Endogenous oxygen radicals modulate protein tyrosine phosphorylation and JNK-1 activation in lectin-stimulated thymocytes

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Molecular events mediating the T-lymphocyte response to lectins are still incompletely understood, although much evidence suggests that both the mitogenic and the death-promoting effects of these agents involve the biochemical cascade initiated by the CD3/T-cell antigen receptor (TCR) complex. Reactive oxygen species (ROS) and in particular H_2O_2 have been shown to have a role in cell response to cytokines and growth factors. Here we report that the proliferation of mouse thymocytes in response to the mitogenic lectin concanavalin A (ConA) is strongly and selectively inhibited by the intracellular ROS scavenger *N*-acetylcysteine (NAC) and by diphenyleneiodonium (DPI), a potent inhibitor of NADPH-dependent membrane oxidases activated by surface receptors. A rapid 'burst' of intracellular oxygen radicals was observed in mouse thymocytes stimulated by ConA, with kinetics that paralleled the appearance of tyrosine-phosphorylated proteins. This burst was abrogated by the pretreatment of cells with NAC or DPI. Only a modest increase in intracellular oxygen species was found in thymocytes stimulated by strong cross-linking of TCR together with CD4 or CD28. Pharmacological interference with ROS production in ConA-stimulated thymocytes resulted in a decreased tyrosine phosphorylation of multiple protein species, including a 38 kDa band able to recruit the adapter protein Grb2 and corresponding to the recently identified transducer LAT (linker for activation of T-cells), a molecule involved in linking activated TCR to the production of interleukin 2 and the proliferation of T-cells. Furthermore, ROS inhibition markedly attenuated the activation of stress-activated protein kinase/JNK-1 (c-Jun N-terminal kinase 1) in response to lectins. Taken together, these results identify ROS as important modulators of the signalling cascade initiated by mitogenic lectins in thymocytes and, by extension, as a novel class of mediators downstream of antigen receptors.

Key words: c-Jun N-terminal kinase, lymphocyte activation, redox signalling.

INTRODUCTION

Although traditionally considered to be undesirable mediators of cellular damage, reactive oxygen species (ROS) such as the superoxide anion, H_2O_2 and nitric oxide (NO) have recently been recognized as regulators of important cellular functions (reviewed in [1]). Several pieces of evidence have pointed to the involvement of ROS as signalling intermediates for cytokines and growth factors [2] and as mediators for some of the transforming properties of oncogenic Ras [3]. Oxygen radicals have been proved to be necessary for PDGF (platelet-derived growth factor) receptor kinase activity and mitogenic signalling [4], and for a number of intracellular responses to cytokines such as tumour necrosis factor α and interleukin 1; these responses include the activation of nuclear factor κB (NF-κB) [5], the induction of c-Fos [2] and the activation of c-Jun N-terminal kinase (JNK) [6].

T-cell stimulation through the antigen receptor involves a multiplicity of signalling cascades converging on the transcription of the gene for interleukin 2 and on the expression of its receptor [7]. These cascades require the tyrosine phosphorylation of a number of cellular substrates including the antigen receptor itself, and the membrane recruitment of signalling effectors mediating the activation of Ras and eventually ERK (extracellular signal-regulated protein kinase) kinases. Furthermore, a parallel chain of signalling events leads to the activation of the JNKs, whose activity, in concert with the Ras/ERK pathway signalling components, is required for a full response of T-cells to antigenic stimulation [8].

In mouse lymphocytes, this complex cascade of intracellular events can be efficiently triggered by mitogenic lectins such as concanavalin A (ConA). ConA binds the CD3 component of the T-cell receptor (TCR) and shares most stimulatory properties of anti-CD3 or anti-TCR monoclonal antibodies [9]. Furthermore, unlike most soluble anti-CD3 or anti-TCR antibodies, ConA is fully mitogenic for T-cells at the appropriate concentrations [10], probably owing to the simultaneous involvement of one or more co-stimulatory pathways. So far, however, molecular events downstream of T-cell stimulation by lectins remain incompletely clarified.

Lymphocytes represent a classical target for oxidative stress: ROS are involved in T-cell apoptosis induced by steroids [11], and alterations in cellular redox balance accompany and probably mediate some of the cytopathic effects of HIV on T lymphocytes [12]. Exogenously added H_2O_2 induces in lymphocytes a number of signalling events, including tyrosine phosphorylation and the activation of NF-κB, reminiscent of the cellular response to growth factors and cytokines [13,14].

Although signalling cascades initiated in lymphocytes by antigen receptors and co-receptors have been studied extensively, the possibility that oxygen radicals could have a role as components of the response of immune cells to antigenic stimulation has been investigated very little. It has been shown that, in

Abbreviations used: ConA, concanavalin A; DCF-DA, dichlorofluorescein diacetate; DPI, diphenyleneiodonium; GST, glutathione S-transferase; HBSS, Hanks balanced salt solution; JNK-1, c-Jun N-terminal kinase 1; L-NMMA, L-*N*-monomethylarginine; LAT, linker for activation of T-cells; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; ROS, reactive oxygen species; TCR, T-cell receptor.

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immature B cells, growth-arresting signals initiated by surface immunoglobulins seem to require ROS [15]. In contrast, in T-cells the engagement of CD28, a molecule involved in T-lymphocyte co-stimulation by antigen-presenting cells, has been shown to activate the transcription factor NF-κB in a ROS-dependent fashion [16]. These pieces of evidence suggest that both inhibitory and mitogenic signals elicited by antigens could involve the intracellular production of oxygen radicals.

Here we show that ROS are generated in response to lectins in mouse thymocytes and that oxygen-derived radicals are required for lectin-induced thymocyte proliferation and JNK-1 kinase activity up-regulation and modulate protein tyrosine phosphorylation elicited by TCR engagement. These findings demonstrate a role for ROS in the T-cell response to antigens, with important implications for the understanding of the redox regulation of immune functions.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (3–4 weeks old) used in this study as a source of thymocytes were obtained from the Animal Facility of Università Cattolica del Sacro Cuore (UCSC) Medical School (Rome, Italy).

Chemicals and reagents

ConA, *N*-acetylcysteine (NAC), L-*N*-monomethylarginine (L-NMMA), diphenyleneiodonium (DPI), H_2O_2, aminotriazole, PMA, ionomycin and streptavidin were purchased from Sigma (St Louis, MO, U.S.A.). The fluorescent dye dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Standard Western blotting reagents were from Bio-Rad, [γ-^{32}P]ATP was from DuPont NEN, [^{3}H]methylthymidine was from Amersham Radiochemicals and X-ray films were from Kodak.

