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Mechanism of vanadate-induced activation of tyrosine phosphorylation and of the respiratory burst in HL60 cells

Role of reduced oxygen metabolites

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Vanadate induces phosphotyrosine accumulation and activates O_2 consumption in permeabilized differentiated HL60 cells. NADPH, the substrate of the respiratory burst oxidase, was found to be necessary not only for the increased O_2 consumption, but also for tyrosine phosphorylation. The effect of NADPH was not due to reduction of vanadate to vanadyl. Instead, NADPH was required for the synthesis of superoxide, which triggered the formation of peroxovanadyl [$V^{(4+)}$-OO] and vanadyl hydroperoxide [$V^{(4+)}$-OOH]. One or both of these species, rather than vanadate itself, appears to be responsible for phosphotyrosine accumulation and activation of the respiratory burst. Accordingly, the stimulatory effects of vanadate and NADPH were abrogated by superoxide dismutase. Moreover, phosphorylation was activated in the absence of NADPH by treatment with $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH, generated by treatment of orthovanadate with KO_2 or H_2O_2 respectively. The main source of the superoxide involved in the formation of $V^{(4+)}$-OO and $V^{(4+)}$-OOH is the NADPH oxidase. This was shown by the inhibitory effects of diphenylene iodonium and by the failure of undifferentiated cells, which lack oxidase activity, to undergo tyrosine phosphorylation when treated with vanadate and NADPH. By contrast, exogenously generated $V^{(4+)}$-OO induced marked phosphorylation in the undifferentiated cells, demonstrating the presence of the appropriate tyrosine kinases and phosphatases. A good correlation was found to exist between induction of tyrosine phosphorylation and activation of the respiratory burst, suggesting a causal relationship. Therefore an amplification cycle appears to exist in cells treated with vanadate, whereby trace amounts of superoxide initiate the formation of $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH. These peroxides promote phosphotyrosine formation, most likely by inhibition of tyrosine phosphatases. Accumulation of critical tyrosine-phosphorylated proteins then initiates a respiratory burst, with abundant production of superoxide. The newly formed superoxide catalyses the formation of additional $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH, thereby magnifying the response. Since vanadium derivatives are ubiquitous in animal tissues, $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH could be formed *in vivo* by reduced O_2 metabolites, becoming potential endogenous tyrosine phosphatase inhibitors. Because of their potency, peroxides of vanadate may be useful as probes for the study of protein phosphotyrosine turnover.

INTRODUCTION

When activated by invading microbes or their products, phagocytes undergo a number of nearly synchronous reactions intended to defend the host organism. These include chemotaxis, phagocytosis, exocytosis of the contents of three types of secretory organelles and a respiratory burst [1,2]. Most of the O_2 consumed during this respiratory burst is converted by the NADPH oxidase to superoxide ($O_2^{\cdot-}$), which can in turn generate a variety of reduced O_2 metabolites that are potent bactericidal and tumoricidal agents [3,4]. The mechanism whereby the oxidase is stimulated when neutrophils are exposed to chemoattractants is not completely understood. It is believed, however, that protein phosphorylation plays a central role in the activation process. This notion is supported by the finding that stimulation by chemoattractants is accompanied by increased phosphoprotein accumulation [4,5] and by the exquisite ATP-dependence of the activation of the respiratory burst [6,7].

Protein kinase C has been suggested to be primarily responsible for the phosphorylation that leads to activation of the NADPH oxidase [4]. Indeed, selective activation of this kinase by addition of phorbol esters or exogenous diacylglycerol effectively triggers superoxide generation [8,9]. Nevertheless, evidence obtained using a variety of potent antagonists suggests that protein kinase C cannot fully account for the activation of the oxidase by physiological stimuli (summarized in [5]). Because receptor-mediated activation of the burst is virtually eliminated by omission of ATP, it is reasonable to assume that kinases other than protein kinase C are involved in this process. Several lines of evidence have suggested that tyrosine phosphorylation may be an important step signalling activation of the oxidase. First, tyrosine-phosphorylated proteins were found to accumulate in neutrophils stimulated with chemotactic peptides or leukotrienes [10,11], and in cells primed with granulocyte/macrophage colony-stimulating factor [12]. Tyrosine phosphorylation was also observed when superoxide generation was initiated in permeabilized cells by addition of guanosine 5′-[γ-thio]triphosphate (GTP[S]) [13]. Secondly, the respiratory burst induced by soluble chemoattractants and by opsonized zymosan could be blocked by inhibitors of tyrosine kinase activity [14,15]. This inhibition appears to be specific, in that other responses elicited by these stimuli, such as exocytosis and actin polymerization, were essentially unaffected [14,15]. Third, a burst of O_2 consumption was triggered in permeabilized neutrophils by addition of vanadate, an inhibitor of tyrosine phosphatases [16]. The burst was accompanied by and tentatively attributed to an

Abbreviations used: DPI, diphenylene iodonium; GTP[S], guanosine 5′-[γ-thio]triphosphate; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; V^{4+}, vanadyl; V^{5+}, vanadate; $V^{(4+)}$-OO, peroxovanadyl; $V^{(4+)}$-OOH, vanadyl hydroperoxide.

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increase in phosphotyrosine accumulation. Similar results were obtained using electroporated or streptolysin-O-permeabilized HL60 cells [17], a promyelocytic leukemia cell line which can differentiate into a neutrophil-like phenotype when treated with dimethyl sulphoxide.

In permeabilized cells, such as those used to assess the effects of vanadate, the endogenous nucleotide pool is rapidly depleted, and exogenous NADPH is required and is routinely added to measure the activity of the oxidase [18]. In the experiments described above, NADPH was also present when tyrosine phosphorylation was determined in parallel, in order to maintain comparable conditions. However, while studying the effects of vanadate on HL60 cells, it became apparent that the extent of tyrosine phosphorylation depended on the availability of NADPH. Significant phosphorylation was detectable only in the presence of the nucleotide (see below). The purpose of the experiments described in the present paper is to understand the mechanism underlying this unexpected observation. The results indicate that conversion of vanadate into a reduced metabolite is required for the observed phosphotyrosine accumulation and for the concomitant stimulation of the NADPH oxidase. Such reduced vanadate species appear to be much more potent, and possibly specific, inhibitors of tyrosine phosphatase, and may provide a useful tool for the study of the protein phosphotyrosine turnover.

