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Pharmacological comparison of LTB_4-induced NADPH oxidase activation in adherent and non-adherent guinea-pig eosinophils

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1 Leukotriene B_4 (LTB_4) stimulation of guinea-pig peritoneal eosinophils, induced a biphasic activation of the NADPH oxidase composed of a rapid (<3 min) phase mediated by non-adherent cells and a sustained (3–120 min) phase mediated by CD11b/CD18 adherent eosinophils. Studies were undertaken to compare the intracellular mechanism that mediate these responses.

2 SB 203580 and PP1, inhibitors of p38 mitogen-activated protein (MAP) kinase and the src-family protein tyrosine kinases, respectively caused concentration-dependent attenuation of both the rapid (SB203580: $pD_2 = -6.31$; PP1: $pD_2 = -5.50$) and sustained (SB203580: $pD_2 = -6.50$; PP1: $pD_2 = -5.73$) phases. Similarly, the MAP kinase kinase-1 inhibitor, PD098059 produced partial inhibition of the both phases of superoxide generation.

3 The protein kinase C (PKC) inhibitors Ro-31 8220, GF 109203X and Gö 6976 attenuated the rapid NADPH oxidase response (pD_2s $= -6.10, -6.72, -6.15$ respectively) and, to a lesser extent, (pD_2s $= -5.54, -6.02, -6.51$ respectively) the sustained phase.

4 An inhibitor of phosphatidylinositol 3-kinase (PtdIns 3-kinase), wortmannin caused concentration dependent attenuation of the sustained ($pD_2 = -8.68$) but not rapid phase of superoxide generation. In contrast, the syk kinase inhibitor, piceatannol abolished the rapid ($pD_2 = -6.43$) but not sustained respiratory responses.

5 This study demonstrates that LTB_4-induced superoxide generation from adherent and non-adherent eosinophils is mediated via both common (p38 MAP kinase, MEK-1, PKC and the src kinases) and divergent intracellular pathways (syk kinases and PtdIns 3-kinase). This suggests the possibility of therapeutic intervention to selective attenuate activation of adherent tissue eosinophils. *British Journal of Pharmacology* (2001) **134,** 797–806

Keywords: Eosinophils; signalling; CD11b/CD18 adhesion; NADPH oxidase activation; MAP kinases; protein kinase C; phosphatidylinositol 3-kinase; src kinases; syk kinase

Abbreviations: LTB_4, leukotriene B_4; MAP kinase, mitogen-activated protein kinase; MEK-1, MAP kinase kinase-1; PKC, protein kinase C; PtdIns 3-kinase, phosphatidylinositol 3-kinase; VLA4, very late antigen-4

Introduction

Although eosinophils were thought to be primarily involved in immune defence against parasitic infection it is now recognized that they are important in the inflammation associated with a number of allergic and non-allergic diseases such as atopic dermatitis, asthma, rhinitis and Crohn's disease. The inflammation associated with these disorders is mediated following activation of tissue eosinophils which secrete a host of cytotoxic species including granule proteins and the activation of the NADPH oxidase complex (Giembycz & Lindsay, 1999). The NADPH oxidase (E.C. 1.23.45.3) catalyzes the single electron reduction of molecular O_2 to superoxide (O_2^-), a powerful oxidizing and reducing agent (Babior *et al*., 1973). In the presence of superoxide dismutase, O_2^- dismutes to hydrogen peroxide (H_2O_2) which can be subsequently converted into range of highly toxic free radicals (Lindsay & Giembycz, 1997).

Human and guinea-pig eosinophils in suspension (non-adherent) undergo a rapid and transient activation of the NADPH oxidase to a range of physiological soluble and particulate stimuli including leukotriene B_4 (LTB_4), platelet activating factor (PAF), fMLP, complement factor 5a, interleukin-8, eotaxin and opsonized particles (Lindsay & Giembycz, 1997). Furthermore, pre-incubation with sub-threshold concentrations of PAF has been demonstrated to prime the subsequent NADPH oxidase response to opsonized particles (Tool *et al*., 1992) and fMLP (Zoratti *et al*., 1992). More recent studies have demonstrated NADPH oxidase activation following human eosinophils adhesion to tissue culture plates coated with a range of extracellular matrix proteins (e.g. fibronectin, fibrinogen, collagen, laminin) and bovine serum albumin (BSA) (Dri *et al*., 1991; Horie & Kita, 1994; Lynch *et al*., 1999). Under these conditions, binding and activation of the β_2-integrin, CD11b/CD18 causes a slowly developing and sustained generation of oxidants (Laudanna *et al*., 1993; Horie & Kita, 1994; Nagata *et al*., 1995; Lynch *et al*., 1999).

Presently, little is known of the intracellular pathways that underlie the activation of the NADPH oxidase in eosinophils whilst the majority of these studies have been performed in 'suspended' cells (Giembycz & Lindsay, 1999). Studies using

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adherent eosinophils are complicated by the need to differentiate between the pathways that increase receptor-ligand binding ('inside-out' signalling) and those that mediate receptor-ligand-induced responses ('outside-in' signalling) such as cell spreading, and subsequent degranulation and activation of the NADPH oxidase (Nathan, 1987).

In previous studies of the intracellular mechanisms that mediate LTB_4-induced NADPH oxidase activation, we have shown that this is partially mediated by lyn kinase, PKC and PLA_2 but occurs essentially independently of changes in the intracellular [Ca^{2+}], phospholipase D, PtdIns 3-kinase and ERK1/2 (Perkins *et al*., 1995; Lindsay *et al*., 1998a,b; Lynch *et al*., 2000). However, these studies were performed upon non-adherent guinea-pig eosinophils in which LTB_4 produces a rapid and transient (<3 min) oxidant generation which is probably unrepresentative of the *in vivo* environment where eosinophils would be adherent to other cells and the extracellular matrix. In this study, we have therefore employed pharmacological inhibitors to compare the mechanism of LTB_4-induced NADPH oxidase activation in adherent and non-adherent guinea-pig eosinophils.