Biotin-conjugated monoclonal hamster anti-(mouse TCR), hamster anti-(mouse CD4) and hamster anti-(mouse CD28) were obtained from Pharmingen. The monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology; affinity-purified rabbit anti-Vav polyclonal IgG and goat anti-(JNK-1) polyclonal IgG were from Santa Cruz Laboratories.

Escherichia coli strains expressing the glutathione S-transferase (GST)–Grb2/SH2 fusion protein (residues 60–158) and the GST–Jun fusion protein (residues 5–89) were kindly provided by Dr A. Pawson (Toronto, Ontario, Canada) and Dr Y. Wu (Stanford, CA, U.S.A.) respectively. Fusion proteins were purified from bacterial lysates induced with β-D-thiogalactopyranoside (Advanced Biotechnology) with glutathione-conjugated Sepharose 4B (Pharmacia, Uppsala, Sweden) in accordance with the manufacturer's recommendations.

Cell preparation and culture

Single-cell suspensions of mouse thymocytes were prepared by standard procedures [17]. Cells were washed twice in PBS and incubated for 6–8 h in Dulbecco's modified Eagle's medium (Eurobio) without fetal-calf serum, with or without 30 mM NAC, pH 7.4, at 37 °C in air/CO_2 (19:1). Immediately before stimulation, cell viability was always greater than 90% as measured by Trypan Blue exclusion. HeLa human cervical carcinoma cells (A. T. C. C., Manassas, VA, U.S.A.) were grown in RPMI/10% fetal-calf serum (Eurobio) and harvested in the exponential growth phase.

Thymocyte proliferation assay

For the thymidine incorporation assay, thymocytes were seeded in 96-well flat-bottomed plates at 5×10^5 cells per well in 200 μl of RPMI-1640 medium (Eurobio) containing 10% (v/v) fetal-calf serum, glutamine, antibiotics and 50 μM 2-mercaptoethanol. Mitogenic stimuli (6 μg/ml ConA or 40 ng/ml PMA plus 0.6 μg/ml ionomycin) and freshly prepared NAC, pH 7.4, were added at the beginning of the culture. After 40 h, microcultures were labelled with [^{3}H]thymidine (1 μCi per well; Amersham Radiochemicals) and incubated for a further 10 h before being semi-automatically harvested and subjected to liquid-scintillation counting.

Measurement of ROS production

Control and antioxidant-pretreated thymocytes were stimulated at a density of 10^8 cells/ml in Hanks balanced salt solution (HBSS), with either 300 μg/ml ConA or 100 μg/ml streptavidin after cell-surface labelling with biotin-conjugated antibodies (1 μg/100 μl of HBSS for 30 min on ice) or PBS (no difference from isotype control antibodies) in a water bath at 37 °C. Cells were then pulsed with DCF-DA (10 μg/ml) for 5 min; the fluorescence of cells falling in the lymphocyte gate was quickly analysed by flow cytometry (Epics XL-MCL; Coulter Corporation) (emission at 488 nm, 525 nm bandpass filter). Time of stimulation was 5 min unless indicated otherwise. In some experiments, cells were pretreated with NAC (8 h, 30 mM), L-NMMA (1 h, 500 μM), DPI (30 min, 20 μM) or H_2O_2 (10 min, 500 μM) before stimulation with ConA.

Preparation of protein lysates

For the analysis of protein tyrosine phosphorylation and JNK-1 activity, cells were stimulated with ConA or biotin-conjugated antibodies plus streptavidin (Sigma) as described above, for 2 or 20 min. After stimulation, cells were spun down at 10000 ***g*** for 20 s at 4 °C and the pellets were lysed in 400–500 μl of lysis buffer [150 mM NaCl/50 mM Tris/HCl (pH 8)/5 mM EDTA/0.1% NaN_3] containing 1% (v/v) Triton X-100, 1 mM PMSF and 1 mM sodium orthovanadate (Sigma). After incubation for 15 min in lysis buffer, nuclei and unlysed cells were removed by centrifugation at 4 °C for 10 min at 10000 ***g*** and supernatants were stored at −80 °C.

Analysis of tyrosine-phosphorylated proteins

Protein tyrosine phosphorylation was evaluated by anti-phosphotyrosine immunoblotting. To assess total protein tyrosine phosphorylation under different conditions, 25 μl (approx. 50 μg of proteins) of thymocyte lysate was resuspended in 6 × SDS loading buffer, boiled for 5 min and resolved by SDS/PAGE. Proteins were electroblotted to nitrocellulose (Schleicher and Schuller or Bio-Rad) and membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline containing Tween 20 (TBST) for 2 h at room temperature (20 °C). Filters were then incubated for 2 h at room temperature with 4G10 anti-phosphotyrosine antibody (diluted 1:5000) in TBST, followed by horseradish-peroxidase-conjugated goat anti-mouse antibodies (Bio-Rad); immunocomplexes were detected with an enhanced chemiluminescence system (Amersham).

Grb2-binding phosphoproteins were isolated as described [18]. In brief, protein lysates (approx. 3×10^7 cells equivalent) were

incubated with 5–10 μg of Sepharose-bound GST–Grb2/SH2 or GST for 3 h at 4 °C with continuous agitation. Protein–protein complexes were isolated by centrifugation at 10000 ***g*** for 30 s and washed three or four times with lysis buffer, resuspended in SDS loading buffer and subjected to SDS/PAGE. Tyrosine-phosphorylated proteins associated with GST–Grb2 were detected as described above. In some experiments, autoradiograms were digitized and band intensities (row volume) were quantified with a GelDoc system (Bio-Rad) equipped with a densitometry software (Quantity One, version 4.1). Vav phosphorylation was evaluated by anti-phosphotyrosine Western blotting of anti-Vav (Santa Cruz Biotechnology) immunoprecipitates, as described [18].

JNK-1 activity assay

JNK-1 kinase was purified from thymocyte lysates by a standard immunoprecipitation procedure with the use of a specific goat antibody that was not cross-reactive with JNK-2 (Santa Cruz Biotechnology) and Protein G–Sepharose (Sigma); kinase activity was assessed as described in [19] with small modifications. In brief, JNK-1 immunoprecipitates or isotype control immunoprecipitates were washed three times in lysis buffer and once in kinase buffer [25 mM Hepes (pH 7.5)/25 mM $MgCl_2$/2 mM dithiothreitol/100 μM sodium orthovanadate] and finally resuspended in 25 μl of kinase buffer containing 50 μM ATP, 10 μCi of [γ-^{32}P]ATP and 5 μg of Sepharose-bound GST–Jun (5–89) as substrate. The reaction was performed at 30 °C for 30 min with occasional agitation and stopped with 6 μl of 6 × SDS loading buffer. Samples were then boiled, subjected to SDS/PAGE [10 % (w/v) gel] and blotted to nitrocellulose. Filters were stained with Ponceau S to ensure an equal amount of substrate in all lanes and were exposed to X-ray films for 2–12 h. Finally, after membrane blocking in TBST/5 % (w/v) dried milk, the amount of immunoprecipitated JNK-1 was evaluated by Western blotting with anti-(JNK-1) antibody (diluted 1:500 in TBST), followed by horseradish-peroxidase-conjugated rabbit anti-goat secondary antibody (Pierce) and detection by enhanced chemiluminescence.

