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NEUTROPHIL "PRIMING" INDUCED BY ORTHOVANADATE: EVIDENCE OF A ROLE FOR TYROSINE PHOSPHORYLATION

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Abstract—The mechanism of neutrophil "priming" is unknown. In this study the level of tyrosine phosphorylation within intact neutrophils, using orthovanadate, have been manipulated. It has been demonstrated that this procedure both increased tyrosine phosphorylation of a number of protein substrates, including a prominent band at 74 kDa, and also primed the neutrophil oxidase response with a time and orthovanadate concentration-dependency, which were consistent with a role for tyrosine phosphorylation. No effect of orthovanadate on cytosolic-free Ca^{2+} concentration or actin polymerization was detected. Inhibition of tyrosine phosphorylation by genistein prevented "priming" by orthovanadate. This data thus provided evidence of a role for tyrosine phosphorylation in neutrophil "priming".

Key words: neutrophils; priming; tyrosine phosphorylation; signalling; signal transduction; tyrosine kinase

The ability of polymorphonuclear neutrophilic leukocytes (neutrophils) to protect against infecting micro-organisms is dependent on both triggering the response of the non-mitochondrial oxidase and on its "priming", whereby the response to stimulation is enhanced [1]. Although the signalling events which accompany direct activation of the neutrophil oxidase are becoming established [2–4], the intracellular events which accompany priming remain unclear. However, priming by low concentrations of f-met-leu-phe† and substance P, or TNF-α and GM–CSF is not accompanied by an elevation of cytosolic free Ca^{2+} [5–8], but is accompanied by tyrosine phosphorylation in neutrophils [9–12]. The possibility thus exists that tyrosine phosphorylation plays an important role in the priming mechanism [11]. In this paper the use of orthovanadate to elevate tyrosine phosphorylation levels [13] within neutrophils is reported, and it is demonstrated, for the first time, that there is a direct causal link with neutrophil priming. Evidence that the concentration and timing of the orthovanadate-induced "priming" is consistent with a role for tyrosine phosphorylation in neutrophil "priming" is presented, and it is shown that the effect was abolished by genistein, an inhibitor of tyrosine kinase activity.

MATERIALS AND METHODS

Chemicals. Sodium orthovanadate, -f-met-leu-phe, luminol and cytochalasin B were from Sigma (Poole, U.K.), peroxidase-conjugated sheep anti-mouse antibody, nitrocellulose immunoblotting membranes (Hybond C+) and ECL Western blotting detection system were from Amersham (Amersham, U.K.). All other chemicals were from Aldrich (Poole, U.K.).

Oxidase activation. Neutrophils (5×10^6 cells/mL) isolated from the peripheral blood of healthy volunteers, as previously described [14], were suspended in HEPES-buffered Krebs medium containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM HEPES and 0.1% bovine serum albumin (fraction V), pH 7.4. The viability of neutrophils was always greater than 95%. Luminol dependent chemiluminesence was measured as described previously [15].

Fluorescent labelling of neutrophils. The fluoresecent labelling of neutrophils with an FITC-conjugated anti-phosphotyrosine antibody (PT66) was carried out as previously described [16]. Neutrophils were allowed to adhere to glass slides and then stimulated with orthovanadate or a control for 10 min at 37° before fixation in 4% paraformaldhyde; permeabilization with 0.2% Tween 20 and staining with the FITC conjugated anti-phosphotyrosine antibody cells were observed using fluorescent microscopy.

Phospho-tyrosine detection. Laemmeli sample buffer (100 μL) [17] was added to approximately 5×10^6 cells, and boiled for 5 min. The sample proteins were then separated by 8% SDS–PAGE and electroblotted onto nitrocellulose [18]. Western blotting was performed as described previously [19], using mouse monoclonal anti-phosphotyrosine antibody (clone PT66; Sigma, Poole, U.K.). Immunoreactive bands were detected using Amer-

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† Abbreviations: -f-met-leu-phe, formylmethionyl-leucyl-phenylalanine; TNF-α, tumour necrosis factor-α; GM–CSF, granulocyte–monocyte colony stimulating factor; FITC, fluorescein-isothiocyanate, PLC-gamma, phospholipase C; IP_3, inositol trisphosphate; PLD, phospholipase D.

sham's ECL Western blotting detection system and quantified by scanning densitometry.

