Signal Transduction and Activation of the NADPH Oxidase in Eosinophils

Mark A Lindsay+, Mark A Giembycz

Thoracic Medicine, Imperial College School of Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK

Activation of the eosinophil NADPH oxidase and the subsequent release of toxic oxygen radicals has been implicated in the mechanism of parasite killing and inflammation. At present, little is known of the signal transduction pathway that govern agonist-induced activation of the respiratory burst and is the subject of this review. In particular, we focus on the ability of leukotrine B_4 to activate the NADPH oxidase in guinea-pig peritoneal eosinophils which can be obtained in sufficient number and purity for detailed biochemical experiments to be performed.

Key words: leukotriene B_4 - eosinophil - NADPH oxidase - signal transduction

The NADPH oxidase (E.C. 1.23.45.3) catalyses the single electron reduction of molecular O_2 to superoxide (O_2^-), a powerful oxidising and reducing agent (Fig. 1) (Babior et al. 1973). In the presence of superoxide dismutase, O_2^- dismutates to hydrogen peroxide (H_2O_2) which can be subsequently converted into hypobromous acid in the presence of eosinophil peroxidase (a highly basic protein stored within specific eosinophil granules) and bromide (Weiss et al. 1986) (Fig. 1). Alternatively, in the presence of ferrous ions, O_2^- and H_2O_2 interact to form the membrane-perturbing hydroxyl radical ($OH^{\cdot}$), one of the most unstable oxidising species known (Fig. 1). Other pathways of free radical formation have also been described including the reaction of O_2^- with nitric oxide to form peroxynitrite which provides an additional, iron-independent route of $OH^{\cdot}$ formation together with nitrogen dioxide radicals (Fig. 1). Hypobromous acid is able to interact with H_2O_2 to form singlet oxygen, the biological significance of which is currently unclear (Fig. 1). Activation of the NADPH oxidase and the subsequent production of toxic oxygen radicals is thought to be important to the role of eosinophils during host defence (Butterworth & Thorne 1993). However, it is now appreciated that NADPH oxidase activation maybe cytotoxic to many mammalian cells, particular those of the gut, skin and lung, a finding that has implicated eosinophils in the pathogenesis of a number of non-parasitic inflammatory disorders, including Crohn's disease, atopic dermatitis and allergic asthma (Butterfield & Leiferman 1993). Indeed, the activity of the NADPH oxidase is significantly higher in eosinophils that in other phagocytes (Yamashita et al. 1985, Petreccia et al. 1987, Sedgwick et al. 1988, Yagisawa et al. 1996).

At present, little is known of the intracellular mechanisms responsible for NADPH oxidase activation in eosinophils. This is in contrast to neutrophils, where studies of the mechanism of O_2^- release by the chemotactic peptide, formyl-methyl-leucyl-phenylalanine (fMLP) have suggested the participation of phospholipase A_2- (PLA_2), phospholipase C- (PLC), phospholipase D- (PLD) protein kinase C- (PKC), phosphatidylinositol 3-kinnase- (PI-3K) and tyrosine kinase-dependent pathways (possibly those leading to mitogen activated protein kinase stimulation) (Bokoch 1995). This lack of knowledge relates primarily to the difficulty in obtaining sufficient numbers of cells, particular human eosinophils. Thus, we and others have overcome this problem by using guinea-pig eosinophils as a model system, which can be harvested from the peritoneum in sufficient numbers for detailed biochemical studies.

Human and guinea-pig eosinophils 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) (Palmbald et al. 1984, Maghni et al. 1991, Rabe et al. 1992, Subramanian et al. 1992, Perkins et al. 1995), platelet activating factor (PAF) (Shute et al. 1990, Wymann et al. 1995), fMLP (Palmblad et al. 1984, Kroegal et al. 1990, Wymann et al. 1995), complement factor 5a (C5a)(Wymann et al. 1995), interleukin-8 (IL-8) (Wymann et al. 1995), eotaxin (Elsner et al. 1996, Tenscher et al. 1996) and opsonized particles (Koenderman et al. 1990, Shute et al. 1990). Furthermore, pre-incubation