RESULTS

Effects of antioxidants on mouse thymocyte proliferation

Plant lectins such as ConA are mitogenic for human and murine T lymphocytes [20] and have been used extensively to study the activation events initiated in T-cells after engagement of the T-cell antigen receptor (TCR). As an initial step to evaluate the potential role of oxygen radicals in the intracellular response to lectins, we examined whether antioxidant drugs would interfere with the activation of mouse C57Bl/6 thymocytes *in vitro* by optimal mitogenic amounts of ConA. As shown in Figure 1(A), thymocyte proliferation in response to ConA was markedly decreased by the cell-permeant ROS scavenger NAC. The mitogenic effects of ConA were also completely prevented by sub-micromolar (500 nM) concentrations of DPI, a potent inhibitor of ligand-activated membrane-bound NADPH oxidases. Both NAC and DPI had a much less pronounced effect on the T-cell proliferation elicited by the combination of PMA and the Ca^{2+} ionophore ionomycin (Figure 1B), suggesting that the above treatments were not aspecifically (i.e. not specifically related to ROS scavenging and signal modulation) toxic for T-cells and that not all activation pathways have an equal requirement for ROS.

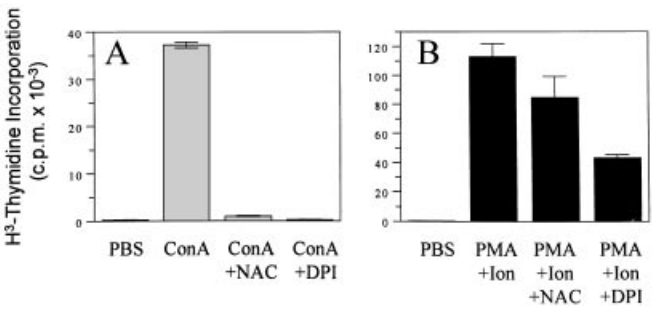

Figure 1 Antioxidant agents inhibit thymocyte proliferation in response to ConA

(**A**) C57Bl/6 thymocyte suspensions were prepared as described in the Materials and methods section. Cells were plated at density of 5×10^5 per well (96-well flat-bottomed plates; Corning) in RPMI/10 % (v/v) fetal-calf serum, then stimulated with 6 μg/ml ConA alone or in combination with 30 mM NAC, pH 7.4, or 500 nM DPI. After 40 h microcultures were labelled with [^{3}H]thymidine; the incorporation of radioactivity was measured 10 h later (see the Materials and methods section). Values are means ± S. D. for triplicate cultures. Background incorporation (unstimulated thymocytes) was 251 ± 28 c.p.m. The results are representative of two independent experiments. (**B**) Cells were treated as in (**A**) except that 40 ng/ml PMA and 0.6 μg/ml ionomycin (Ion) were used instead of ConA. Note that the inhibitory effects of NAC and DPI on the proliferation of thymocytes were much less pronunced than in cultures stimulated with ConA. Values are means ± S.D. for triplicate cultures. Results are representative of two independent experiments.

Generation of ROS in lectin-stimulated thymocytes

Because the effects of NAC and DPI on ConA-stimulated proliferation indirectly suggested a specific role for ROS in the activation of thymocytes by lectins, the production of oxygen radicals immediately (5 min) after challenging thymocytes with a high concentration of ConA was monitored by oxidation of the H_2O_2-sensitive fluorescent probe DCF-DA [5].

A cytofluorimetric analysis of DCF-loaded thymocytes revealed a strong increase in intracellular ROS concentration on cell stimulation with ConA (268 %), as indicated by the marked shift to the right of the mean intensity of cell fluorescence (Figure 2A, panel a). A small increase (+43 %) in ROS production was also documented in thymocytes stimulated by antibody-mediated TCR plus CD4 cross-linking (Figure 2A, panel b) but not by TCR cross-linking alone (results not shown). Intrinsic pro-oxidant properties of ConA were excluded by analysing the fluorescence of TCR-negative HeLa cells stimulated with ConA and probed with DCF (results not shown). Furthermore, the pretreatment of thymocytes with excess (1 mM) H_2O_2 induced a marked increase in intracellular ROS concentration, as indicated by the strong increment in mean cell fluorescence, and abolished differences between ConA-treated and control samples, suggesting that lectin stimulation did not effect DCF-DA cellular loading and/or metabolism (Figure 2A, panel c). ROS generation was also detected at concentrations of ConA lower than 300 μg/ml (results not shown); however, high concentrations of ConA were used throughout the study to facilitate the biochemical analysis of early T-cell activation events.

As shown in Figure 2(B), ROS production in ConA-stimulated thymocytes increased rapidly (in 5–10 min) and reached a plateau with no or little apparent decrease over 30 min. Cell pretreatment with radical scavenger and the glutathione precursor NAC effectively decreased intracellular ROS in ConA-stimulated cells (Figure 2C, panel c). A similar effect (Figure 2C, panel d) was obtained by a short (30 min) preincubation of cells with DPI, a potent inhibitor of flavonoid-containing enzymes such as