Measurement of cytosolic-free Ca^{2+}. Neutrophil cytosolic-free Ca^{2+} concentration was determined using fura-2 and dual wavelength fluorimetry [20]. Excitation at 340 and 380 nm and fluorescence emission at 505 nm was achieved and recorded using a Spex fluorolog III (Glen Spectra, Stanmore, U.K.).

RESULTS

Orthovanadate induced tyrosine phosphorylation in neutrophils

Incubation with orthovanadate (1 mM, 37°, 10 min) caused tyrosine phosphorylation of cytosolic proteins in neutrophils (Fig. 1a). The prominent tyrosine phosphorylated proteins had molecular

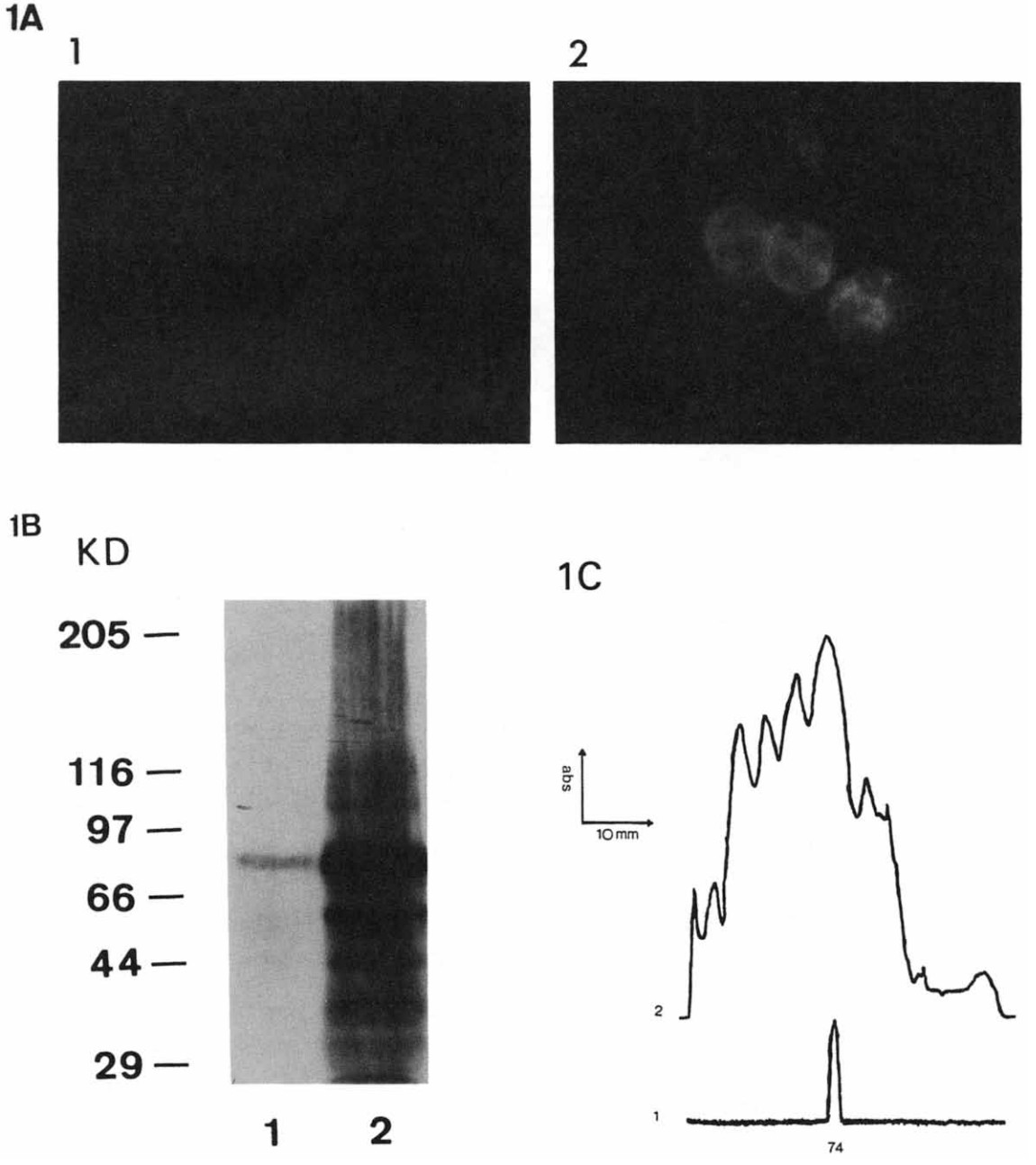
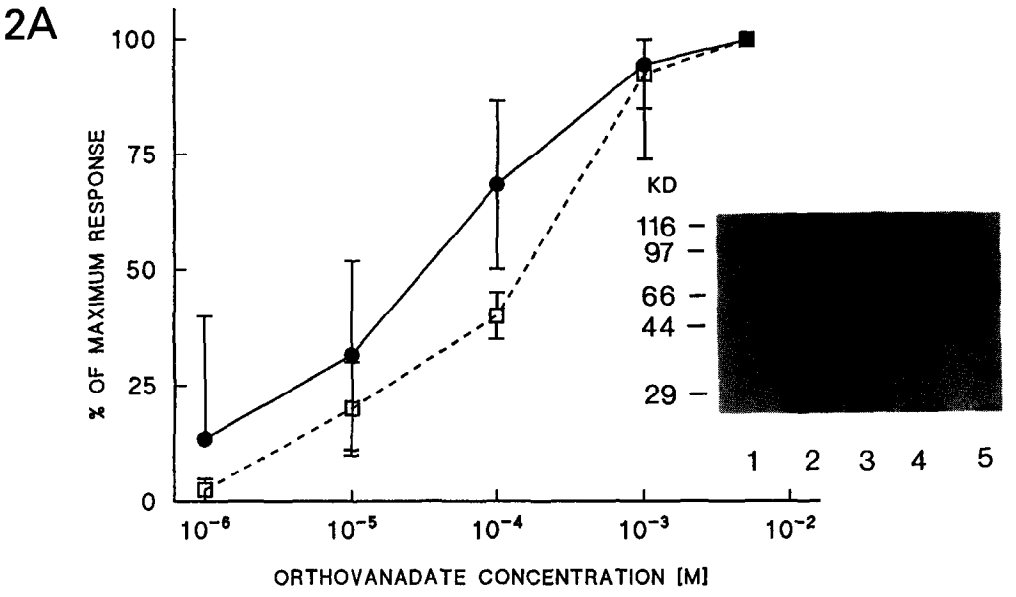