Methods

Induction, harvesting and purification of guinea-pig peritoneal eosinophils

Eosinophils were elicited into the peritoneum of male Dunkin-Hartley guinea-pigs (~1 kg) as previously described (Lynch *et al*., 2000). Following harvesting and purification, cells were pooled, washed twice and resuspended in HEPES buffer (HBSS + 10 mM HEPES, pH 7.4 + 0.1% BSA (fatty acid free)).

Measurement of superoxide anion generation and adhesion

Eosinophils (5×10^6 ml^{-1}) were incubated with 10 μM Calecein-AM in HEPES buffer for 30 min at 37°C, washed three times and resuspended in the same buffer at 5×10^6 ml^{-1}. Aliquots (20 μl) of the cell suspension then were incubated in HEPES buffer (+1 mM $CaCl_2$/1 mM $MgCl_2$), supplemented with 25 μM lucigenin and the relevant inhibitor/antibody in a total volume of 200 μl and seeded onto 96-well tisue culture plates coated with either BSA (0.1% w v^{-1}) or FCS. Superoxide anion generation was monitored using superoxide dismutase inhibitable lucigenin-enhanced chemiluminescence (Gyllenhammar, 1987) with a plate reading luminometer (Lucy II, Labtech Ltd., Uckfield, U.K.) and determined from the peak response (0–2 min for rapid phase; 10–60 min for sustained phase). Comparison of the rate of superoxide production generated by xanthine/xanthine oxidase using chemiluminescence and ferricytochrome *c* reduction showed that a RLU was equivalent to production of approximately 0.8 nmol superoxide min^{-1} 10^6 cells^{-1}. At the appropriate time points, the number of adherent cells was determined by measuring the fluorescence of cellular Calecein-AM. Briefly, total fluorescence was measured at the outset of the experiment (reading 1) and then at pre-determined time intervals (see text; reading 2) using a Biolite F1 plate reader ($\lambda_{excitation} = 485 \pm 20$ nm; $\lambda_{emission} = 530 \pm 25$ nm). Reading 2 was taken after non-adherent cells had been removed from the culture plates by gently washing in HEPES buffer and the percentage of adherent eosinophils then was calculated by multiplying the ratio of fluorescence (reading 2/reading 1) by 100. Studies of the magnitude of the respiratory burst conducted in the presence and absence of eosinophils loaded with Calcein-AM showed that this fluorescent indicator had no adverse effects upon oxidant production or viability (data not shown).

Drugs and analytical reagents

PD 098059, SB 203580, piceatannol, wortmannin, Ro-31 8220, GF109203X and Gö 6976 were obtained from Calbiochem (Nottingham). Flat clear-bottomed, white-walled 96-well tissue culture-treated plates, Ficoll-Paque and Calecein AM were purchased from Costar Ltd (Buckinghamshire, U.K.), Pharmacia (Uppsala, Sweden) and Molecular Probes (Eugene, OR, U.S.A.), respectively. Blocking antibodies (6.5E, KIM255, MAX68P, MOPC21) to adhesion receptors and the inhibitor of the src-family of protein tyrosine kinases, PP1 (CP 118556), were kindly donated by

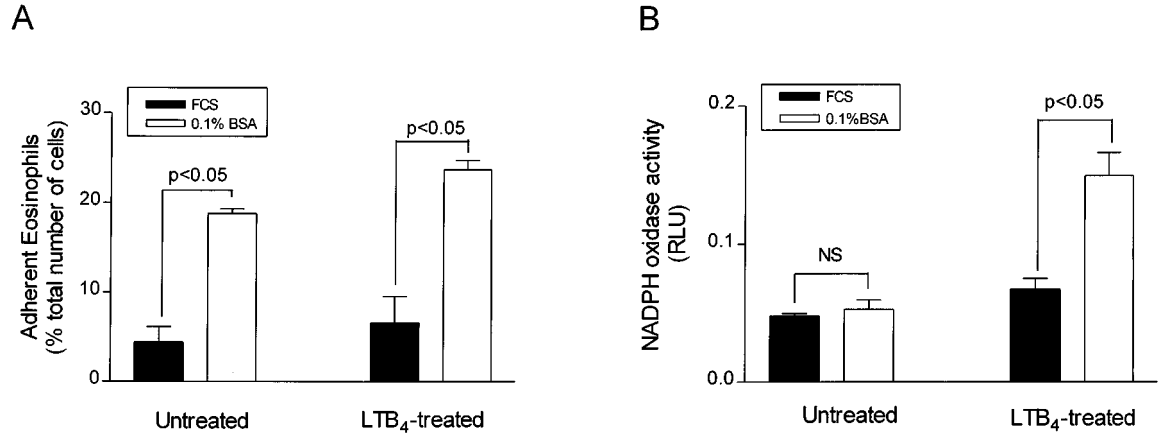

Figure 1 Effect of LTB_4 on guinea-pig eosinophil adhesion and superoxide anion generation. Eosinophils were incubated upon 96-well plates coated with either BSA (0.1%) or fetal calf serum (FCS) in the absence or presence of LTB_4 (100 nM) at 37°C for 60 min. Maximum adhesion (percentage of the total number of cells, A) and the peak of NADPH oxidase activity (B) were determined. The results are the mean ± s.e.mean of five independent experiments.

Celltech Ltd (Slough, U.K.) and Pfizer (Groton, Connecticut, U.S.A.) respectively. All other reagents were purchased from Sigma (Poole, Dorset, U.K.).