MATERIALS AND METHODS

Materials

HL60 cells were purchased from the American Type Culture Collection. Fetal bovine serum was from Flow Laboratories. L-Glutamine and penicillin/streptomycin were from Gibco. Molecular mass standards, phenylmethanesulphonyl fluoride, ATP (K^+ salt), EGTA, NADPH, dimethyl sulphoxide, Nonidet P40, Ponceau S stain, phosphoserine, phosphotyrosine, Coomassie Blue, Hepes, catalase, superoxide dismutase (SOD), cytochrome *c*, phorbol 12-myristate 13-acetate (PMA) and potassium superoxide were obtained from Sigma. Catalase was also purchased from Boehringer Mannheim. Albumin and glutathione were from Calbiochem. The monoclonal anti-phosphotyrosine antibody PY20 IgG 2B was obtained from ICN. H_2O_2 was purchased from Fisher Scientific. Vanadyl sulphate and sodium orthovanadate were from Aldrich Chemicals. Diphenylene iodonium (DPI) was a gift from Dr. A. Cross (Department of Biochemistry, University of Bristol, Bristol, U.K.). Complete medium RPMI 1640 was prepared by the University of Toronto Media Preparation Service. Bicarbonate-free medium RPMI 1640 was from Sigma.

Solutions

Bicarbonate-free medium RPMI 1640 was buffered to pH 7.3 with 25 mM-Hepes-Na. Unless otherwise specified, KCl permeabilization medium contained (in mM): 140 KCl, 1 $MgCl_2$, 2 NADPH, 1 EGTA, 10 Hepes-K (pH 7.0), 1 ATP, 10 glucose and sufficient $CaCl_2$ to give a final free Ca^{2+} concentration of 100 nM, calculated as described in [19]. Peroxovanadyl [$V^{(4+)}$-OO] was prepared by mixing equivalent concentrations of sodium orthovanadate and potassium superoxide (10 mM) for 15 min at 37 °C. Residual $O_2^{\cdot-}$ was then removed by addition of SOD (5 μg/ml). Vanadyl hydroperoxide [$V^{(4+)}$-OOH] was prepared by mixing equimolar concentrations of sodium orthovanadate and H_2O_2 (10 mM), followed by incubation for 15 min at 22 °C. To remove residual H_2O_2, catalase (200 μg/ml) was then added. This procedure is similar to that described by Fantus *et al.* [20] to generate 'pervanadate'. Aliquots of $V^{(4+)}$-OO and $V^{(4+)}$-OOH were used immediately after addition of SOD or catalase respectively. In some experiments, 10 mM-orthovanadate was preincubated with 2 mM-glutathione for 5 min at 22 °C, and a portion of this mixture was immediately added to the cell suspension.

HL60 cell culture

HL60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), streptomycin (100 units/ml) and penicillin (100 mg/ml). The cells were passaged at starting densities of $(2.5–3.5) \times 10^5$ cells/ml and maintained in suspension culture at 37 °C in an air atmosphere containing 5% CO_2. The cultures were diluted every 3–4 days so that the cell density did not exceed $(1–2) \times 10^6$ cells/ml. To induce granulocytic differentiation, the cells were inoculated at 3.5×10^5 cells/ml of medium in spinner flasks and treated with 1.3% (v/v) dimethyl sulphoxide for 6–7 days. The cells were harvested by centrifugation and resuspended at a density of 10^7 cells/ml in bicarbonate-free Hepes-buffered RPMI 1640 medium to be used for experiments. Unless indicated otherwise, all the experiments were performed using differentiated cells.

Cell permeabilization procedures

For electroporation, intact cells were sedimented and resuspended in ice-cold permeabilization solution at 10^7 cells/ml. Aliquots (0.8 ml) of this suspension were then transferred to a Bio-Rad Pulser cuvette and permeabilized using two discharges of 1.0 kV from a 25 μF capacitor. The cells were sedimented using an Eppendorf 5415 Microfuge and resuspended in fresh ice-cold permeabilization solution between pulses. The cells were kept on ice and used within 15 min of permeabilization.

Phosphotyrosine detection

Where indicated, cells were permeabilized and suspended in the KCl permeabilization medium. After a 2 min incubation at 37 °C in the presence or absence of 2 mM-NADPH, vanadate or one of its reduced congeners was added as indicated and the suspension was incubated for a further 5 min. Phenylmethanesulphonyl fluoride (100 μM) was added, the samples were sedimented and the pellets were immediately solubilized in boiling Laemmli sample buffer. Immunoblotting was performed as outlined in [21]. Briefly, following electrophoresis and transfer to nitrocellulose, the proteins were visualized by staining with Ponceau S (0.2%, w/v) in 3% (w/v) trichloroacetic acid, and destained in water. The blot was then incubated with phosphotyrosine-specific antibodies and washed. The monoclonal anti-phosphotyrosine antibody was detected by goat anti-mouse antibody conjugated to alkaline phosphatase. Alkaline phosphatase staining was then performed as recommended by the kit manufacturer (Bio-Rad).

Oxygen consumption

O_2 consumption was measured polarographically with a model 53 biological monitor (Yellow Springs Instrument Co.) as described [18], using a cell density of 4×10^6/ml. O_2 consumption was calculated using a solubility coefficient of 0.024 ml of O_2/ml at 37 °C, the temperature used for all the experiments.

Superoxide production

$O_2^{\cdot-}$ generation was measured in disposable 1 cm plastic cuvettes at 37 °C by continuous spectrophotometric measurement of the SOD-inhibitable reduction of ferricytochrome *c* at 550 nm [22]. A cell density of 4×10^6/ml was used and the samples were stirred magnetically throughout the assay. $O_2^{\cdot-}$ production was calculated using an absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$.

Other methods

The immunoblots, O_2 consumption traces or superoxide production recordings illustrated in the Figures are representative of a minimum of three separate experiments. Unless otherwise specified, data are presented in the text as the means ± S.E.M. of the numbers of determinations indicated in parentheses.

RESULTS

Mechanism of vanadate-induced phosphotyrosine accumulation

Fig. 1(*a*) analyses the effects of vanadate and NADPH, alone or in combination, on protein tyrosine phosphorylation in electropermeabilized differentiated HL60 cells. The presence of tyrosine-phosphorylated polypeptides was established by immunoblotting using monoclonal antibodies. The specificity of the PY20 IgG 2B antibody had been previously demonstrated by competitive displacement from the blots by phosphotyrosine, but not by phosphoserine or phosphothreonine [16]. As reported earlier [17], extensive phosphotyrosine accumulation was observed when vanadate (50 μM) and NADPH (2 mM) were present simultaneously. The increase was apparent throughout the range of molecular mass resolved by 10% polyacrylamide gels (e.g. see Fig. 2), but was particularly noticeable in the 35–150 kDa range. In contrast, only marginal levels of phosphotyrosine were detectable in samples incubated in the presence of NADPH or vanadate alone (Fig. 1*a*; representative of five similar experiments). Somewhat different labelling patterns are apparent in some of the experiments illustrated (see Figs. 1–3).