Fig. 1. Orthovanadate induced tyrosine phosphorylation in neutrophils. (A) Fluorescent labelling of phosphotyrosine in neutrophils with FITC conjugated anti-phosphotyrosine antibody (PT66). (B) A typical anti-phosphotyrosine immunoblot showing phosphotyrosine proteins after 10 min (37°) followed by -f-met-leu-phe (10 nM) for 1 min (1) and orthovanadate (1 mM) for 10 min (37°) followed by -f-met-leu-phe (10 nM) for 1 min (2). (C) Densitometry scans of the anti-phosphotyrosine immunoblot shown in (B), where 1 and 2 refer to lanes 1 and 2 in (B).



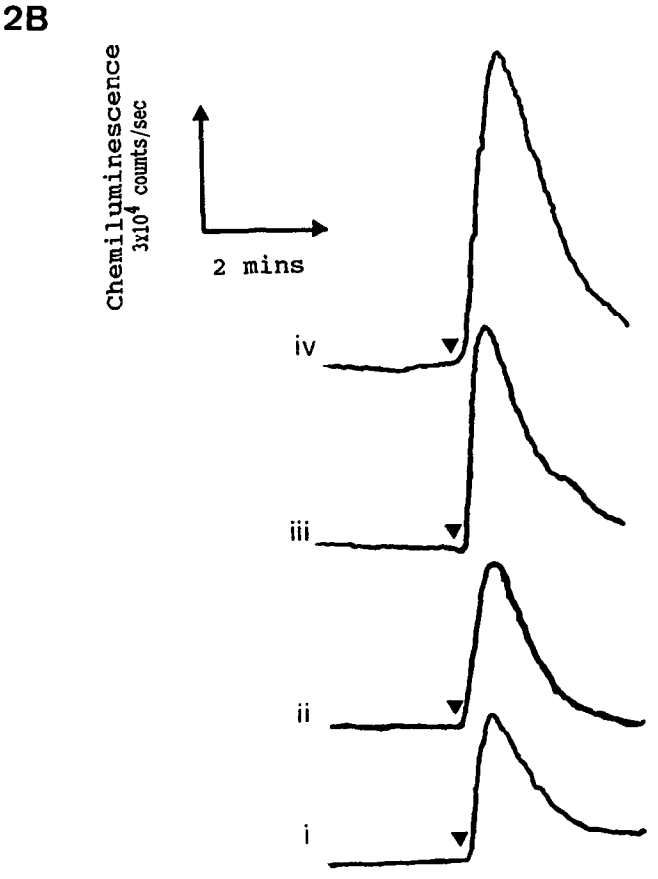
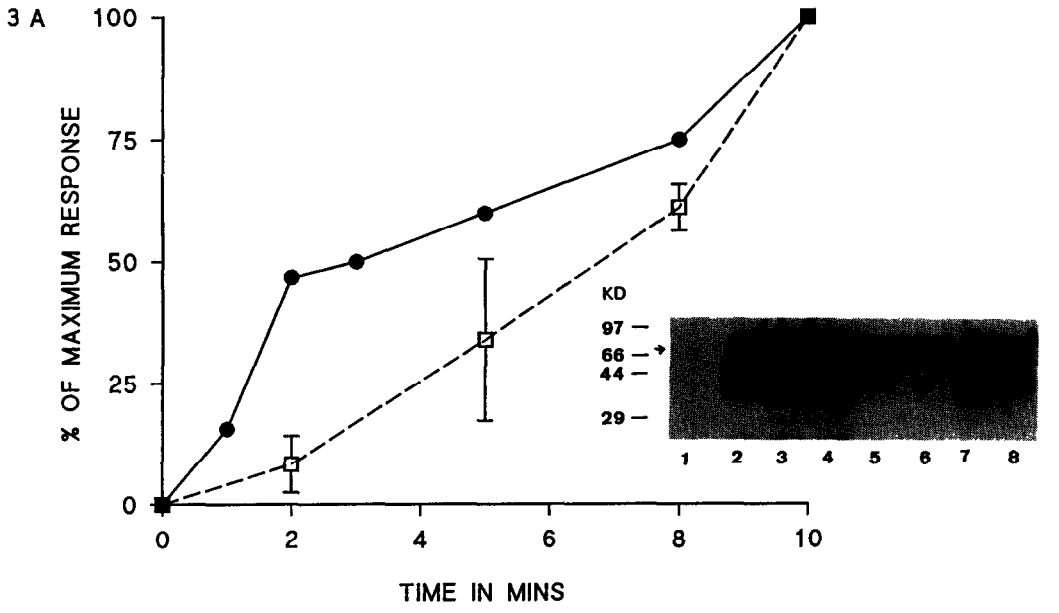


Fig. 2. Relationship of orthovanadate induced tyrosine phosphorylation and neutrophil priming. (A) Dose–response curves showing the effect of increasing orthovanadate concentration (10 min, 37°) on tyrosine phosphorylation of the 74 kDa protein (●) and neutrophil priming, calculated as the ratio of response from primed to unprimed cells (□). The bar shows the SEM for data from five experiments and three experiments for priming and tyrosine phosphorylation, the data is expressed as a percentage of the maximum response for comparison. The insert shows a typical tyrosine phosphorylation immunoblot; lane 1 is untreated cells, lanes 2–5 are 1 mM, 100, 10 and 1 μM orthovanadate. (B) Priming effect of orthovanadate on neutrophil luminol chemiluminesence, where i is the response to -f-met-leu-phe (10 nM) and ii–iv are the responses after pre-incubation with 1, 100 μM and 1 mM, orthovanadate (10 min, 37°), respectively.