Statistical analysis

Data points and values in the text and figure legends represent the mean ± s.e.mean of '*n*' independent determinations taken from different cell preparations. Concentration-response curves were analysed by least-squares non-linear iterative regression with the 'PRISM' curve fitting program (GraphPad software, CA, U.S.A.) and pD_2 values were subsequently interpolated from curves of best-fit. Where appropriate, data were analysed non-parametrically using the Wilcoxon matched pairs test. The null hypothesis was rejected when $P<0.05$. The data presented within the graphs has been normalized where maximal adhesion and superoxide release is 100%.

Results

Development of a model of LTB_4-induced NADPH oxidase activation in adherent and non-adherent guinea-pig eosinophils

Incubation of guinea-pig eosinophils for 60 min upon 96-well tissue culture plates coated with 0.1% (w v^{-1}) BSA resulted in spontaneous (18 ± 1%: $P<0.05$) adhesion when compared to cells seeded onto plates treated with FCS (4 ± 2%) (Figure 1A). Interestingly, non-stimulated cells generated a very low concentration of superoxide anions (~0.05RLU) upon both FCS and BSA surfaces, suggesting that adhesion *per se* was unable to activate the NADPH oxidase (Figure 1B). However, prior adhesion was required for LTB_4 (100 nM)-induced oxidant production since this was observed only from cells seeded on BSA-coated plates (Figure 1B).

The time-course of LTB_4-induced NADPH oxidase activation (by lucigenin-enhanced chemiluminescence) was biphasic, composed of a rapid phase, which peaked at approximately 45 s before dropping to baseline at 120 s, and a sustained phase, which was maximal at approximately 30 min (Figure 2A). Examination of the sustained oxidant generation showed that this correlated with spontaneous adhesion suggesting that a relationship may exist between these responses (Figure 2B). To test this hypothesis and to determine the mechanism of eosinophil adhesion, we examined the effect of blocking antibodies to the β_1-integrin (CD29), VLA_4 (CD29/CD49d) and the β_2-integrin (CD18), Mac-1 (CD11b/CD18) upon adhesion and NADPH oxidase activation (Figure 3). Incubation with blocking antibodies to CD18 (6.5E) and CD11b (KIM 225) suppressed adhesion, by 43 ± 3% (*$P<0.05$) and 18 ± 12% (*$P<0.05$) and oxidant generation, by 57 ± 2% (*$P<0.05$) and 57 ± 22% (*$P<0.05$), respectively (Figure 3). The combination of 6.5E and KIM225 also significantly enhanced the attenuation of superoxide release

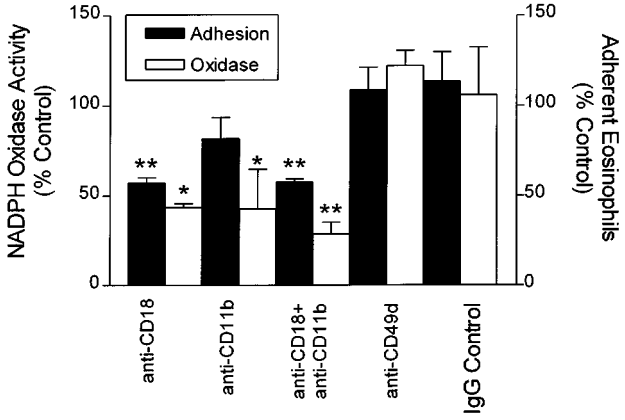

Figure 3 Effect of integrin blocking antibodies upon LTB_4-induced eosinophil adhesion and sustained oxidase activation in guinea-pig eosinophils. Following pre-incubation for 5 min at 37°C with blocking antibodies (10 $\mu g\ ml^{-1}$) to CD18 (6.5E), CD11b (KIM225), CD49d (Max68P) or an isotype-matched control antibody (MOPC21), eosinophils were stimulated with LTB_4 (100 nM) and incubated for 60 min (sustained phase) upon 96-well plates coated with BSA (0.1%). Eosinophil adhesion at 60 min and the peak of NADPH oxidase activity were monitored and expressed as a percentage of control (untreated) cells. The results are the mean ± s.e.mean of five independent experiments using cells from different guinea-pigs. ** $P<0.01$ and * $P<0.05$; significant inhibition relative to control.

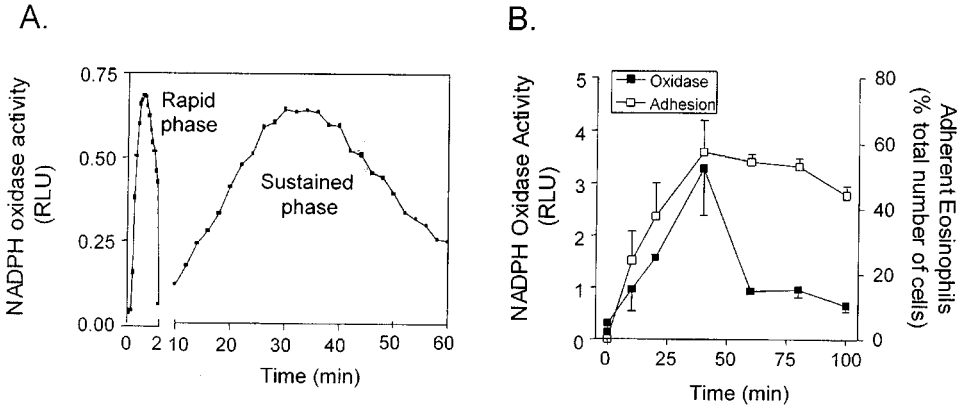

Figure 2 Time-course of eosinophil adhesion and NADPH oxidase activation from LTB_4-activated guinea-pig eosinophils. LTB_4-stimulated cells were incubated upon 96-well plates coated with BSA (0.1%) and the time-course of adhesion (□) (percentage of total number of cells) and NADPH oxidase (■) (RLU) monitored at 37°C over a period of 60–100 min. The results are the mean ± s.e.mean of five independent experiments using cells from different guinea-pigs.