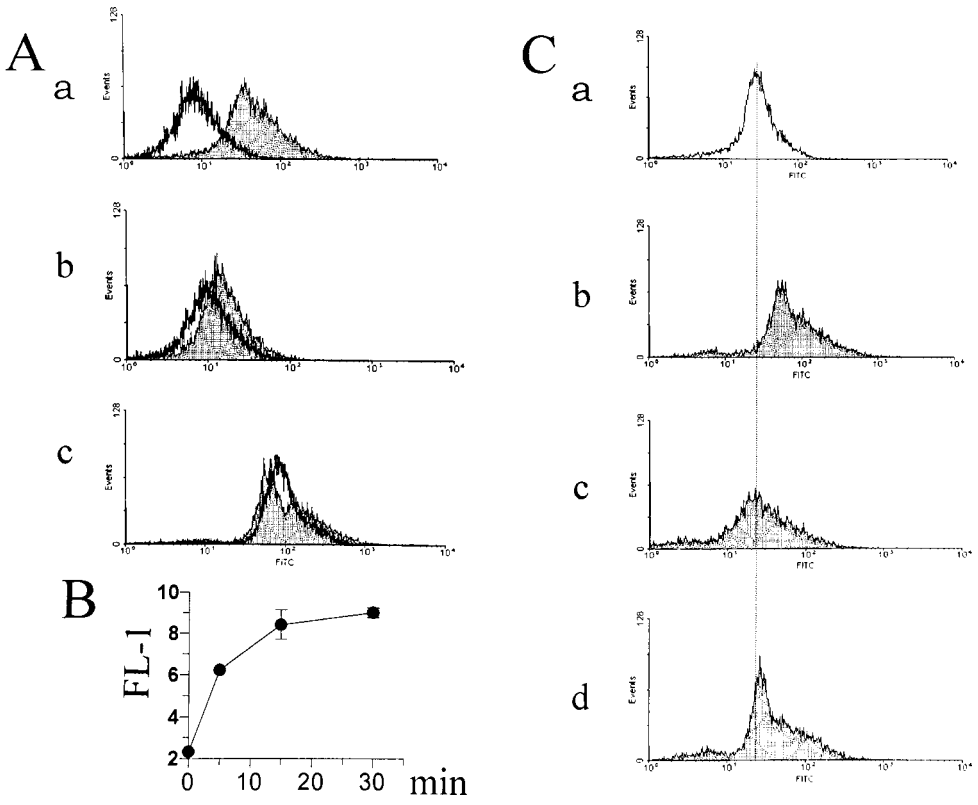

Figure 2 Increased intracellular ROS in mouse thymocytes stimulated through TCR, and modulation by antioxidants

(**A**) (**a**) Mouse thymocytes were resuspended at 10^8 cells/ml, prewarmed at 37 °C for 2 min and treated with either PBS (open histogram) or 300 μg/ml ConA (shaded histogram) for 5 min. DCF-DA (10 μg/ml) was then added for 5 min. Finally, cells were diluted 1:10 and analysed immediately by flow cytometry. (**b**) Thymocytes (2×10^7) were incubated with 1 μg of anti-(mouse TCR) and 2 μg of anti-(mouse CD4) (shaded histogram) or PBS alone (open histogram) for 30 min on ice. After removal of the unbound antibodies, cells were resuspended in 200 μl of HBSS and stimulated with 100 μg/ml streptavidin for 5 min. Analysis of ROS generation was performed as in (**a**). (**c**) Cells were pretreated with 1 mM H_2O_2 for 10 min before stimulation with ConA as in panel (**a**). All results are representative of at least two independent experiments. (**B**) Time course of ROS production in response to ConA. Cells were stimulated with ConA and probed for 5 min with DCF-DA after 5, 15 or 30 min. Cell harvesting and cytofluorimetric analysis were performed as described above. Values are means ± S.E.M. of duplicate samples. Results are representative of two independent experiments. FL-1, fluorescence-1. (**C**) Thymocytes were pretreated with NAC [30 mM for 8 h (**c**)] or DPI [20 μM for 30 min (**d**)] or left untreated. Cells were stimulated in the presence of the inhibitors with ConA or in the presence of ConA alone (**b**) or PBS alone (**a**) and processed as in Figure 1(A). Mean cell fluorescence (in arbitrary units) was 29 in (**a**), 70 in (**b**), 25 in (**c**) and 37 in (**d**). Results are representative of several independent experiments.

NADPH oxidase [21] and NO synthase [22], whereas the NO-synthase-specific inhibitor L-NMMA had no effect (results not shown).

Effects of endogenous oxygen radicals on signalling events mediated by protein tyrosine phosphorylation

Oxidants such as H_2O_2 and diamide have been reported to trigger protein tyrosine phosphorylation in many cell types including T lymphocytes [13,23,24]. The growth-factor-like activity of oxidant species is due mainly to the oxidation and inhibition of multiple tyrosine phosphatases, leading to the unbalanced accumulation of phosphate groups on protein tyrosine residues [25]. In an attempt to identify intracellular targets for oxygen species generated by the exposure of cells to lectins, we first evaluated the intensity and pattern of protein tyrosine phosphorylation in thymocytes differently stimulated through the TCR pathway. An anti-phosphotyrosine immunoblot analysis of total lysates obtained from cells stimulated with anti-TCR plus anti-CD4 antibodies or ConA revealed very similar profiles of protein phosphorylation, with bands at 120, 85, 75 and 38 kDa most prominently induced by all the treatments after 2 min of stimulation (Figure 3A). However, in ConA-stimulated cells, in which a marked increase in intracellular peroxides accompanied TCR triggering (Figure 2), phosphorylation of the 75 and 38 kDa protein species was prolonged, these bands being still evident after 20 min of stimulation but seeming substantially dephosphorylated in cells treated with anti-receptor antibodies (Figure 3A; compare lanes 4 and 6).

Equal protein loading in all lanes was confirmed by reversible staining of the nitrocellulose filter with Ponceau S (results not shown).

In apparent contrast with previous reports, exogenous H_2O_2 alone was insufficient to elicit any detectable protein tyrosine phosphorylation in resting thymocytes (Figure 3A, lanes 7 and 8), probably owing to the lower H_2O_2 concentration used in this experiment and to high levels of antioxidant enzymes expressed in this cell type.

The adaptor protein Grb2 links phosphorylated growth factor receptors, including lymphocyte antigen receptors, to Ras activation by mediating the membrane recruitment of Ras exchange factor SOS [26]. To assess the phosphorylation of specific signalling components relevant to lymphocyte activation by TCR, we took advantage of a GST fusion protein containing the SH2 (Src homology 2) phosphotyrosine-binding domain of