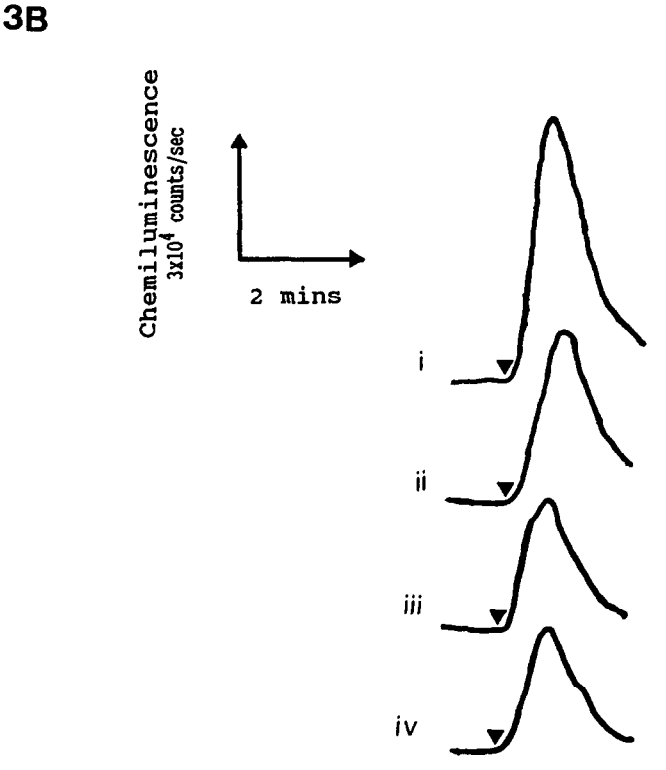


Fig. 3. Temporal relationship of orthovanadate induced tyrosine phosphorylation and neutrophil priming. (A) Time course of orthovanadate induced tyrosine phosphorylation of the 74 kDa protein (●) and priming (□). The bar shows the SEM for data from five experiments and three experiments for priming and tyrosine phosphorylation, the data is expressed as a percentage of the maximum response for comparison. The insert shows a typical immunoblot of tyrosine phosphorylation in response to orthovanadate (1 mM). Lane 1 is the control and lanes 2–8 pre-incubation, where the times were 15, 10, 8, 5, 3, 2 and 1 min, respectively. (B) The effect of increasing orthovanadate incubation time on luminol chemiluminescence in response to -f-met-leu-phe (10 nM). The traces show typical experiments where i–iv are 10, 8, 2, 0 min pre-incubation times with orthovanadate (1 mM, 37°), respectively.

masses of approximately 118, 112, 94, 74, 48 and 40 kDa (Fig. 1b), with the 74 kDa protein having the greatest fold increase in tyrosine phosphorylation. Maximal and half-maximal tyrosine phosphorylation of this protein occurred at 5 mM and 50 μM orthovanadate, respectively (Fig. 2a). Maximum tyrosine phosphorylation of the p74 protein was reached within 10 min (Fig. 3a).

Orthovanadate induced "priming" of neutrophils

Incubation of orthovanadate (0.01–5 mM, 10 min, 37°) did not activate the neutrophil oxidase directly. However, hypochlorite ion production in response to subsequent stimulation by f-met-leu-phe (10 nM) was increased by between 1.1- and 3.0-fold (Fig. 2b). The possibility of non-intentional priming during the experiment could not be excluded and may have contributed to the variation in priming between cell preparations. However, the concentration required for maximum and half-maximum priming were found to be consistently around 5 mM and 100 μM, respectively (Fig. 2a). The concentration relationship for priming was similar to that for tyrosine phosphorylation. The time required for priming by orthovanadate was also similar to that for tyrosine phosphorylation, the period of pre-incubation with orthovanadate required for maximum priming being approximately 10 min (Fig. 3b).