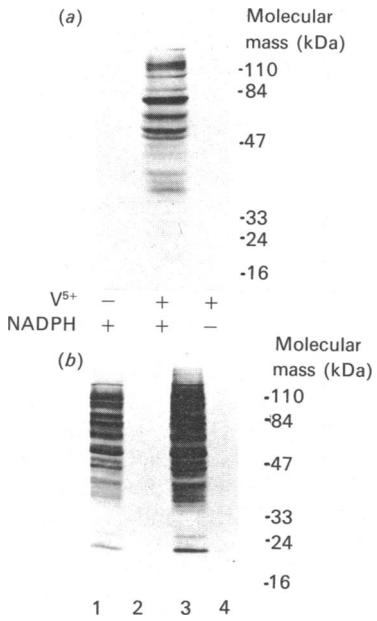

Fig. 1. Effect of NADPH on vanadate-induced phosphotyrosine accumulation in HL60 cells

(*a*) Electropermeabilized cells were pre-incubated for 2 min at 37 °C in the presence (+) or absence (−) of 2 mM-NADPH as indicated. Where indicated, 50 μM-sodium orthovanadate (V^{5+}) was then added and the samples were incubated for a further 5 min at 37 °C. (*b*) Electroporated cells were preincubated for 2 min at 37 °C in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 2 mM-NADPH. Next, 100 μM of V^{5+} (lane 1), vanadyl sulphate (V^{4+}; lanes 2 and 3), or V^{5+} pretreated with glutathione (GSH) (lane 4) as described in the Materials and methods section were added and the suspension was incubated for a further 5 min at 37 °C. After electrophoresis and blotting, polypeptides containing phosphotyrosine were detected using a monoclonal antibody and alkaline phosphatase staining. Typical immunoblots are shown.

These are due to slight differences in the degree of differentiation (e.g. day 6 versus day 7), the amount of vanadate derivative used or the duration of the electrophoretic transfer to the nitrocellulose.

In the physiological pH range, vanadate can exist in multiple oxidation states [23]. At the appropriate redox potential, the pentavalent vanadate can be reduced to vanadyl (VO^{2+}), a tetravalent form that has been reported to be responsible for some of the biological actions of vanadium, including inhibition of ribonuclease and *p*-nitrophenol phosphatase (see [23] for review). Reduction of vanadate to vanadyl takes place in the presence of a number of reducing agents common to cells, such as glutathione [24]. Because the redox potential of NAD(P)H is even more negative than that of reduced glutathione, we tested the possibility that vanadyl was the active species responsible for increased phosphotyrosine formation in HL60 cells. For this purpose, permeabilized cells were exposed to equimolar concentrations of vanadate (with NADPH), vanadate pretreated with reduced glutathione, or vanadyl (with or without NADPH). To minimize oxidation of vanadyl by atmospheric O_2, solutions of vanadyl sulphate were prepared immediately before use. As shown in Fig. 1(*b*), neither vanadyl alone nor vanadate pretreated with glutathione could support phosphotyrosine accumulation in the absence of NADPH. In the presence of the nucleotide, vanadyl was at least as effective as the pentavalent vanadate. Thus the permissive effect of NADPH on tyrosine phosphorylation is not simply due to direct reduction of vanadate to vanadyl.

As an alternative mechanism, we considered the possibility that the effects on vanadate-catalysed phosphorylation were not exerted directly by NADPH, but rather by a product of its metabolism. In granulocytic cells, the NADPH oxidase is the main pathway for NADPH utilization, and $NADP^+$ and $O_2^{\cdot -}$ are the primary metabolites generated. In a reaction studied in detail by Fridovich and his colleagues (see [25,26] for reviews), $O_2^{\cdot -}$ has in fact been found to interact with vanadate, generating peroxovanadyl ($V^{(4+)}$-OO; reaction 1):

$$V^{(5+)} + O_2^{\cdot -} < \text{-----} > V^{(4+)}\text{-OO} \quad (1)$$

Peroxovanadyl can in turn remove hydrogen from NADPH, producing vanadyl hydroperoxide ($V^{(4+)}$-OOH; reaction 2):

$$V^{(4+)}\text{-OO} + \text{NAD(P)H} < \text{-----} > V^{(4+)}\text{-OOH} + \text{NAD(P)}^{\cdot} \quad (2)$$

In the presence of protons, vanadyl hydroperoxide can decompose, producing H_2O_2 and regenerating vanadate (reaction 3):

$$V^{(4+)}\text{-OOH} + H^+ < \text{-----} > V^{(5+)} + H_2O_2 \quad (3)$$

It is therefore conceivable that $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH are the species responsible for the observed accumulation of protein phosphotyrosine. To test this prediction, the formation of the peroxides of vanadate was precluded either by scavenging superoxide with cytochrome *c* or by catalysing its dismutation with SOD. Permeabilized HL60 cells were preincubated for 1 min with 5 μg of SOD/ml or 50 μM-cytochrome *c* and then challenged with vanadate/NADPH as described above. The results of such experiments are illustrated in Fig. 2(*a*). Phosphotyrosine accumulation was greatly inhibited by removal of superoxide, suggesting that $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH play a role in this process. From the above reactions (1)–(3), it is also expected that the amount of $V^{(4+)}$-OOH accumulated will decrease when H_2O_2 is removed from the medium, driving the reversible reaction (3) to the right. Decomposition of $V^{(4+)}$-OOH would decrease the extent of tyrosine phosphorylation if this species is inhibitory to the phosphatase(s). This prediction was borne out by the experiments shown in Fig. 2(*a*). The presence of 10 μg of catalase/ml greatly diminished the extent of phosphorylation caused by vanadate/NADPH. The preparations of catalase

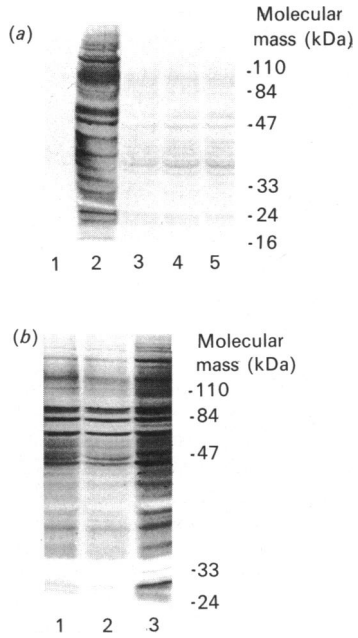

Fig. 2. Role of reduced O_2 metabolites in phosphotyrosine accumulation

(*a*) Electropermeabilized cells were pretreated for 1 min at 37 °C with 5 μg of SOD/ml (lane 3), 50 μM-cytochrome *c* (lane 4), 10 μg of catalase/ml (lane 5), or left untreated (lanes 1 and 2). The cells were then incubated further for 2 min at 37 °C with 2 mM-NADPH. Vanadate (50 μM) was subsequently added to all samples except that in lane 1. After a final 5 min incubation at 37 °C, the reaction was stopped and the samples were used for immunoblot analysis. (*b*) Electroporated cells were suspended in permeabilization buffer and preincubated with (lane 1) or without (lanes 2 and 3) 2 mM-NADPH for 2 min at 37 °C. Next, 100 μM-V^{5+} (lane 1), -$V^{(4+)}$-OO (lane 2) or -$V^{(4+)}$-OOH (lane 3) was added and cells were incubated for a further 5 min at 37 °C. The samples were used for electrophoresis and immunoblotting as described in the Materials and methods section. The immunoblots are representative of at least three similar experiments.

offered by some suppliers (e.g. Sigma) can be contaminated by SOD [25]. However, we obtained similar results using the more purified catalase from Boehringer Mannheim. Together, these observations provide indirect evidence which suggests that $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH are the active species promoting tyrosine phosphorylation when NADPH is added simultaneously with vanadate.