+Corresponding author. Fax: +44-171-351. 5675. E-mail: m.lindsay@ic.ac.uk
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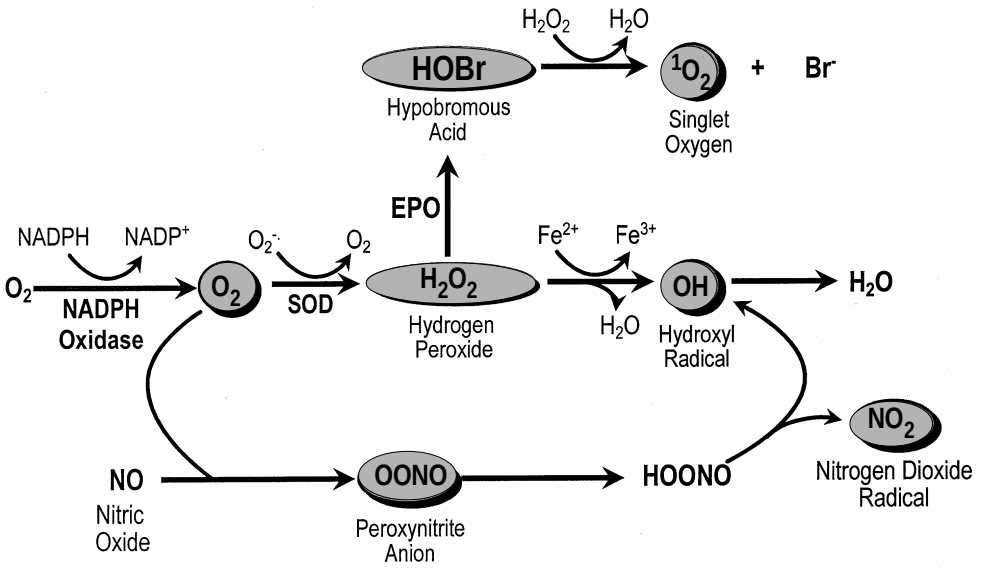

Fig. 1: generation of reactive oxygen species in eosinophils.

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 a similar priming in human eosinophils adherent to tissue culture plates coated with a range of extracellular matrix proteins (e.g. fibronectin, fibrinogen, collagen, laminin) and fetal calf serum. Under these conditions, the cytokines tumor necrosis factor-α (TNF-α), granulocyte macrophage-colony stimulating factor (GM-CSF), which are unable to stimulate the NADPH oxidase in 'non-adherent' cells, produce a slowly developing and sustained generation of O_2^- (Dri et al. 1991, Horie & Kita 1994). However, since there are no studies concerning the biochemical mechanism of NADPH oxidase activation in adherent eosinophils, this review will focus predominately upon those studies on 'non-adherent' cells. In particular, we will concentrated upon recent studies of the mechanism of LTB_4-induced NADPH oxidase activation in guinea-pig eosinophils (Perkins et al. 1995, Lindsay et al. 1995a, b).

STRUCTURE AND ASSEMBLY OF THE NADPH OXIDASE

In neutrophils, an active NADPH oxidase complex assembles at the phagocytic and plasma membranes following activation (Segal & Abo 1993) (Fig. 2). At least five proteins are required for the formation of an active oxidase complex: the membrane-bound cytochrome b_{558} (consisting of two subunits, gp91^{phox} and p22^{phox}) and the cytosolic proteins, p47^{phox}, p67^{phox} and a small GTP-binding protein, Rac-1 or Rac2 (Casimer & Teahan 1994, Bokoch 1994). Recently, two additional components have been identified, these being the cytosolic protein, p40^{phox}, that appears to be associated with p67^{phox} (Wientjes et al. 1993, Tsunawaki et al. 1994) and the membrane associated small GTP-binding protein, Rap1a (Gabig et al. 1995). Under resting conditions, the cytosolic components exist as a 240-300 kDa oligomer (Park et al. 1992, 1994). Following activation, translocation of these components to the membrane-bound cytochrome b_{558} and assembly of the active oxidase complex is thought to be mediated by a mechanism involving both protein binding through Src homology 3 (SH3) domains and phosphorylation of p47^{phox} (Rosrosan & Leto, 1990, McPhail 1994, Park & Ahn, 1995, Demendez et al. 1996).