Mechanism of orthovanadate "priming"

Orthovanadate primed without modulation of the cytosolic-free Ca^{2+} concentration. At priming concentrations of orthovanadate (1 mM, 10 min, 37°) no change in cytosolic-free Ca^{2+} concentration was

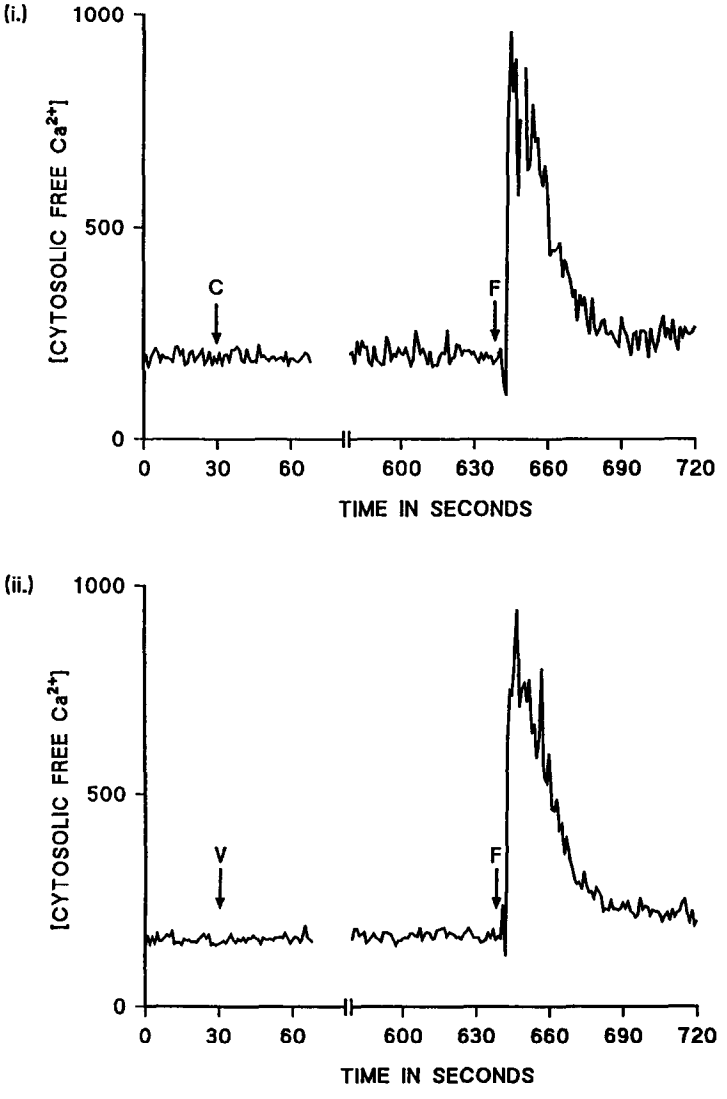


Fig. 4. The effect of orthovanadate on cytosolic free Ca^{2+}. Neutrophils were pre-incubated with either Krebs medium or with orthovanadate (1 mM) added at the arrows marked C and V, respectively. After 10 min incubation (37°) -f-met-leu-phe, (10 nM) was added at the arrows marked F. The traces show typical results.