Further evidence to support this mechanism was obtained by generating $V^{(4+)}$-OO and $V^{(4+)}$-OOH *in vitro*, by a process independent of cellular oxidases and NADPH. Vanadate was incubated with equimolar concentrations of KO_2 or H_2O_2 to generate $V^{(4+)}$-OO or $V^{(4+)}$-OOH respectively. Excess superoxide and H_2O_2 were then eliminated by addition of SOD and catalase respectively. Permeabilized cells were incubated for 5 min with 100 μM of the vanadate derivatives. This value probably overestimates the actual concentrations of $V^{(4+)}$-OO and $V^{(4+)}$-OOH, as it assumes that the reactions proceeded to completion and that the products are stable. As shown in Fig. 2(*b*), extensive phosphotyrosine accumulation was detected in permeabilized cells treated with $V^{(4+)}$-OO and $V^{(4+)}$-OOH. The levels of phosphorylation obtained by treatment with $V^{(4+)}$-OO were comparable with those obtained in cells incubated with the equivalent concentration of vanadate and 2 mM-NADPH, and the levels attained in cells treated with $V^{(4+)}$-OOH were even higher. It is important to stress that $V^{(4+)}$-OO and $V^{(4+)}$-OOH induced phosphotyrosine accumulation in the absence of added NADPH, consistent with the notion that a metabolite of NADPH, and not the nucleotide itself, is required to promote phosphorylation. In addition, it is noteworthy that phosphorylation was observed in these experiments in the presence of SOD and catalase. This observation indicates that the enzymes do not directly interfere with the phosphorylation/dephosphorylation reactions, and suggests that their inhibitory action noted in Fig. 2(*a*) is due to a specific effect on reduced O_2 metabolites. Results similar to those obtained with KO_2 were also observed in neutrophils treated with vanadate preincubated with superoxide, generated by treating xanthine with xanthine oxidase (results not shown).

That NADPH activates phosphotyrosine accumulation through formation of $V^{(4+)}$-OO or $V^{(4+)}$-OOH assumes the formation of superoxide by the NADPH oxidase or a related enzyme. Two approaches were developed to test this hypothesis directly. First, we studied the consequences of inhibiting the oxidase on the phosphorylation triggered by vanadate and NADPH. For this purpose we utilized DPI, an inhibitor of the respiratory burst oxidase thought to interact with its flavoprotein component [27]. The potency of this compound as an inhibitor of the oxidative burst in HL60 cells is illustrated in Fig. 3(*a*). Addition of 1 μM-DPI following stimulation of the oxidative burst with 10 nM-PMA, an activator of protein kinase C, resulted in a drastic decrease in the rate of O_2 consumption. In three experiments the rate of O_2 consumption induced by PMA was determined to be 2.9 ± 0.17 nmol of O_2/min per 10^6 cells before and 0.17 ± 0.068 nmol of O_2/min per 10^6 cells after the addition of DPI. By inhibiting the generation of superoxide, DPI was therefore expected to inhibit $V^{(4+)}$-OO and $V^{(4+)}$-OOH formation and possibly also the accumulation of phosphotyrosine. As illustrated in Fig. 3(*c*), this prediction was fulfilled; in cells treated with DPI, only marginal levels of phosphotyrosine were detected in response to vanadate/NADPH (Fig. 3*c*).

A second line of evidence supporting the involvement of the NADPH oxidase in the formation of $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH and a role for the latter compounds in phosphorylation was obtained using undifferentiated HL60 cells. Unlike their dimethyl-sulphoxide-differentiated counterparts, undifferentiated HL60 cells are reported to possess only marginal NADPH oxidase activity [28]. The observation that 10 nM-PMA was unable to significantly affect the rate of O_2 utilization in these cells (Fig. 3*b*) is in agreement with these findings. The basal rate of O_2 consumption in unstimulated undifferentiated cells averaged 0.13 ± 0.02 nmol/min per 10^6 cells ($n = 3$). A significant fraction of the basal consumption was sensitive to addition of azide (Fig. 3*b*), and therefore probably reflects mitochondrial O_2 utilization. Oxygen consumption after addition of the phorbol ester averaged 0.19 ± 0.04 nmol/min per 10^6 cells ($n = 3$), which is statistically not significantly different from the basal rate. Because these cells show virtually no detectable NADPH oxidase activity, their capacity to support vanadate/NADPH-induced increases in protein phosphotyrosine was examined. Fig. 3(*d*) illustrates that treatment with 100 μM-vanadate and 2 mM-NADPH, concentrations which have been shown to elicit pronounced responses in both neutrophils and differentiated HL60 cells, failed to generate substantial phosphotyrosine accumulation in undifferentiated cells (Fig. 3*d*). It might be argued that the failure of these cells to elicit an increase in protein phosphotyrosine content is due to lack of active tyrosine kinases or of the appropriate phosphatases. That this is not the case, however, was demonstrated using $V^{(4+)}$-OO, generated exogenously by co-incubation of KO_2 and orthovanadate, as described above. As illustrated in Fig. 3(*d*), addition of $V^{(4+)}$-OO in the absence of NADPH yielded a marked accumulation of tyrosine-phosphorylated polypeptides in undifferentiated cells.