($72\pm7\%$: $^{**}P<0.01$) but not eosinophil adhesion ($42\pm2\%$). In contrast, blocking antibodies to CD49d (MAX68P) and a non-specific, isotype-matched control antibody (MOPC21) had no significant affect upon either adhesion or respiratory burst. These results, therefore, suggested that spontaneous eosinophil adhesion to BSA-coated plates is mediated via the β_2 integrin, CD11b/CD18 but not the β_1 integrin, VLA-4 and that adhesion is required for the sustained phase of LTB_4-induced NADPH oxidase activation.

Comparison of the mechanism of NADPH oxidase activation in adherent and non-adherent eosinophils

The initial studies suggest that the rapid phase of LTB_4-induced respiratory burst is mediated by non-adherent eosinophils whilst the dependence of the sustained phase of oxidant production upon CD11b/CD18-mediated adhesion implies that this is due to adherent cells. A pharmacological approach was therefore adopted to examine and compare the mechanism of LTB_4-induced NADPH oxidase activation in adherent- and non-adherent eosinophils by examination of the action of various inhibitors upon the rapid and sustained phases of LTB_4-induced oxidant generation, respectively.

Role of mitogen-activated protein kinases

To examine the role of MEK-1 and p38 MAP kinase, the selective inhibitors PD098059 and SB203580, respectively, were employed. Figure 4(A) illustrates that PD098059 produced a partial but significant inhibition of both the rapid and sustained phases, this being maximal at 3 μM (rapid: $56\pm3\%$, sustained: $48\pm10\%$) (Figure 4(B)). Similarly, SB203580 produced a near identical concentration-dependent attenuation of oxidant production of both the rapid and sustained phase with maximal inhibition of $\sim70\%$ (at 10 μM) and pD_2 values of -6.31 ± 0.1 and -6.5 ± 0.4, respectively (Figure 4(C,D)). Neither PD098059 nor SB203580 suppressed CD11b/CD18-dependent adhesion.

Role of phosphatidylinositol 3-kinase

Wortmannin, a potent inhibitor of PtdIns 3-kinase (Arcaro & Wymann, 1993) had no effect upon the rapid phase of oxidant production or adhesion (Figure 5(A,B)). However, a role for PtdIns 3-kinase in the mechanism of oxidant release from adherent cells was suggested following the demonstration of potent concentration-dependent attenuation of the sustained response (pD_2 of -8.68 ± 0.14; Figure (5B)).

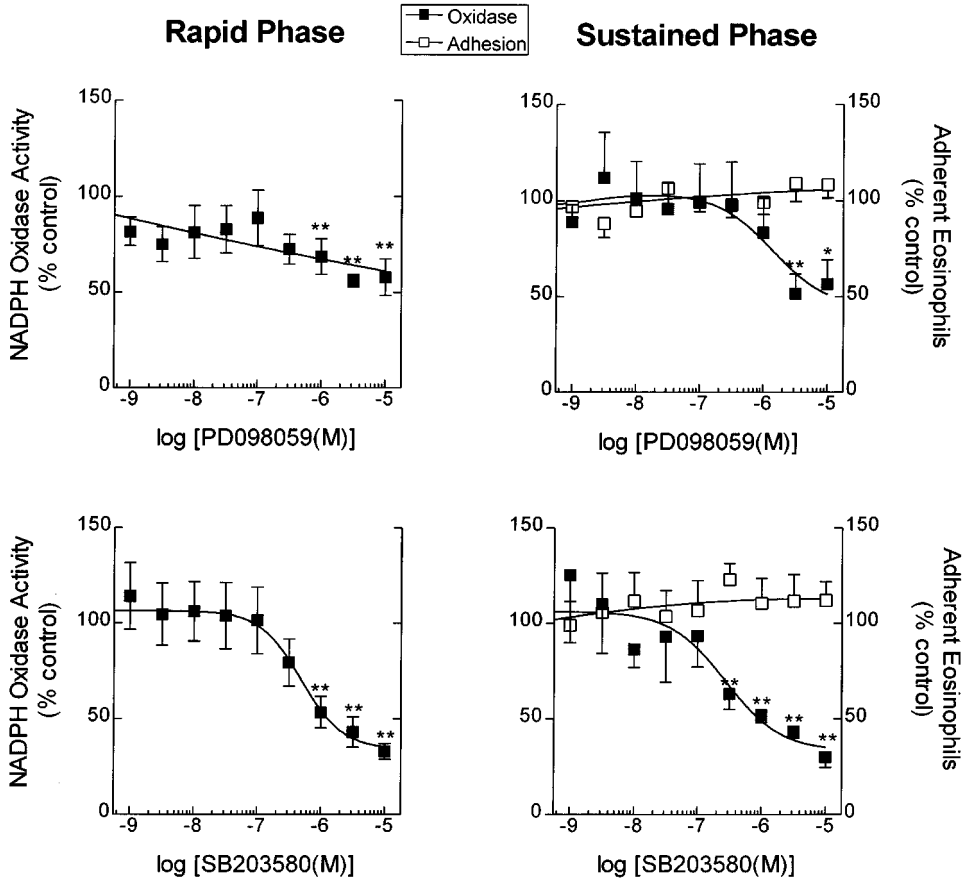

Figure 4 Effect of PD098059 and SB203580 upon rapid and sustained NADPH oxidase activation, and adhesion. Following pre-incubation for 5 min at 37°C with buffer or the indicated concentration of inhibitor, cells were incubated upon 96-well plates coated with BSA (0.1%). Following stimulation with LTB_4 (100 nM), NADPH oxidase activity and adhesion were measured for either 2 min (rapid phase) or 60 min (sustained phase). Eosinophil adhesion and the peak of NADPH oxidase activity was expressed as percentage of control (untreated) cells. The results are the mean$\pm$s.e.mean of five independent experiments using different donors. $^{*}P<0.05$; ** $P<0.01$; significant inhibition of LTB_4-induced response by the indicated concentration of inhibitor compared to controls.