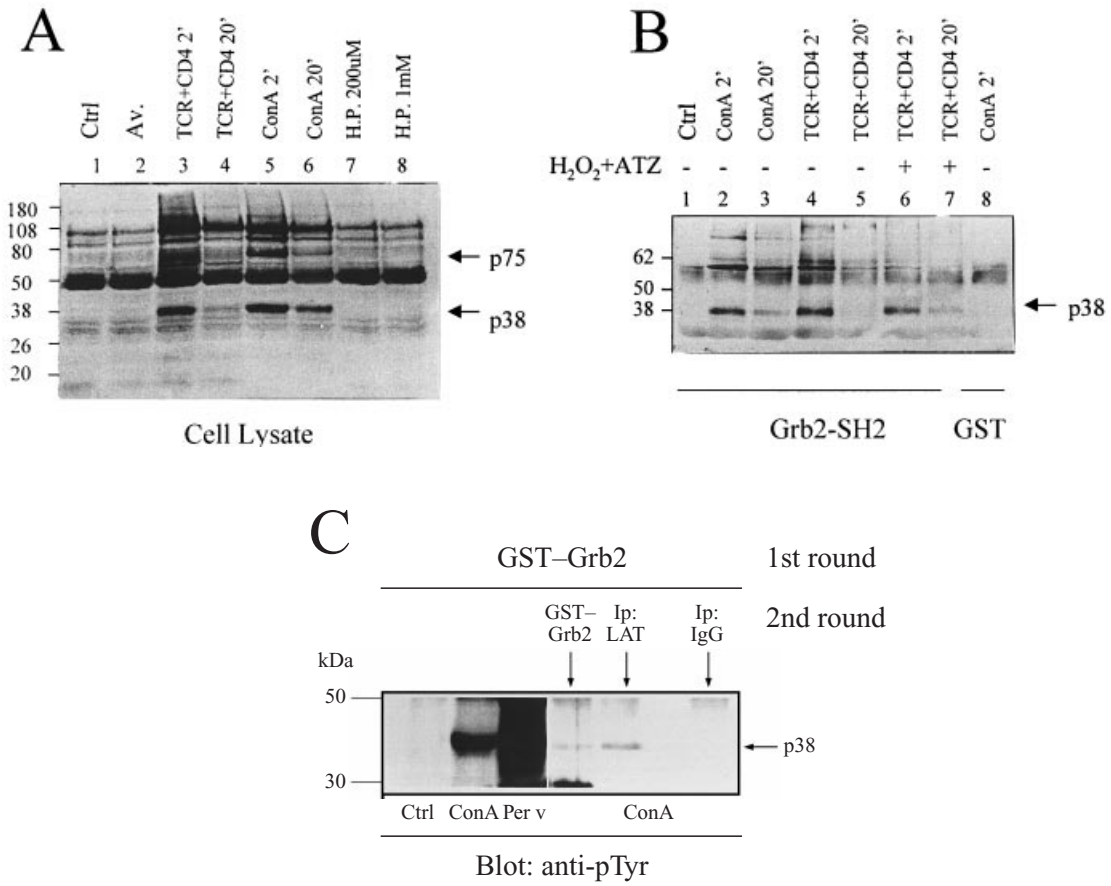

Figure 3 Tyrosine phosphorylation in mouse thymocytes stimulated through TCR

(**A**) Cells were stimulated as described in the Materials and methods section, then pelleted and lysed in 1% (v/v) Triton X-100. Total cell lysates were resolved by SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose and probed with anti-phosphotyrosine antibody 4G10 (diluted 1:5000). The positions of molecular mass markers are indicated (in kDa) at the left. Two prominent bands of 75 and 38 kDa are indicated by arrows. Abbreviations: Av., streptavidin alone; H.P., H_2O_2; 2', 2 min; 20', 20 min. Equal loading throughout the blot was verified by Ponceau S protein staining (results not shown). Densitometric values for p38 bands were: lane 1, 0.8 units; lane 2, 0 units; lane 3, 15.1 units; lane 4, 4.5 units; lane 5, 26.7 units; lane 6, 14.8 units; lane 7, 0 units; lane 8, 0 units. (**B**) Phosphoproteins involved in Ras activation were pulled down with a Sepharose-bound GST fusion protein containing the SH2 domains of Grb2 or GST alone as control (Ctrl), and detected by anti-phosphotyrosine immunoblotting. The 38 kDa phosphoprotein, probably representing phosphorylated LAT (see the text), is indicated by an arrow. In lanes 6 and 7, cells were preincubated with 50 mM aminotriazole (ATZ) for 1 h followed by 200 μM H_2O_2 for 10 min before stimulation with anti-TCR plus anti-CD4. Equal loading throughout the blot was verified by Ponceau S staining of the GST–Grb2 fusion protein band. Abbreviations: 2′, 2 min; 20', 20 min. The positions of molecular mass markers are indicated (in kDa) at the left. (**C**) Identification of p38 as phospho-LAT. Phospho-p38 was pulled down with Sepharose-conjugated GST–Grb2, as in (**B**), from unstimulated cells (Ctrl, lane 1), cells stimulated with ConA (lane 2) or cells exposed to the protein tyrosine phosphatase inhibitor pervanadate (Per v) for 5 min (lane 3). Grb2-binding proteins from ConA-stimulated cells were either loaded directly on the gel (lane 2) or solubilized in 0.5% SDS at 100 °C for 10 min, diluted 1:5 and subjected to a second round of purification with GST–Grb2 (lane 4), anti-LAT (lane 5) or control IgG (lane 6). All samples were assessed by anti-phosphotyrosine (anti-pTyr) immunoblotting. The arrow indicates p38 bound to GST–Grb2 or 're-precipitated' with anti-LAT. Abbreviation: Ip, immunoprecipitation.

the adaptor protein Grb2 to isolate Grb2 binding proteins potentially involved in linking the triggering of the TCR to Ras/MAPK kinase activation. GST–Grb2 fusion protein was used to absorb lysates obtained from antibody- or lectin-stimulated thymocytes (Figure 3B). A prominent Grb2-binding protein of 38 kDa was detected by anti-phosphotyrosine immunoblotting; its phosphorylation was more pronounced in ConA-treated cells than in thymocytes treated only with anti-TCR plus anti-CD4. This difference was especially evident after 20 min of stimulation (Figure 3B; compare lanes 3 and 5), when the protein still conserved significant reactivity with anti-phosphotyrosine antibody in lectin-treated cells but was almost completely unstained in the antibody-treated sample. The disappearance of the 38 kDa phosphoprotein band after 20 min of stimulation was not due to its sequestration in the Triton-insoluble cell fraction, because it could not be recovered from the cell pellet by extraction with SDS plus deoxycholate (results not shown), and was most probably due to dephosphorylation by one or more tyrosine phosphatases. Almost no difference in p38 phosphorylation/Grb2 binding between the different treatments was observed at an early time point (2 min). Interestingly, cell exposure to exogenous H_2O_2 after inhibition of the H_2O_2 cellular scavenger catalase by aminotriazole significantly delayed p38 dephosphorylation after TCR/CD4 cross-linking, thereby mimicking the effect of ConA (Figure 3B, lanes 5 and 7).