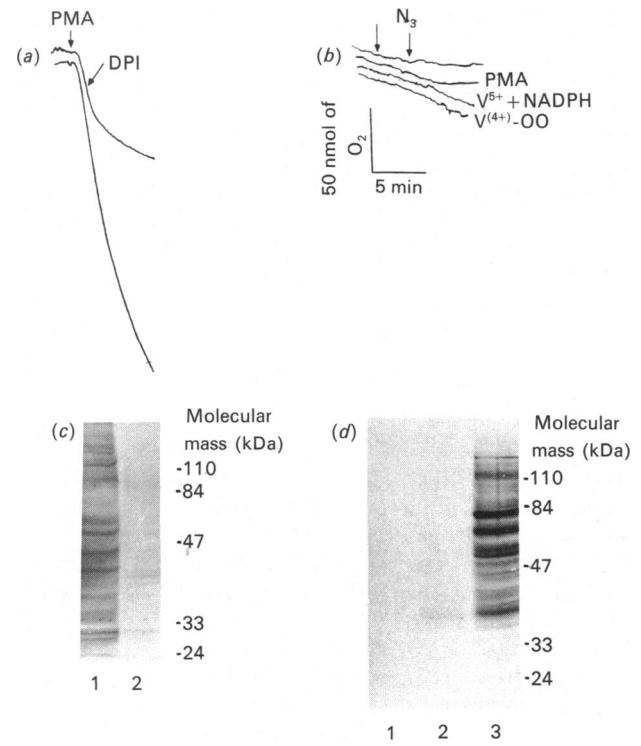

Fig. 3. Role of the NADPH oxidase in vanadate-induced phosphotyrosine accumulation

(*a*) Oxygen consumption measurements. Differentiated cells in permeabilization medium with 2 mM-NADPH were stimulated with 10 nM-PMA where indicated. DPI (1 μM) was added at the arrow to the top trace only. (*b*) Oxygen utilization measurements. Electroporated undifferentiated cells in permeabilization medium were treated with 100 μM-V^{5+}, 100 μM-$V^{(4+)}$-OO or 10 nM-PMA where indicated by the unlabelled arrow. NADPH (2 mM) was present in all traces. The top trace was treated with 2 mM-azide only, at the second (labelled) arrow. The time and O_2 consumption scale applies to both (*a*) and (*b*). (*c*) Anti-phosphotyrosine immunoblot of differentiated cells pretreated 1 min at 37 °C with (lane 2) or without (lane 1) 1 μM-DPI followed by stimulation with 2 mM-NADPH and 50 μM-vanadate as in Fig. 1. (*d*) Anti-phosphotyrosine immunoblot of undifferentiated cells treated for 2 min at 37 °C with (lanes 1 and 2) or without (lane 3) 2 mM-NADPH, followed by a 5 min incubation with 100 μM-V^{5+} (lane 2) or 100 μM-$V^{(4+)}$-OO (lane 3). Traces and immunoblots are representative of three experiments.

Together, the evidence obtained with cells treated with DPI and with undifferentiated cells is consistent with the notion that $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH are the species responsible for phosphotyrosine accumulation and that these species are generated by reduction of pentavalent vanadate by superoxide originating from the NADPH oxidase.

Mechanism of vanadate-stimulated O_2 consumption

We had previously demonstrated that micromolar concentrations of vanadate elicited a burst of O_2 utilization in permeabilized neutrophils and differentiated HL60 cells [16,17]. Because of its strict NADPH-dependence, this burst was attributed to the activation of the NADPH oxidase. However, perusal of the reaction sequence described by Fridovich and colleagues (reactions 1–3 above) raises another possibility. These authors have indicated that the $NAD(P)^{\cdot}$ generated by reaction (2) above can in turn reduce O_2, yielding superoxide and $NAD(P)^+$ (reaction 4):

$$NAD(P)^{\cdot} + O_2 < \text{-----} > NAD(P)^+ + O_2^{\cdot -} \qquad (4)$$

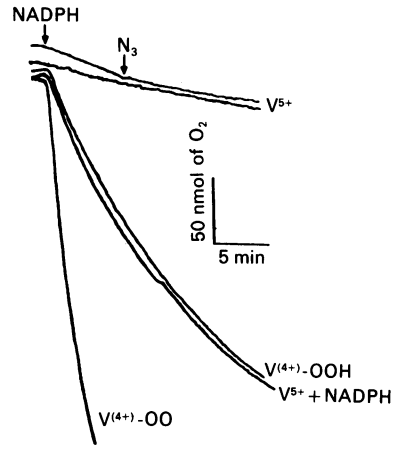

Fig. 4. Effects of vanadate derivatives on O_2 consumption

Electroporated cells in permeabilization medium were preincubated in the presence or absence of 2 mM-NADPH for 2 min at 37 °C and subsequently treated with 50 μM-V^{5+}, 50 μM-$V^{(4+)}$-OO or 50 μM-$V^{(4+)}$-OOH for a further 5 min. Samples were then sedimented, resuspended in fresh permeabilization medium and used for O_2 consumption measurements as described in the Materials and methods section. NADPH (2 mM) was added to all traces at the arrow. Azide (2 mM) was added to the top trace only, at the second arrow. The traces are representative of three similar experiments.

From the global analysis of reactions (1)–(4), it is apparent that a chain reaction can be initiated by vanadate and traces of cellular superoxide whereby, in the presence of NAD(P)H, O_2 is utilized to form H_2O_2. Thus it is conceivable that the measured burst in O_2 utilization is indicative of the occurrence of this chain reaction, without involvement of the cellular NADPH oxidase. Several experiments were undertaken to distinguish between these possibilities.

If O_2 is consumed primarily as a result of the vanadate-catalysed chain reaction, the removal of vanadate from the cell suspension should terminate the response instantly. Conversely, O_2 utilization resulting from activation of the NADPH oxidase in response to increased cellular levels of protein phosphotyrosine should persist after removal of vanadate, for the length of time required for tyrosine dephosphorylation and oxidase inactivation. To discern between these alternatives, permeabilized cells were pretreated with NADPH and vanadate for 2 min at 37 °C, and then sedimented and resuspended in medium free of vanadate for measurement of O_2 utilization (Fig. 4). Re-addition of NADPH to such cells resulted in a brisk elevation of the rate of O_2 consumption (trace labelled V^{5+} + NADPH in Fig. 4), despite the fact that vanadate was not present during the measurement. The persistent O_2 consumption does not result from trapping or incomplete washing of vanadate. This was demonstrated by pre-incubating cells in the presence of vanadate alone, without NADPH. As shown in Fig. 4, such pretreatment failed to induce O_2 utilization, despite re-addition of NADPH (trace labelled V^{5+}; the mean of three experiments was 0.14 ± 0.03 nmol of O_2/min per 10^6 cells, compared with a basal value of 0.12 ± 0.03 nmol of O_2/min per 10^6 cells). Thus a slowly reversible change was effected on the cells by the pre-incubation with vanadate and NADPH, consistent with activation of the cellular oxidase by phosphorylation.