Role of src and syk cytosolic protein tyrosine kinases

PP1 (Hanke *et al*., 1996) and piceatannol (Geahlen & Mclaughlin, 1989) were employed to determine the role of the src and syk family of protein tyrosine kinases in NADPH oxidase activation. PP1 (10 nM – 100 μM) was found to produce comparable concentration-dependent inhibition of both the rapid ($pD_2 = -5.5 \pm 0.37$) and sustained ($pD_2 = -5.73 \pm 0.12$) phase of oxidant production (Figure 6(A,B)). In contrast, piceatannol selectively inhibited the rapid but not the sustained phase of LTB_4-induced respiratory burst with a pD_2 of -6.43 ± 0.09 (Figure 6(C,D)). Neither PP1 nor piceatannol affected eosinophil adhesion. These results indicate that both src and syk tyrosine kinases are involved in oxidase activation from non-adherent cells whilst only the src kinases appear to be of importance in adherent cells.

Role of protein kinase C

In an effort to identify the role of PKC isoenzymes in the mechanism of oxidant generation, the action of the inhibitors Ro 31-8220, GF 109203X and Gö 6976 were examined. Ro-31 8220 (10 nM – 100 μM), a broad spectrum inhibitor (Davis *et al*., 1989), suppressed both the rapid and sustained phase of oxidase activity in a concentration dependent manner with pD_2's of -6.1 ± 0.45 and -5.54 ± 0.44, respectively (Figure 7(A,B)). However, although this compound caused complete attenuation of superoxide production from non-adherent cells at 10 μM, it produced only partial inhibition ($77 \pm 8\%$) of the sustained response at the highest concentration employed (100 μM). Similar results were found using GF 109203X (10 nM – 100 μM), an inhibitor of both the conventional cPKC and nPKC isoforms (Martiny-Baron *et al*., 1993). Thus, although GF 109203X caused comparable attenuation of the rapid ($pD_2 = -6.724 \pm 0.25$) and sustained ($pD_2 = -6.02 \pm 0.17$) phases respectively, the latter response was inhibited by $58 \pm 14\%$ at concentrations (10 μM) that totally inhibited the rapid phase (Figure 7(C,D)). Interestingly, Gö 6976 (Martiny-Baron *et al*., 1993), which is reported to selectively inhibit cPKC isoforms, caused comparable suppression of the two phases with pD_2 values of -6.15 ± 0.16 (rapid) and -6.51 ± 0.22 (sustained) (Figure 7(E,F)). However, this compound failed to completely attenuate oxidant release at the highest concentrations employed (10 μM) giving inhibition of $76 \pm 5.8\%$ and $58 \pm 13.3\%$ versus the rapid and sustained responses respectively.

All three compounds had no inhibitory effect upon adhesion. On the contrary, a partial increase was observed with all three inhibitors with maximum adhesion obtained at micromolar concentrations (Ro 31-8220: $153 \pm 36\%$ at 30 μM; GF 109203X: $183 \pm 50\%$ at 30 μM; Gö 6976: $145 \pm 40\%$ at 10 μM) indicating that PKC may be involved in a negative feedback mechanism which regulates CD11b/CD18 avidity (Figure 7(B,D,F)).

Discussion

Previous results suggest that LTB_4 can evoke a rapid, transient and concentration-dependent generation of H_2O_2 from eosinophils in suspension (Perkins *et al*., 1995; Lindsay *et al*., 1998a,b; Lynch *et al*., 2000). Using guinea-pig eosinophils incubated upon 96-well plates coated with BSA, near identical rapid superoxide release, as measured by lucigenin-enhanced chemiluminescence was observed. In addition, these cells produced a sustained respiratory burst with a time-course that paralleled CD11b/CD18-mediated NADPH oxidase activation (*via* 'outside-in' signalling) in human eosinophil (Lynch *et al*., 1999). Subsequent studies showed that, like human eosinophils, unstimulated guinea-pig eosinophils adhered spontaneously *via* a mechanism that was at least partially dependent upon binding through CD11b/CD18. However, in contrast to studies with human cells (Lynch *et al*., 1999), spontaneous adhesion *per se* failed to