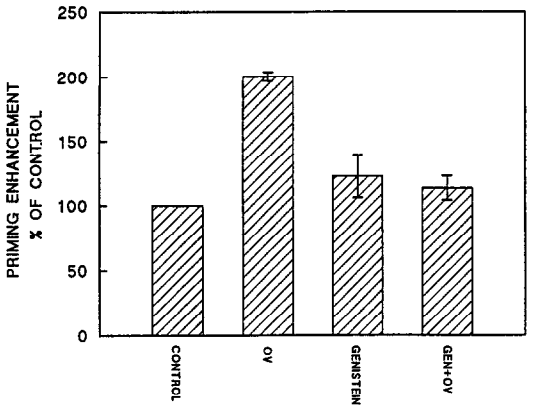


Fig. 5. The effect of the tyrosine kinase inhibitor genistein (6.25 μg/mL, 15 min, 37°) on the priming induced by orthovanadate. The response to -f-met-leu-phe (10 nM) after pre-incubation with orthovanadate, 1 mM, 10 min, 37° (OV), after genistein, 6.25 μg/mL, 15 min, 37° (GENISTEIN) and after orthovanadate plus genistein (GEN + OV) is shown. The bars show the range for data from at least two experiments, expressed as a percentage of the control response.

detected (Fig. 4). Furthermore, pre-incubation with priming levels of orthovanadate had no effect on the Ca^{2+} transient subsequently triggered by the peptide f-met-leu-phe (1 μM) (Fig. 4). The possibility that orthovanadate priming involved modulation of the cytoskeletal actin polymerization status was also discounted. Cytochalasin B, which prevented actin polymerization, failed to effect orthovanadate priming. Furthermore, priming concentrations of orthovanadate did not change the amount of actin in the Triton-X 100 insoluble cytoskeleton.

A role for tyrosine phosphorylation in orthovanadate-induced priming was, however, supported by the use of the tyrosine kinase inhibitor genistein. Tyrosine phosphorylation and orthovanadate-induced priming were both completely abolished by genistein (6.25 μg/mL, 10 min, 37°) without affecting cell viability (Fig. 5).

DISCUSSION

In this study it has demonstrated that orthovanadate, which causes tyrosine phosphorylation of a number of protein substrates, including a prominent band at 74 kDa, also primed the neutrophil oxidase response. The timing and orthovanadate concentration required for "priming" were correlated with phosphorylation of this 74 kDa protein. Furthermore, inhibition of tyrosine phosphorylation prevented "priming" by orthovanadate. These data are therefore consistent with a role for tyrosine phosphorylation of this protein in neutrophil priming.

In this study vanadate was used to induce tyrosine phosphorylation, although the mechanism by which this occurred is not entirely established. However, it may have occurred via the generation of peroxovandyl ions (V^{4+}-OO) and/or vanadyl hydroperoxide (V^{4+}-OOH) as a result of an interaction of orthovanadate with O_2^-, generated by basal NADPH oxidase activity [21–23]. These ions have been shown to inhibit protein tyrosine phosphatases [24] and also to oxidise critical sulphhydryl groups leading to the activation of some tyrosine kinases [25]. The increase in phosphotyrosine proteins after treatment with orthovanadate may have resulted from either a combination of protein tyrosine kinase activation and/or inhibition of phospho-tyrosine phosphatases.

The data presented here implicate the phosphorylation of a number of protein substrates, especially a 74 kDa protein. Tyrosine phosphorylation of a similarly sized protein (71–76 kDa) accompanied priming of the neutrophil oxidase in response to TNF-α, GM–CSF [11] and substance P [10], and also the activation of macrophages by zymozan [26] and aggregation of platelets [27]. This may suggest a central role for this protein, although its identity and mechanism has yet to be established. Although activation of PLC-gamma by tyrosine phosphorylation has been demonstrated [28, 29], it is unlikely to be playing a role here. No evidence was found for an alteration in cytosolic-free Ca^{2+} handling. Thus, indicating that PLC-gamma activity was not increased sufficiently for IP_3 generation to release Ca^{2+} from stores within neutrophils. It has also been reported that PLD activity is also modulated by tyrosine phosphorylation [30] and that, in HL60 cells and neutrophils, peroxyvanadate activates PLD [30–32]. Increased PLD activity could produce "priming" by the production of phosphatidic acid and or diacylglycerol, which may act synergistically with a subsequent Ca^{2+} signal for activation of protein kinase C [32] or by direct modulation of the NADPH oxidase [33, 34].

Although the mechanism awaits full elucidation, the results presented here strongly implicate a role for tyrosine phosphorylation in the mechanism of "priming" of the neutrophil oxidase response.

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