Further evidence in favour of the latter mechanism was obtained by pretreating cells with $V^{(4+)}$-OO or $V^{(4+)}$-OOH in the absence of NADPH. Following this pretreatment, the cells were sedimented and resuspended in medium devoid of vanadium derivatives. Cells pretreated in this manner responded to the

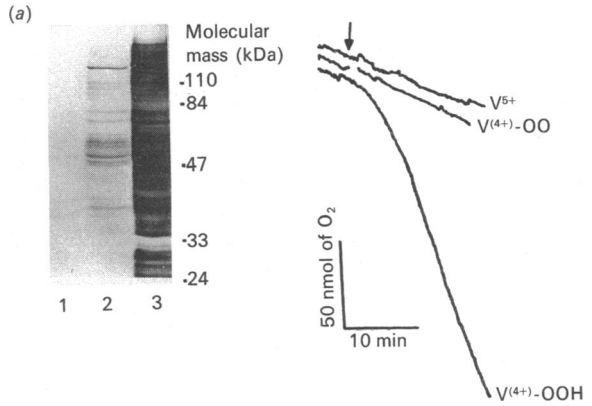

Fig. 5. Effect of peroxides of vanadate on intact cells

(*a*) Anti-phosphotyrosine immunoblot of intact cells treated for 12 min at 37 °C with 100 μM-V^{5+} (lane 1), -$V^{(4+)}$-OO (lane 2) or -$V^{(4+)}$-OOH (lane 3). (*b*) O_2 consumption measurements. Intact cells were treated with 100 μM-V^{5+}, -$V^{(4+)}$-OO or -$V^{(4+)}$-OOH as indicated. Note that the time scale for this Figure is more compact than those of previous Figures. The immunoblots and traces are representative of three similar experiments.

addition of NADPH with a vigorous respiratory burst [Fig. 4; traces labelled $V^{(4+)}$-OO and $V^{(4+)}$-OOH]. Pretreatment with $V^{(4+)}$-OO elicited a response comparable with that triggered by vanadate/NADPH (1.35 ± 0.068 and 1.36 ± 0.074 nmol of O_2/min per 10^6 cells respectively; $n = 3$). A considerably greater response was generated by $V^{(4+)}$-OOH pretreatment (3.31 ± 0.068 nmol of O_2/min per 10^6 cells; $n = 3$). As discussed below in more detail, the relative magnitude of the O_2 consumption responses produced by vanadate and its derivatives was proportional to the levels of tyrosine phosphorylation (cf. Fig. 2*b*), furthering the correlation between these events.

A requirement for the NADPH oxidase in the O_2 consumption response was also demonstrated using undifferentiated cells. As documented earlier, such cells lack significant oxidase activity. Accordingly, pretreatment with either vanadate/NADPH or $V^{(4+)}$-OO at concentrations shown to elicit responses in differentiated cells (Fig. 4) did not significantly increase the rate of O_2 consumption in undifferentiated cells (Fig. 3*b*). In three determinations the basal rate averaged 0.13 ± 0.02 nmol of O_2/min per 10^6 cells. Rates of 0.23 ± 0.19 and 0.33 ± 0.44 nmol of O_2/min per 10^6 cells were recorded for cells pretreated with vanadate/NADPH and $V^{(4+)}$-OO respectively.

Analysis of the end products of the reaction provides a further means of identifying the mechanism responsible for O_2 consumption in cells treated with vanadate derivatives. According to the reaction sequence (1)–(4), H_2O_2 is expected to accumulate, while superoxide will be formed only in catalytic amounts. In contrast, superoxide is the primary product of O_2 reduction by the NADPH oxidase. We therefore undertook measurements of superoxide formation as the SOD-sensitive reduction of cytochrome *c* [22]. Because the presence of the cytochrome had been earlier shown to preclude stimulation by vanadate (Fig. 2), it was necessary to treat permeabilized cells with vanadate or its derivatives before performing the superoxide assay. In three experiments, superoxide generation averaged 0.024 ± 0.006 nmol of $O_2^{\cdot-}$/min per 10^6 cells in untreated cells. After stimulation with 10 nM-PMA, a maximal rate of 2.06 ± 0.063 nmol of $O_2^{\cdot-}$/min per 10^6 cells was attained. By comparison, cells pretreated with vanadate/NADPH, $V^{(4+)}$-OO and $V^{(4+)}$-OOH displayed rates of 0.84 ± 0.22, 0.88 ± 0.15 and 2.14 ± 0.019 nmol of $O_2^{\cdot-}$/min per 10^6 cells respectively. Cells pretreated with vanadate alone (without NADPH), failed to produce significant amounts of superoxide upon addition of NADPH (0.018 ± 0.013 nmol of $O_2^{\cdot-}$/min per 10^6 cells). Thus the relative potency of the vanadate derivatives to elicit superoxide generation parallels that observed for O_2 consumption. Moreover, the absolute amount of superoxide generated is of the same order as the amount of O_2 consumed (approx. 1.5-fold lower), suggesting that a major fraction of O_2 utilized is converted to superoxide, probably by the NADPH oxidase.

Two further tests were undertaken to confirm that activation of the NADPH oxidase was indeed occurring in cells stimulated with vanadate derivatives. First we measured the effect of inhibition of the oxidase on the burst of O_2 utilization elicited by vanadate plus NADPH. The flavoprotein antagonist DPI (1 μM) was found to obliterate the O_2 consumption burst initiated by vanadate (50 μM) in the presence of NADPH. In three experiments the O_2 consumption rate averaged 1.34 ± 0.078 nmol of O_2/min per 10^6 cells and 0.25 ± 0.051 nmol of O_2/min per 10^6 cells in the absence and presence respectively of DPI. Similar results were obtained when superoxide generation was measured. In cells pretreated with 50 μM-vanadate, superoxide production averaged 0.91 ± 0.15 nmol of $O_2^{\cdot-}$/min per 10^6 cells. The rate was lowered to 0.065 ± 0.012 nmol of $O_2^{\cdot-}$/min per 10^6 cells after the addition of DPI. Because DPI is thought to act specifically on the oxidase, such findings strongly support the notion that vanadate-stimulated O_2 consumption is occurring largely via the NADPH oxidase.

If vanadate stimulates O_2 consumption by a mechanism independent of the NADPH oxidase, activation of the latter by the physiological route should produce a separate, additive, response. Conversely, if the stimulation produced by vanadate is due to activation of the physiological pathway, little additional effect is expected upon addition of other stimuli of the oxidase. The respiratory response elicited by 0.1 μM-PMA, a maximally stimulatory dose, was not significantly augmented by the subsequent addition of 50 μM-vanadate; in four experiments the O_2 consumption rate averaged 6.99 ± 1.27 nmol of O_2/min per 10^6 cells in the presence of PMA alone and 7.71 ± 1.46 nmol of O_2/min per 10^6 cells after the addition of vanadate. Likewise, 10 nM-PMA added to a cell suspension which had been maximally activated by 500 μM-vanadate did not further potentiate the response. The average rate of O_2 consumption in the presence of vanadate was determined to be 13.04 ± 1.91 nmol/min per 10^6 cells before and 13.84 ± 0.69 nmol/min per 10^6 cells after the addition of PMA. Clearly, the responses are not additive. It appears, therefore, that PMA and vanadate stimulate a common pathway of O_2 consumption. Since it has been well documented that PMA activates the NADPH oxidase [3,4], it is most likely that this is the mechanism by which O_2 is consumed in vanadate-activated cells.