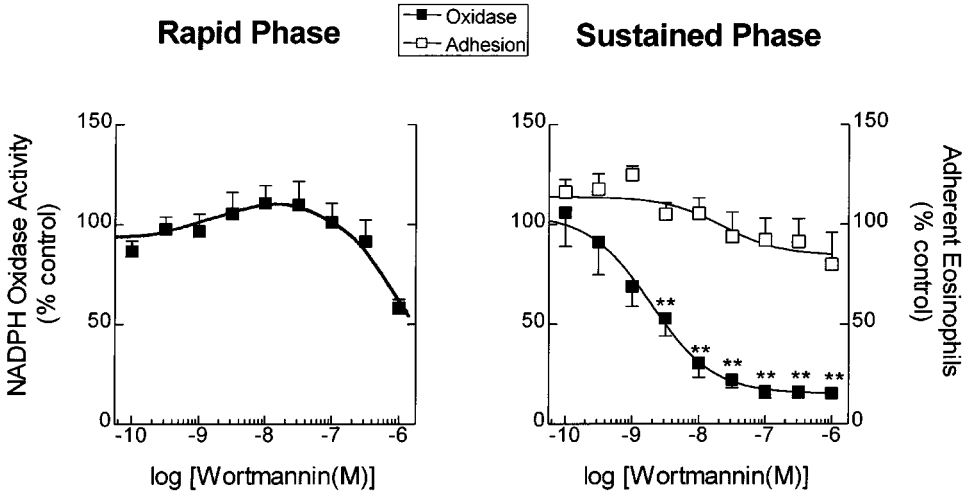

Figure 5 Effect of wortmannin upon rapid and sustained NADPH oxidase activation, and adhesion. Cells were pre-incubated upon 96-well plates coated with BSA (0.1%) for 5 min at 37°C with buffer or the indicated concentration of inhibitor. Following stimulation with LTB_4 (100 nM), NADPH oxidase activity and adhesion were measured for either 2 min (rapid phase) or 60 min (sustained phase). Eosinophil adhesion and the peak of NADPH oxidase activity was expressed as percentage of control (untreated) cells. The results are the mean ± s.e.mean of five independent experiments using different donors. ** $P < 0.01$; significant inhibition of LTB_4-induced response by the indicated concentration of inhibitor compared to controls.

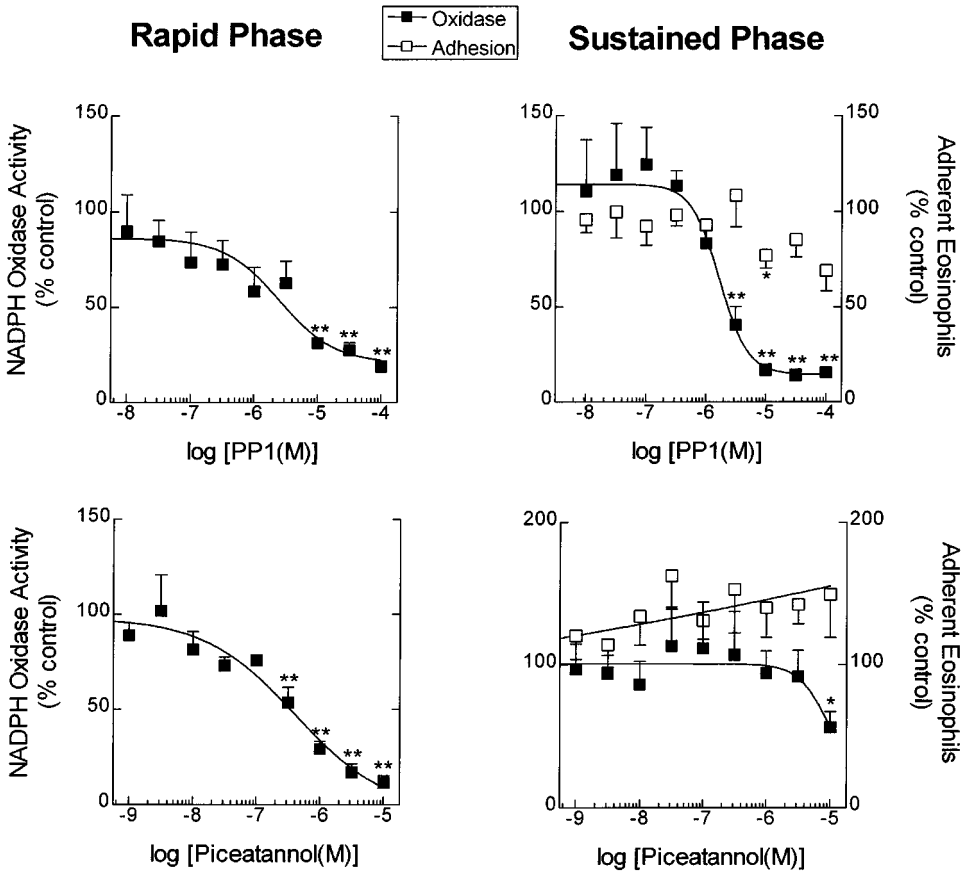

Figure 6 Effect of PP1 and piceatannol upon rapid and sustained NADPH oxidase activation, and adhesion. Cells were pre-incubated upon 96-well plates coated with BSA (0.1%) for 5 min at 37°C with buffer or the indicated concentration of inhibitor. Following stimulation with LTB_4 (100 nM), NADPH oxidase activity and adhesion was measured for either 2 min (rapid phase) or 60 min (sustained phase). Eosinophil adhesion and the peak of NADPH oxidase activity was expressed as percentage of control (untreated) cells. The results are the mean ± s.e.mean of five independent experiments using different donors. * $P<0.05$; ** $P<0.01$; significant inhibition of LTB_4-induced response by the indicated concentration of inhibitor compared to controls.

induce NADPH oxidase activation. Although these observations could have resulted from differences in the species (guinea-pig vs human), it is also likely that the source of the cells (peritoneum vs circulating) may have profound effects upon their responsiveness. Thus, it appears that in addition to adhesion, the tissue guinea-pig derived peritoneum eosinophils require a second stimuli (in this case LTB_4) to produce a sustained oxidant response. Physiologically, this may prevent inappropriate activation of tissue eosinophils except in the presence of inflammatory stimuli. Alternative, it might be envisaged that the LTB_4-induced response results from its priming of the adherence induced NADPH oxidase response. However, this priming might also be expected to increase adhesion, which is not observed.

Overall, it therefore appears that exposure of guinea-pig eosinophils to LTB_4 induces a biphasic activation of the NADPH oxidase composed of a rapid release of O_2^- which correlates with that observed previously in non-adherent cells, and a sustained phase of O_2^- generation which is dependent upon CD11b/CD18-mediated eosinophil adhesion. In subsequent studies, an attempt was made to examine, pharmacologically, the pathway(s) that mediates NADPH oxidase activation. These demonstrated a role for p38 MAP kinase, MEK-1, src kinases and PKC in the respiratory burst evoked in both cellular responses. In contrast, sustained oxidant production is selectively attenuated by PtdIns 3-kinase inhibitors, whilst the rapid response is dependent upon syk. In addition, a putative role for PKC isoforms in negative regulation of CD11b/CD18-mediated adhesion was suggested.