The 38 kDa Grb2-binding protein corresponds to the 38 kDa protein band previously noticed in the total cell lysates, and probably represents the recently cloned p38/LAT (linker for activation of T-cells), a molecule necessary for interleukin 2 production and cell proliferation in response to TCR triggering [27]. In fact an anti-LAT specific antiserum, but not a control antiserum, immunoprecipitated the p38 phosphoprotein bound to Grb2/GST after resolubilization in SDS (Figure 3C). Moreover, immunoblotting of ConA-stimulated total protein lysates

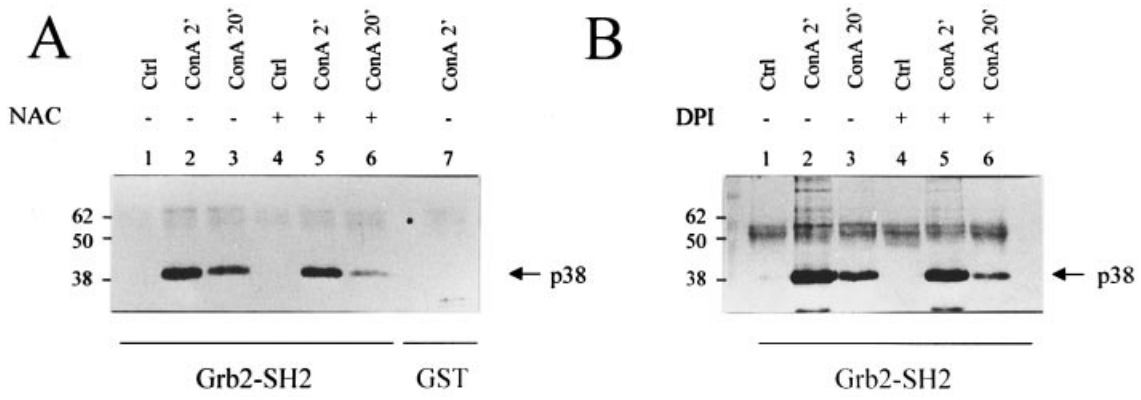

Figure 4 Antioxidants accelerate p38 dephosphorylation in ConA-stimulated thymocytes

(**A**) Thymocytes (2×10^7) pretreated with PBS or NAC (30 mM, 8 h) were stimulated with ConA (300 μg/ml) and Grb2-binding phosphoproteins were isolated by precipitation with Sepharose-bound GST–Grb2/SH2 and analysed by anti-phosphotyrosine immunoblotting. Densitometric values for p38 bands were: lane 1, 0 units; lane 2, 29.6 units; lane 3, 17.9 units; lane 4, 0 units; lane 5, 25.4 units; lane 6, 7.9 units; lane 7, 0 units. (**B**) Cells were pretreated with either 20 mM DPI in DMSO or DMSO alone for 30 min before stimulation with ConA followed by lysis and isolation of Grb2-binding phosphoproteins. Densitometric values for p38 bands in this experiments were: lane 1, 0 units; lane 2, 49.9 units; lane 3, 28.5 units; lane 4, 0 units; lane 5, 41.4 units; lane 6, 16.9 units. Note that antioxidants (both NAC and DPI) significantly decreased p38 phosphorylation after 20 min of stimulation, with little effect at an early time point. All presented results were confirmed over two or more independent experiments.

with either anti-LAT or anti-phosphotyrosine antisera yielded perfectly overlapping 38 kDa bands (results not shown).

To ascertain whether p38 tyrosine phosphorylation/dephosphorylation was modulated by endogenous ROS in ConA-treated cells, we purified Grb2-binding proteins from cells stimulated with either ConA alone or ConA plus the antioxidant NAC or the NADPH oxidase inhibitor DPI. Phosphoproteins were detected by anti-phosphotyrosine immunoblotting. As shown in Figure 4, both NAC and (to a smaller extent) DPI accelerated the dephosphorylation of p38 in comparison with mock-treated controls (compare lanes 3 and 6 in both panels); again, there was little effect on the maximum protein phosphorylation as evaluated after only 2 min of stimulation. Densitometric quantification of anti-phosphotyrosine staining revealed that p38 dephosphorylation was significantly more rapid in NAC- and DPI-treated samples (70 % and 60 % decrease respectively after 20 min) in comparison with mock-treated cells (40 % decrease between 2 and 20 min of stimulation). Treatment with antioxidant did not affect cell viability (Trypan Blue exclusion) and had no effect on the tyrosine phosphorylation of the adaptor protein/exchange factor Vav (results not shown).

JNK modulation by ROS in ConA-stimulated T lymphocytes

JNK/SAPK (stress-activated protein kinase) was initially identified as a c-Jun kinase responsive to UV radiation, oxidative stress and the oncoprotein Ras [28]. JNK also has a crucial role in the signalling cascade leading to T-cell activation and proliferation in response to immunogenic stimuli [8,29]. These characteristics qualify JNK as a potential target for ROS in lectin-stimulated thymocytes.

The kinase activity of immunoprecipitated JNK-1 was markedly increased by treatment for 20 min with ConA, whereas activation after 2 min of treatment was marginal (Figure 5A, panel a). ConA had no effect on the amount of immunoprecipitated kinase (Figure 5A, panel b), suggesting that increased substrate phosphorylation resulted from post-translational modification of the enzyme. To investigate the role of ROS in JNK-1 activation by ConA, the effect of DPI on JNK activity was evaluated in both resting and lectin-stimulated thymocytes (Figure 5B, panel a). Pretreatment of cells with DPI decreased JNK activity significantly, and in a dose-dependent manner, after stimulation with ConA (Figure 5B, panel a, lanes 3–5), which is consistent with the hypothesis of a role for oxygen radicals in mediating JNK activation by lectins. Unstimulated kinase activity was also decreased by DPI, although to a smaller extent, in comparison with mock-treated cells (Figure 5B, panel a; compare lanes 1 and 2).

Weaker JNK up-regulation by ConA (compare Figures 5B and 5A) is probably due to the presence of DMSO (vehicle control for DPI); this effect, although probably unrelated to ROS generation (see the Discussion section), might reflect altered transmembrane signalling in response to surface receptor triggering [30]; it is currently under investigation.

NAC was also found to prevent JNK up-regulation by ConA (results not shown) but the interpretation of this result is complicated by the fact that NAC itself strongly activated the kinase. A similar effect was also observed with the reducing agent dithiothreitol (results not shown).