It is clear from the data presented above that the O_2 consumption rate observed at 500 μM-vanadate was greater than the maximal rate elicited by PMA. This is due to the presence of an additional process of O_2 consumption, independent of the oxidase, which is in fact observed in the absence of cells and is particularly apparent at high vanadate concentrations [16]. This component is also NADPH-dependent and may indeed represent the chain reaction described by Liochev & Fridovich [25,26].

Effects of $V^{(4+)}$-OO and $V^{(4+)}$-OOH on intact cells

We had previously reported that vanadate can stimulate phosphorylation and O_2 consumption in permeabilized cells, but is relatively ineffective towards intact cells [16,17]. This precludes its use for the study of processes that require membrane integrity, such as the control of cytosolic Ca^{2+} or pH, or the transmembrane

potential. Because it became apparent that reduced vanadate metabolites are much more potent inducers of phosphotyrosine accumulation than vanadate itself, we decided to test whether responses could be elicited in intact cells by either $V^{(4+)}$-OO or $V^{(4+)}$-OOH. Typical results are illustrated in Fig. 5(*a*). As reported, vanadate itself (100 μM) had practically no effect on phosphotyrosine accumulation (Fig. 5*a*), nor did it significantly increase the rate of O_2 utilization. In three experiments, O_2 utilization averaged 0.13 ± 0.018 nmol of O_2/min per 10^6 cells in control and 0.14 ± 0.019 nmol of O_2/min per 10^6 cells in vanadate-treated cells. A comparable concentration of $V^{(4+)}$-OO only modestly enhanced phosphorylation and did not significantly stimulate O_2 utilization (0.13 ± 0.008 nmol/min per 10^6 cells; $n = 3$). $V^{(4+)}$-OOH, however, induced a substantial increase in phosphoprotein content and, after a lag of approx. 4 min, clearly stimulated O_2 utilization (Fig. 5*b*). In three experiments the maximal respiratory rate averaged 0.88 ± 0.042 nmol of O_2/min per 10^6 cells. Again, it is important to note the parallel behaviour of tyrosine phosphorylation and activation of the respiratory burst.

DISCUSSION

The experiments summarized in this report were undertaken to determine why the presence of NADPH is necessary for vanadate to enhance phosphotyrosine accumulation in granulocytic cells. Experiments using reduced glutathione or vanadyl sulphate indicated that direct reduction of the pentavalent vanadate by the nucleotide was not involved (Fig. 1*b*). We therefore considered the possibility that vanadate interacts instead with a product of the cellular metabolism of NADPH. NADPH is widely used by cells to drive reductive anabolic pathways, notably the synthesis of fatty acids and steroids, and can be used by mitochondria for respiratory metabolism, although with much less efficiency than NADH. However, granulocytes are not very active in the synthesis of fat or steroids and possess comparatively few mitochondria. In contrast, a pyridine nucleotide oxidase which utilizes NADPH more avidly than NADH is abundant in these cells [3]. This enzyme, which is virtually quiescent in resting granulocytes, is responsible for the respiratory burst when these cells are stimulated. The primary biochemical event during the respiratory burst is the univalent reduction of O_2 to superoxide, with the concomitant oxidation of NADPH. Superoxide, in the presence of NAD(P)H, can convert vanadate to both $V^{(4+)}$-OO and $V^{(4+)}$-OOH through a chain reaction that has been elegantly conceived and documented by Fridovich and collaborators [25,26]. Several lines of evidence strongly suggest that one or both of these peroxides of vanadate are responsible for the observed stimulation of tyrosine phosphorylation. First, in cells treated with vanadate and NADPH, phosphotyrosine accumulation was prevented by dismutation or re-oxidation of superoxide, or by removal of H_2O_2 with catalase (Fig. 2*a*), procedures that are expected to prevent the formation of $V^{(4+)}$-OO and $V^{(4+)}$-OOH. Second, the stimulatory effects of vanadate/NADPH on phosphorylation could be mimicked by addition of exogenously synthesized $V^{(4+)}$-OO or $V^{(4+)}$-OOH, in the absence of added NADPH (Fig. 2*b*). Third, inhibition of the cellular NADPH oxidase by DPI greatly suppressed the stimulatory effects of vanadate/NADPH (Fig. 3). Fourth, undifferentiated cells, which possess little NADPH oxidase activity, responded poorly to the addition of vanadate/NADPH, despite the presence of the required kinases and phosphatases, as shown by the direct addition of $V^{(4+)}$-OO (Fig. 3). The two latter findings not only indicate that superoxide is necessary for the induction of phosphorylation by vanadate, but additionally point to the NADPH oxidase as its primary source.

It is unclear whether $V^{(4+)}$-OO or $V^{(4+)}$-OOH is the species responsible for inducing phosphotyrosine accumulation. While $V^{(4+)}$-OO is generated by reaction of vanadate with superoxide (reaction 1), this species is rather unstable in aqueous buffers [25]. Moreover, superoxide itself is unstable in protic solvents, where it can be converted to H_2O_2 [25]. Thus, in those experiments where $V^{(4+)}$-OO was generated by addition of KO_2, some $V^{(4+)}$-OOH could conceivably have been formed. Because the latter species seems to be considerably more potent than $V^{(4+)}$-OO (e.g. Fig. 2), it may be solely responsible for the observed biological effects. However, dismissal of $V^{(4+)}$-OO as the biologically active species appears premature at present.

Although we have not directly investigated the mechanism of action of $V^{(4+)}$-OO and/or $V^{(4+)}$-OOH on phosphorylation, it is likely that they are involved primarily in the inhibition of phosphotyrosine phosphatase activity. This notion is supported by earlier studies where pulse–chase experiments demonstrated decreased rates of tyrosine dephosphorylation in cells treated with vanadate/NADPH [16]. In the same study, addition of vanadate plus NADPH was found to have no effect on the accumulation of thiophosphorylated proteins when ATP[S] replaced ATP [16]. Because the thiophosphate bond is hydrolysed very slowly, the rate of thiophosphoprotein formation is dictated largely by the activity of the kinases, with a minor contribution by the phosphatases. Thus the failure of vanadate/NADPH to affect thiophosphorylation was interpreted to indicate that the effect observed using ATP as the substrate is exerted mainly at the phosphatase level. This conclusion assumes that ATP[S] is a suitable substrate for tyrosine kinases.

