activations are different. (i) JNK activation by PDTC was refractory to inhibitors of protein tyrosine kinases, protein kinase C, or Ca^{2+} signaling, whereas the JNK activation mediated by either T cell receptor-CD3/CD28 antibodies or PMA plus ionophore was inhibited to a variable extent by such inhibitors. (ii) TRE and IL-2 promoter-mediated *trans*-activation by PMA and ionophore was sensitive to the expression of *trans*-dominant negative versions of *ras* and *raf* which failed to inhibit the AP-1 *trans*-activation induced by PDTC. (iii) Transient phosphorylation of ERK-2, detected at early times of activation with PMA, was not observed after treatment with PDTC.

Although the expression of c-Ha-*ras* N17 dominant negative did not affect the *trans*-activation of AP-1 by PDTC (Fig. 2*A*), this was augmented by the expression of a constitutively active v-Ha-*ras* (data not shown). Since activated Ha-*ras* has been shown to partially activate JNK (10), it is likely that the additional effect displayed by activated *ras* on the activation of AP-1

by PDTC is mediated through JNK. Hence, the Ras/Raf/ERK pathway can modulate the PDTC-induced signaling to AP-1, although it did not appear to mediate the effect of the antioxidant.

An interesting feature of ERK regulation arising during the analysis of its phosphorylation in extracts of Jurkat cells cotreated with PMA and PDTC was the persistent activated state of ERK-2. Whether this prolonged activation is related to the synergism observed on the activation of AP-1 by both agents (23, 24) remains to be investigated further. Since PAC-1 is a phosphatase rapidly expressed after T cell activation which has been shown to inhibit ERK-2 phosphorylation and activation (34, 42), we have analyzed its involvement in the activation induced by PMA plus PDTC. However, our studies on *PAC-1* gene expression indicated that *PAC-1* mRNA levels induced by PMA were not significantly affected by PDTC (Fig. 3*B*). Other explanations that could account for the constitutive activation of ERK include the possibility that PAC-1 phosphatase activity was post-transcriptionally inhibited by PDTC, or more likely, that other phosphatases could be inhibited by the antioxidant. In this regard, the expression of mitogen-activated protein kinase phosphatase-1 in T lymphocytes has recently been shown to block PMA-induced ERK activity and to inhibit IL-2 promoter-dependent transcription in response to PMA plus ionophore (48). Since mitogen-activated protein kinase phosphatase-1 has also been shown to dephosphorylate JNK (49), it will be of interest to study the activity and expression of this phosphatase in response to antioxidants.

In HeLa cells PDTC has been shown to induce c-*fos* and c-*jun* transcription (23). Although we have found induction of both c-*fos* and c-*jun* by PDTC in Jurkat cells, important kinetic differences are observed when both cell types are compared. Thus, in HeLa cells PDTC triggers c-*fos* gene expression faster than c-*jun*, with a transient induction in both cases (23). However, in Jurkat cells PDTC-mediated c-*jun* expression was prolonged and preceded that of c-*fos*. Therefore, PDTC can operate by different mechanisms depending on the cell type analyzed.

The SRE within the c-*fos* promoter has been shown to act as a primary antioxidant element that can be activated by PDTC in HeLa cells (23). However, as discussed above, the mechanisms by which the antioxidant acted in Jurkat cells does not appear to be mediated by the Ras/Raf/ERK pathway. Hence, PDTC seems to be one of the stimuli that can induce c-*fos* in the absence of ERK activation. Since JNK has been shown to phosphorylate TCF/Elk-1, the major transcription factor involved in SRE activation, it is possible that PDTC can activate c-*fos* transcription through the phosphorylation of TCF by JNK. Additional experiments, including in-gel kinase analysis of GST-Elk-1 in extracts of cells treated with antioxidants, will be required to elucidate this issue.

We have found that structurally unrelated antioxidant agents activate JNK in Jurkat T cells. However, the kinetics and strength of this activation as well as the abilities to stimulate AP-1 displayed by the different antioxidants are different. Thus, PDTC *per se* exerts a potent and prolonged JNK activation and activates both AP-1 DNA binding activity and the TRE-dependent *trans*-activation. BHA displays a weak but sustained activation of JNK; and *N*-acetylcysteine, which has been shown to induce AP-1 binding but not significant TRE *trans*-activation (23), exerts a transient activation of JNK. Moreover, *N*-acetylcysteine was the strongest antioxidant stimulating JNK. Therefore, sustained activation of JNK appears to be a mechanism through which some antioxidants can operate, but it is not a general mechanism that mediates the signaling of all of the antioxidants. Likewise, these results suggest that additional signaling, different from JNK activation, must be

required to activate TRE-dependent transcription, since *N*-acetylcysteine, which is not able to activate it *per se* (23, and data not shown), activates JNK in a fashion similar to that of PMA plus ionophore.

It is important to note that both prooxidant and antioxidant conditions lead to the activation of AP-1. JNK appears to be tuned differentially under both conditions. Thus, it would be possible that those antioxidants that trigger sustained activation of JNK might elicit specific antioxidative responses through AP-1-regulated genes, subtly regulated by the duration and the strength of JNK stimulation. In this regard, in PC12 cells the cellular responses elicited by growth factors which lead cells to proliferate or to differentiate seem to be determined by the transient or sustained activation of ERK, respectively (5). On the other hand, we have shown recently that several dithiocarbamates, including PDTC, trigger myeloid differentiation involving the activation of AP-1 (32). Hence, it will be of the utmost interest to determine whether the duration of JNK activation is involved in selectively triggering these cellular responses.

Future experiments will be designed to identify the mechanisms by which antioxidants target JNK activity including their effects on the upstream signaling pathway components that activate JNK/SAPKs and on the phosphatases that regulate its phosphorylation. These studies, which could also contribute to trace the signal transduction mechanisms involved in T lymphocyte activation, are currently in progress.

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