Effects of CD28 cross-linking on intracellular ROS and on protein tyrosine phosphorylation

The T-cell surface antigen CD28 releases important co-stimulatory signals for T lymphocyte activation by antigens or TCR cross-linking. This molecule is required for the optimal proliferative response of mouse thymocytes to lectins [29]; furthermore, the engagement of CD28 on human T-cells produces oxygen radicals and ROS-dependent NF-κB activation [16]. To assess whether and to what extent the effects elicited by ConA in mouse thymocytes might be mediated by CD28, cells were stimulated with a mixture of anti-TCR and anti-CD28 antibodies and ROS generation as well as LAT phosphorylation, assessed as described above. As shown in Figure 6(A), engagement of CD28 together with TCR or TCR plus CD4 failed to induce a amount of ROS comparable to that induced by ConA, although an effect of anti-CD28 reagents on ROS production was observed (Figure 6A). Accordingly, none of the combinations of antibodies mentioned above induced the persistent tyrosine phosphorylation

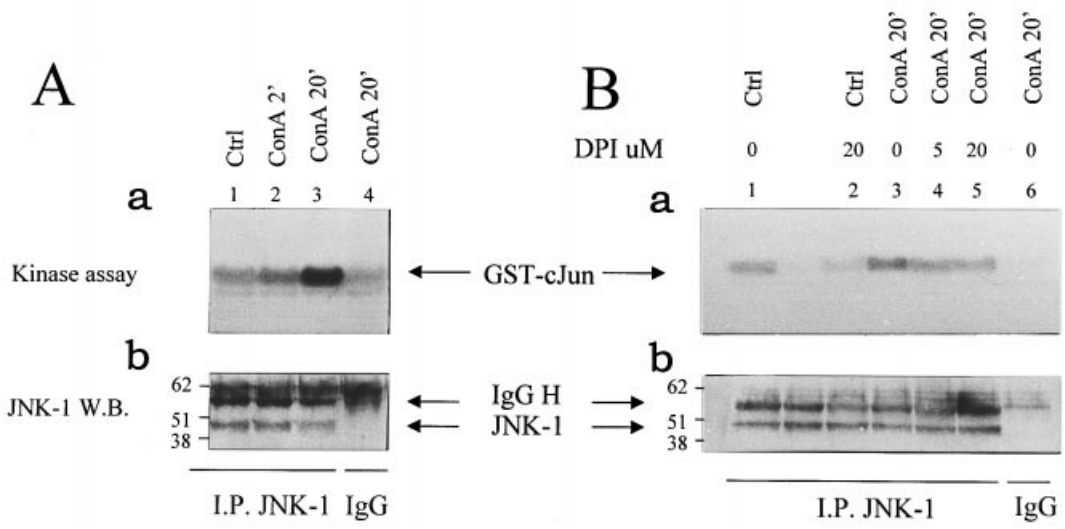

Figure 5 JNK-1 activation by ConA is sensitive to DPI

(**A**) (**a**) JNK-1 activity in ConA-stimulated cells. Cells were stimulated for 0, 2 and 20 min; JNK-1 activity was evaluated as described in the Materials and methods section. Arrow indicates the GST–cJun (5–89) fusion protein used as a specific JNK substrate. (**b**) Western blot (W.B.) analysis of immunopecipitated (I.P.) JNK-1. The protein band (46 kDa) is indicated by the lower arrow, and is absent from the mock immunoprecipitation. Non-specific bands at higher molecular masses are the IgG heavy chains of the precipitating antibody. The positions of molecular mass markers are indicated (in kDa) at the left. (**B**) (**a**) DPI attenuates JNK-1 activation by ConA (300 μg/ml; stimulation for 20 min) in a dose-dependent manner (lane 3, 0 μM; lane 4, 5 μM; lane 5, 20 μM). An effect is also seen on the kinase activity in resting cells (compare lanes 1 and 2). (**b**) An equal amount of immunoprecipitated kinase was present in all the samples except the mock control (lane 6), as assessed by Western blot analysis with anti-(JNK-1) antibody (diluted 1:500; Santa Cruz). Abbreviation: uM, μM. Results shown in this Figure are representative of three independent experiments. Abbreviations in both panels: 2', 2 min; 20', 20 min.

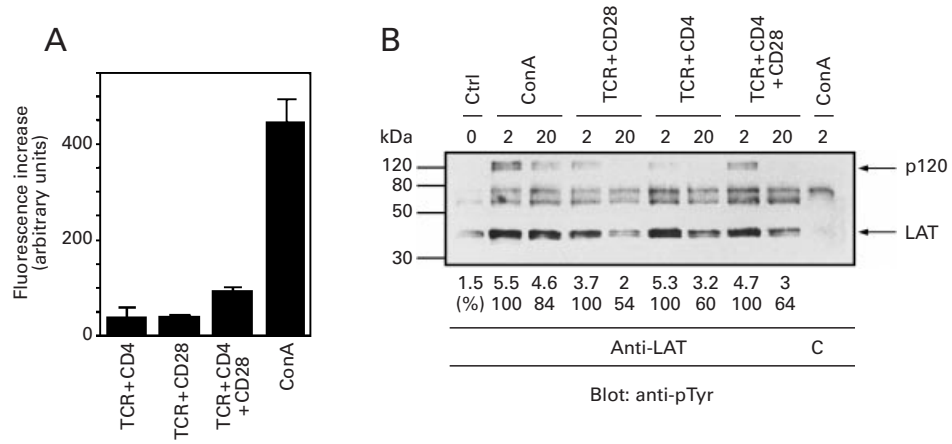

Figure 6 Effects of CD28 cross-linking on intracellular ROS and on LAT tyrosine phosphorylation

(**A**) Cells were stimulated with either ConA or the indicated combinations of biotinylated antibodies followed by avidin. Intracellular ROS were measured after 10 min of stimulation as described in the Materials and methods section. Results are expressed as the increase in fluorescence of DCF-DA (mean fluorescence stimulated minus mean fluorescence unstimulated), and are means $\pm$ S. E. M. for two independent experiments. (**B**) After the indicated treatments, LAT was immunoprecipitated and subjected to anti-phosphotyrosine (anti-pTyr) immunoblotting. Phospho-LAT and the LAT-associated p120 phosphoprotein are indicated at the right. Numbers below the panel indicate, for each treatment, densitometric values for the phospho-LAT band (upper row) and the residual phosphorylation after 20 min as a percentage of the band intensity at 2 min (lower row). Delayed protein dephosphorylation of p38 and p120 was observed only in ConA-treated samples.To confirm equal loading in all lanes, the filter was stripped and re-probed with anti-LAT (results not shown). The positions of molecular mass markers are indicated at the left.

of LAT and LAT-associated p120, observed in cells treated with ConA (Figure 6B).

DISCUSSION

The results presented show that ROS are produced in response to ConA and, to some extent, to TCR plus CD4 or CD28 cross-linking in mouse thymocytes, and that these radical species are required for cell proliferation elicited by lectin and act as modulators of key T-lymphocyte signalling events such as protein tyrosine phosphorylation and the activation of JNK.

Whereas the nature of the oxygen radicals measured in the present study requires further investigation, the effect of DPI on intracellular ROS levels in ConA-stimulated cells indicates that flavin-containing enzymes are potential mediators of oxidative

events initiated by lectin. A DPI-sensitive NADPH oxidase complex containing the small GTPase Rac-1 has been found to produce oxygen radicals in non-phagocytic cells such as fibroblasts [31] and chondrocytes [3], on stimulation by epidermal growth factor, insulin, basic fibroblast growth factor and TNF. Consistently with our observation of p38 hypophosphorylation in thymocytes pretreated with DPI, DPI drastically decreases the induction of protein tyrosine phosphorylation by the respiratory burst in activated neutrophils [32]. It is therefore conceivable that flavonoid-containing NADPH oxidase(s) are activated by ConA in thymocytes, probably through the receptor-mediated activation of rac-1 [33].