A synergistic effect between vanadate and H_2O_2 has been described earlier in adipocytes and hepatoma cells [20,29,30]. Separately, each one of these agents has insulin-mimetic effects, including stimulation of tyrosine phosphorylation. When combined, however, the effects of vanadate and H_2O_2 are larger than predicted by the sum of their individual effects. This was attributed by Fantus and colleagues to the formation of 'pervanadate', which was proposed to activate the insulin receptor tyrosine kinase [20,29]. Because the receptor kinase is itself activated by tyrosine phosphorylation, inhibition of phosphatase activity can result in stabilization of the active form of the kinase. Hence the biological effects of the peroxides of vanadate may all be secondary to inhibition of phosphotyrosine phosphatase activity.

We had previously reported the stimulation of O_2 uptake in neutrophils and HL60 cells treated with vanadate in the presence of NADPH, an effect tentatively attributed to activation of the respiratory burst oxidase [16,17]. However, upon analysis of the chain reaction (1)–(4), it became apparent that O_2 could also be consumed by a cell-independent process, with production of H_2O_2. However, several lines of evidence support the original suggestion that most of the O_2 is utilized by the NADPH oxidase for the synthesis of superoxide, as follows. (*a*) In cells pretreated with vanadate/NADPH and then washed, O_2 consumption could be initiated by re-addition of NADPH in the absence of vanadate. (*b*) Upon addition of NADPH, substantial quantities of superoxide were generated in cells pretreated with vanadate/NADPH, $V^{(4+)}$-OO or $V^{(4+)}$-OOH. On a molar basis, superoxide formation accounted for approx. 67% of the O_2 consumed. The difference may be attributed to other O_2-consuming reactions, or may simply be due to conversion of superoxide to other species (e.g. H_2O_2 or hydroxyl radicals) not detected by the SOD-sensitive cytochrome *c* reduction assay. (*c*) The O_2 consumption burst elicited by vanadate/NADPH, as well as the concomitant surge of superoxide production, were terminated by addition of DPI, an inhibitor of the oxidase [27]. (*d*) Addition of vanadate/NADPH or $V^{(4+)}$-OO to undifferentiated cells failed to generate

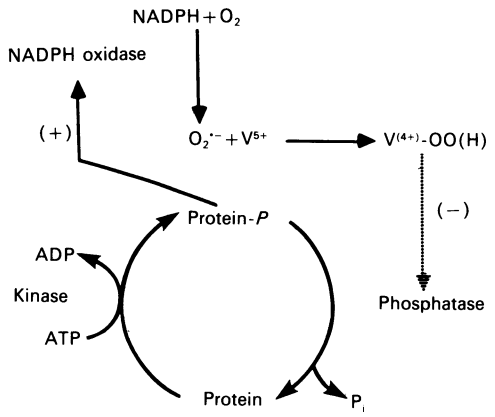

Fig. 6. Schematic representation of the events proposed to occur in cells activated with vanadate (V^{5+}) in the presence of NADPH

See the text for details.

an O_2 consumption burst, most likely because these cells lack a functional NADPH oxidase. (*e*) Finally, at maximally stimulatory concentrations, the effects of PMA and vanadate/NADPH on O_2 utilization were not additive, suggesting activation of a common pathway. Together, these observations implicate the NADPH oxidase as the main mechanism whereby O_2 is consumed in cells stimulated by vanadate derivatives.

In both neutrophils and HL60 cells, an excellent correlation seems to exist between the accumulation of tyrosine-phosphorylated proteins and the activation of the respiratory burst. Both phenomena require permeabilization of the plasma membrane, and show similar concentration-dependence and comparable initiation and termination kinetics [16]. The correlation is extended further by the present findings: the potency sequence for vanadate, $V^{(4+)}$-OO and $V^{(4+)}$-OOH was similar for phosphorylation and for the respiratory burst, and both effects were obtained in intact cells treated with $V^{(4+)}$-OOH. Together with the reported inhibitory effect of tyrosine kinase blockers on superoxide generation [14,15], these observations suggest that phosphorylation of critical proteins on tyrosine residues may suffice to activate the respiratory burst. The following sequence of events can then be envisaged to occur when cells are treated with vanadate (summarized in Fig. 6). Traces of superoxide will convert vanadate into $V^{(4+)}$-OO and, in the presence of NADPH, the latter will abstract hydrogen to become $V^{(4+)}$-OOH. One or both of the peroxides will then inhibit phosphatases and, due to constitutively active tyrosine kinases, protein phosphotyrosine accumulation will result. Phosphorylation of the protein(s) involved in activating the oxidase will promote a respiratory burst, with generation of superoxide. The superoxide created by the stimulated oxidase will in turn markedly increase the concentration of $V^{(4+)}$-OO and $V^{(4+)}$-OOH, greatly amplifying the phosphorylation response and consequently the magnitude of the respiratory burst.

The source of the superoxide required to initiate this cycle of events is not clear. Small amounts of superoxide can be produced by mitochondria or microsomal membranes, which may suffice to trigger the cycle shown in Fig. 6. Alternatively, if vanadate is reduced to vanadyl by NADPH or other cellular reductants, its spontaneous re-oxidation by O_2 can also generate superoxide. Formation of superoxide by re-oxidation of vanadyl with O_2 may also explain why, in the presence of NADPH, vanadyl was a somewhat more potent stimulus than vanadate itself (Fig. 1*b*). However, though sufficient to initiate the reaction, these sources cannot fully account for the phosphorylation effects observed, inasmuch as the response was absent in undifferentiated cells (Fig. 3). It appears, therefore, that full expression of the response requires the activity of the NADPH oxidase.

H_2O_2 has been shown to have insulin-mimetic as well as growth-stimulatory properties in a variety of cell types. In human neutrophils, H_2O_2 was recently reported to activate the respiratory burst [31], while inhibiting chemotaxis and phagocytosis [32]. This stimulation of the oxidase resembles the effects of $V^{(4+)}$-OOH reported here, raising the possibility that the latter compound could be generated by reaction of the added H_2O_2 with an endogenous form of vanadium, which is a ubiquitous natural constituent of most animal tissues [33]. Such a mechanism may be important in tissue damage and hypertrophy at inflammatory sites, where H_2O_2 is generated by phagocytes.

In summary, we have demonstrated that promotion of tyrosine phosphorylation and activation of the respiratory burst by vanadate depend on the presence of NADPH. This is most likely due to the requirement for the formation of superoxide, which in turn converts vanadate into $V^{(4+)}$-OO and $V^{(4+)}$-OOH, which appear to be the biologically active species. The active vanadium species are inhibitors of tyrosine phosphatase activity and probably activate the respiratory burst by inducing the accumulation of one or more phosphorylated proteins. $V^{(4+)}$-OO and $V^{(4+)}$-OOH, which can be generated *in vitro*, appear to be considerably more potent than vanadate itself as phosphotyrosine phosphatase inhibitors. If these derivatives prove to be more selective than vanadate, which can also inhibit E_1–E_2 type ATPases and activate GTP-binding proteins, among other effects, they may become useful probes for the study of phosphotyrosine metabolism.

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