The selective p38 MAP kinase inhibitor, SB203580 attenuation the eosinophil response over a concentration range similar to that observed for a variety of p38 MAP kinase driven responses including CD11b/CD18-dependent adhesion and adhesion-dependent oxidative burst in response to lipopolysaccharide and tumour necrosis factor-α (Detmers *et al.*, 1998), and fMLP-induced NADPH oxidase activation in non-adherent human neutrophils. Although a role in the sustained phase is supported from investigation of CD11b/CD18-dependent oxidant generation in adherent human eosinophils (Lynch *et al.*, 1999) an involvement in the rapid phase of the LTB_4-induced response was surprising since we had previous shown that this agonist does not activate p38 MAP kinase (Lindsay *et al.*, 1998a). However, Lali *et al.* (2000) have recently reported that SB203580 can inhibit phosphoinositide-dependent protein kinase 1, the proximal regulator of protein kinase B/c-Akt (PKB/Akt) (Lali *et al.*, 2000). Since Coffer *et al.* (1998) have reported activation of PKB/Akt in eosinophils stimulated with C5a, PAF and

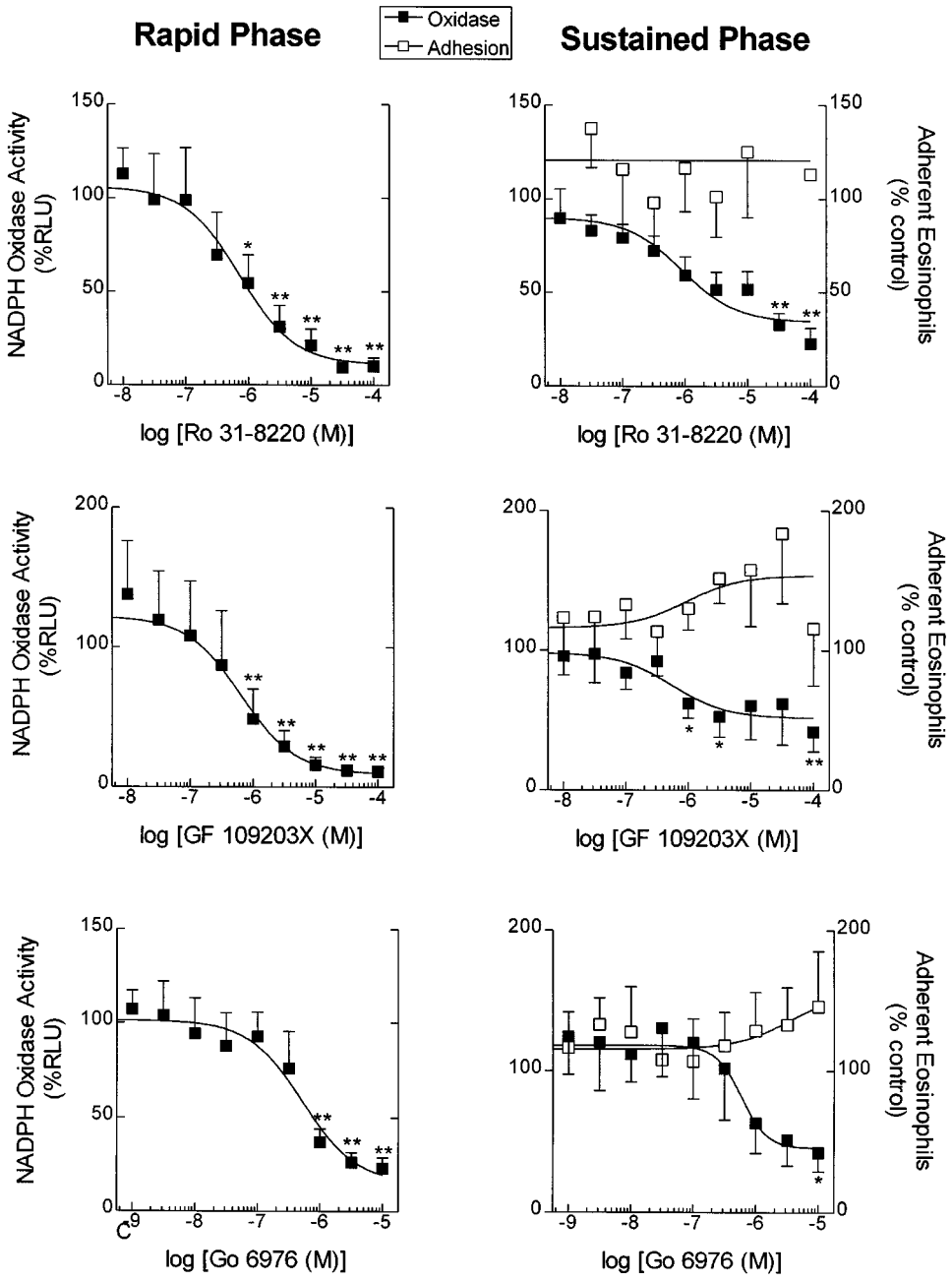

Figure 7 Effect of Ro 31-8220, GF 109203X and Gö 6976 upon rapid and sustained NADPH oxidase activation, and adhesion. Cells were pre-incubated upon 96-well plates coated with BSA (0.1%) for 5 min at 37°C with buffer or the indicated concentration of inhibitor. Following stimulation with LTB_4 (100 nM), NADPH oxidase activity and adhesion were measured for either 2 min (rapid phase) or 60 min (sustained phase). Eosinophil adhesion and the peak of NADPH oxidase activity were expressed as percentage of control (untreated) cells. The results are the mean ± s.e.mean of five independent experiments using different donors. $^*P<0.05$; $^{**}P<0.01$; significant inhibition of LTB_4-induced response by the indicated concentration of inhibitor compared to controls.