The effects of ConA on mouse T-cells are generally attributed to its property of binding and cross-linking the CD3 component of the TCR [10,34], thereby mimicking the physiological TCR ligand represented by the MHC–peptide complex. However, lectins are much more potent mitogens for T lymphocytes than are anti-TCR and anti-CD4 antibodies, possibly owing to the simultaneous activation of one or more co-stimulatory receptors at the T-cell surface. Whereas the involvement of ROS in growth-inhibitory and apoptogenic signals delivered to T-cell hybridomas by TCR stimulation has already been suggested [35], our results showing an impaired proliferative response to ConA in thymocytes in the presence of antioxidant species point to a role for oxygen intermediates in the signalling cascade linking antigenic stimulation to T-cell proliferation. In agreement with this idea is our observation that ConA-stimulated thymocytes produce more ROS than those stimulated by antibody-mediated cross-linking of TCR plus CD4. Although this difference might be due to intrinsic differences in affinity or avidity for TCR components between lectins and antibodies and/or to different capabilities of different stimuli to alter cell structure and to induce receptor capping at the surface of thymocytes, it is also possible that some other surface molecule bound by ConA contributes to ROS production. One good candidate molecule for this function seems to be CD28 because the B7 cognate receptor is involved in the thymocyte response to ConA [36,37] and has also been shown to stimulate the formation of oxygen radicals when triggered by specific antibodies [16]. However, the results shown in Figure 6 suggest that CD28 engagement is not sufficient to reproduce the signalling properties of ConA. It is therefore possible that in ConA-stimulated cells oxygen radicals, triggered by one or more unknown molecules, deliver a 'co-stimulatory' signal that is required for the translation of TCR cross-linking into a fully mitogenic T-cell response (see Figure 1) but is distinguished, at least in part, by CD28. Future work, including studies of cell activation in normal and CD28-deficient mice, will probably help in assessing this hypothesis properly.

Alterations in protein tyrosine phosphorylation/dephosphorylation are among the best-established consequences of cell exposure to oxygen radicals. More recently, antioxidant species such as NAC and catalase have been reported to impede protein tyrosine phosphorylation substantially in smooth-muscle cells stimulated by PDGF [4]. The results presented here show that endogenous ROS produced in response to lectin modulate the tyrosine phosphorylation of at least one molecule relevant to antigen-mediated T-cell activation, the Grb2-binding protein p38/LAT. In fact, (1) in ConA-stimulated thymocytes, ROS production correlates with prolonged phosphorylation or delayed dephosphorylation of p38/LAT; (2) cell pretreatment with H_2O_2 plus aminotriazole prolongs p38 phosphorylation on the cross-linking of TCR and CD4, although is not sufficient to trigger detectable tyrosine phosphorylation in resting cells; and (3) p38 phosphorylation is decreased in lectin-stimulated cells pretreated with antioxidant agents.

Studies on the minimal requirement for TCR engagement during the activation of lymphocytes have pointed to the importance of the spatial and temporal integration of intracellular signals initiated by TCR [38]. In this model, prolonged receptor signalling translates into a lowered threshold for the ligand-induced cell response, an effect also exerted by co-stimulatory signals [39]. In view of the above concepts, we propose that, after stimulation by lectin, endogenous ROS might signal sustained p38 phosphorylation/Ras activation in response to antigen receptor engagement, thereby lowering the threshold for full T-cell activation.

The mechanisms by which endogenous ROS modulate tyrosine phosphorylation in ConA-stimulated T-cells still needs further investigation; however, an involvement of protein tyrosine phosphatases in this phenomenon seems conceivable. This class of enzymes, which counterbalance the action of tyrosine kinases in growth-factor-receptor signalling and contribute to setting triggering thresholds and to the modulation of the intensity and duration of intracellular responses [40], are expecially prone to inactivation by oxygen radicals owing to the oxidation of a critical cysteine residue in their catalytic site [41]. The observation of prolonged tyrosine phosphorylation in thymocytes stimulated by lectins is consistent with the idea that oxygen radicals could serve in these cells as physiological inhibitors for one or more tyrosine phosphatases. In agreement with this hypothesis, we have previously reported prolonged tyrosine phosphorylation of p38/LAT in response to TCR triggering and exaggerated mitogenic response to lectins in mouse thymocytes deficient in the tyrosine phosphatase SHP1/PTP1C [18] (in which PTP stands for protein tyrosine phosphatase). Furthermore, preliminary evidence suggests that the protein tyrosine phosphatase SHP1 is oxidized on cysteine residues after the stimulation of thymocytes with ConA (G. Pani and T. Galeotti, unpublished work).

A relevant result of the present study is that the activation of JNK-1 in ConA-stimulated thymocytes seems to be dependent on the formation of oxygen radicals. This finding is not completely unexpected because ROS are well-known activators of JNK kinase, although the mechanisms underlying this activation are not completely known [42]. Furthermore, endogenous ROS are required for JNK activation by tumour necrosis factor α and interleukin 1 in bovine chondrocytes [6]. Surprisingly, however, the involvement of oxygen species in JNK activation by antigen-mimicking stimuli has not previously been reported. The effects of DPI on JNK stimulation by lectin (Figure 5) suggest that an NADPH-oxidase-like enzyme could act upstream of JNK in T-cells activated through the TCR complex, a hypothesis consistent with emerging evidence on the roles of the small GTPase Rac-1, a putative component of this oxidase, in T-cell signalling [43].

Although the results presented provide strong evidence that ROS are implicated in the molecular and cellular response of T-cells to mitogenic lectins, a number of questions remain still unanswered.

One important point that deserves to be clarified further is whether only mitogenic effects of lectins on thymocytes, or, conversely, also the cytotoxic/apoptogenic ones involve the generation of ROS, and, if so, how redox signalling could lead to opposite physiological outcomes after the stimulation of T-cells.

Another open question is whether effects of oxidant species on protein tyrosine phosphorylation are involved in the redox modulation of JNK activity or whether these signalling compartments are independently regulated by ROS.

Notwithstanding these limitations, the above findings represent significant progress in the understanding of the mechanisms underlying lymphocyte regulation by redox stimuli and could therefore help to explain oxygen-related alterations of immune

functions, such as T-cell tolerance breakdown associated with inflammatory events with ROS leakage in the extracellular milieu [44], or, conversely, blunted lymphocyte activation in the context of large, poorly oxygenated tumours [45].

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