RANTES, it could therefore be envisioned that SB203580 might be acting upon this pathway (Bracke *et al*., 1998).

Unlike previous studies, we showed that a MEK-1 inhibitor caused a partial but significant inhibition of the rapid phase of oxidase activation in non-adherent guinea-pig eosinophils (Lindsay *et al*., 1998b) this conclusion being supported from investigation of the mechanism of fMLP induced respiratory burst in non-adherent neutrophils (Rane *et al*., 1997; Downey *et al*., 1998). Furthermore, in contrast to adherent human eosinophils (Lynch *et al*., 1999), PD098059 significantly attenuated the sustained phase of oxidant activity in guinea-pig eosinophils.

The identification of a role for syk tyrosine kinase in NADPH oxidase activation from non-adherent cells is supported from studies of Fcγ receptor crosslinking in IFN-γ-differentiated U937 cell, mouse bone marrow-derived neutrophils and macrophages as well as human neutrophils (Durden & Liu, 1994; Kiefer *et al*., 1998; Lofgren *et al*.,

1999). In contrast to the rapid response, the syk inhibitor produced no significant attenuation of the sustained phase. Although this is in agreement with the studies of CD11b/CD18-mediated oxidase activation in human eosinophils (Lynch *et al*., 1999), it is a surprising result given that syk kinase has been linked with several signalling pathways that are thought to be modulated by integrins including the src family kinases, lyn and fgr (Yan *et al*., 1997; Miller *et al*., 1999), MAP kinases (Raeder *et al*., 1999) and PtdIns 3-kinase (Beitz *et al*., 1999; Craxton *et al*., 1999).

In earlier studies, we identified the expression of the conventional isoforms, α-, β_I- and β_{II}-, the novel isoforms, δ and ε, and the atypical isoforms, ι-, μ-, ζ- in human eosinophils (Evans *et al*., 1999). Subsequent studies implicated a role for the novel isoforms in the mechanism of CD11b/CD18-mediated NADPH oxidase activation (Lynch *et al*., 1999) and PKCζ during the priming of circulating eosinophils following the late response to allergen challenge (Evans *et al*., 1999). Although, we have identified a role for PKC during oxidant production from both adherent and non-adherent eosinophils, we were unable to identify the individual family members involved since all isoform selective inhibitors produced concentration dependent attenuation of the NADPH oxidase activation.

Consistent with earlier studies linking the respiratory burst and adhesion in eosinophils (Lynch *et al*., 1999) and neutrophils (Arcaro & Wymann, 1993; Vlahos *et al*., 1995; Metzner *et al*., 1997), PtdIns 3-kinase was implicated in the sustained but not the rapid phase of NADPH oxidase activation. Although the mechanism is uncertain, products of PtdIns 3-kinase-induced phosphorylation, such as PtdIns(3,4,5)P_3, have been suggested to mediate cytoskeletal changes such as Rac-mediated membrane 'ruffling' or activation of FAK leading to alteration in cell morphology and cytoskeletal architecture following exposure to growth factors or phorbol esters (Chen & Guan, 1994). Other kinases such as members of the PKC family (Nakanishi *et al*., 1993; Toker *et al*., 1994; Vlahos *et al*., 1995; Bracke *et al*., 1998) and tyrosine kinases such as syk and lyn are thought to be downstream of PtdIns 3-kinase activation, (Ptasznik *et al*., 1996; Beitz *et al*., 1999; Craxton *et al*., 1999) and may indirectly regulate both NADPH oxidase and integrin binding. A report by Burgering & Coffer (1995) described a pleckstrein homology (PH) domain containing PKB/Akt that is thought to be downstream of PtdIns 3-kinase. Indeed, constitutive activation of PtdIns 3-kinase in the monoblastic cell line GM-1, was found to constitutively activate PKB and cause phosphorylation of $p47^{phox}$, the cytosolic component of the NADPH oxidase (Didichenko *et al*., 1996).

Overall, it appears that the action of these inhibitors was not directly associated with integrin binding since they were unable to block adhesion. Indeed, the enhancement of CD11b/CD18-mediated adhesion to BSA-coated plates by all three PKC inhibitors showed that PKC isoforms were important in negatively regulating the adhesive response. GF 109203X was particularly effective thus implicating a role for both conventional and novel isoforms of PKC. Previous studies have demonstrated similar effects of PKC on other functions such as Ca^{2+} transients, homotypic aggregation, oxidative burst and eicosanoid production in guinea-pig and human-eosinophils (Perkins *et al*., 1995; Teixeira *et al*., 1997; Dent *et al*., 1998).

In summary, the results presented herein illustrate that LTB_4-stimulated NADPH oxidase activation is biphasic resulting in an initial rapid phase of O_2^- generation followed by a slow sustained release, which is dependent on the β_2 integrin, CD11b/CD18. The inability of CD11b/CD18 adhesion alone to induce NADPH oxidase activation (*via* outside-in signalling) implies that this pathway is uncoupled and requires the recruitment of additional signalling pathways either by priming or direct stimulation, to produce sustained oxidant generation. Since wortmannin selectively attenuated the NADPH oxidase response in adherent cells, it suggests that activation of PtdIns 3-kinase may be central to the mechanism of re-coupling this response. Furthermore, these observation imply that it might be possible to develop therapeutic interventions to selectively inhibit the activation of adherent tissue eosinophils.

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