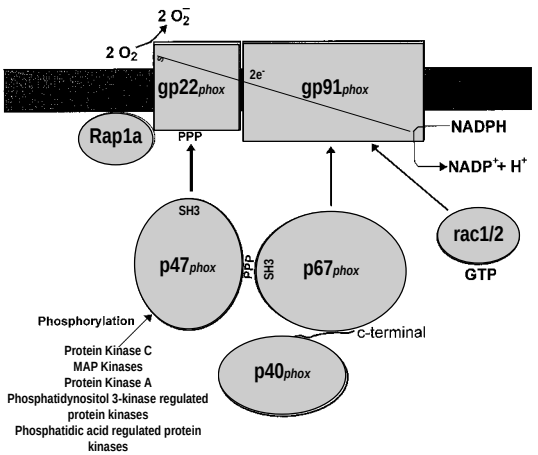

Fig. 2: structure of the NADPH oxidase. PPP: proline rich regions; SH3: src homology domain 3.

In eosinophils, evidence for a similar if not identical mechanism of oxidase assembly and activation is also available. Thus, the cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and membrane components, $p22^{phox}$ and $gp91^{phox}$ have been identified (Segal et al. 1981, Yagisawa et al. 1996, Zhan et al. 1996) whilst $p47^{phox}$ and $p67^{phox}$ have been shown to reconstitute NADPH oxidase activity in cell free systems prepared from both neutrophils and eosinophils fractions (Bolsher et al. 1990).

ROLE OF PHOSPHOLIPASE C, INTRACELLULAR CA^{2+} AND PROTEIN KINASE C

In neutrophils, stimulation of phospholipase C (PLC) is thought to be central to the activation of the NADPH oxidase. PLC catalyses the hydrolysis of phosphatidylinositol (4,5)-bisphosphate to inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can release Ca^{2+} from intracellular stores whilst DAG is known to activate protein kinase C (PKC). Studies in eosinophils have demonstrated a rapid and transient increase in both IP_3 and $[Ca^{2+}]_i$ following exposure of guinea-pig and human eosinophils to LTB_4, PAF and fMLP (Kroegel et al. 1991, Perkins et al. 1995, Wymann et al. 1995). Furthermore, human eosinophils release DAG following stimulation with opsonized particles (Koenderman et al. 1990). However, the generation of O_2-derived free radicals is only marginally suppressed in Ca^{2+}-depleted cells, suggesting that neither IP_3 nor Ca^{2+} play a major role in the activation of the NADPH oxidase (Subramanian et al. 1992, Perkins et al. 1995, Wymann et al. 1995). Similarly, whilst the PKC activators, phorbol esters, are potent and robust stimulants of oxidase activation in guinea-pig and human eosinophils (Petreccia et al. 1987, Perkins et al. 1995), the PKC inhibitors Ro-31 8220 (Perkins et al. 1995) and 1-*O*-hexadecyl-2-*O*-methylglycerol (Rabe et al. 1992) only partially inhibit (by 20 to 30%) agonist-induced H_2O_2 release in guinea-pig eosinophils, suggesting that PKC is not central to this response. Indeed, in human eosinophils exposed to opsonised particles, the rate of oxygen consumption is *augmented* in the presence of inhibitors of PKC (van der Bruggen et al. 1993) implying that one of more of these enzymes can negatively regulate oxidase activation. Collectively, therefore, these data provide persuasive evidence that agonist-induced activation of the NADPH oxidase in eosinophils is mediated by mechanisms that are largely independent of intracellular Ca^{2+} and PKC.

ROLE OF PHOSPHOLIPASE D AND PHOSPHATIDYLINOSITOL 3-KINASE

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) which can subsequently hydrolysed to diradylglycerol (DRG) by phosphatidic acid phosphohydrolase. Since PLD is generally considered to be the predominate pathway for the production of DAG, it was originally thought that PLD mediates NADPH oxidase activation following PKC stimulation (Bonser et al. 1989, Thompson et al. 1990, Kessels et al. 1991). However, recent studies in cell free system have suggested the possible involvement of PA-regulated protein kinases in the mechanism of $p47^{phox}$ phosphorylation and NADPH oxidase activation (McPhail et al. 1995). Attempts to measure PLD activation in eosinophils have produced conflicting results which is probably related to differences in the stimuli used. Thus, although C5a stimulated PLD activation in human eosinophils (Minnicozzi et al. 1990) this was not observed in guinea-pig eosinophils exposed to LTB_4 (Perkins et al. 1995). Unusually, the latter study found that butan-1-ol, an inhibitor of PLD was able to inhibit NADPH oxidase activation. However, it is likely that the action of butan-1-ol was due to its ability to elevate intracellular cyclic AMP, which is known to inhibit the activation of the NADPH oxidase in eosinophils (see below) (Perkins et al. 1995).

Phosphatidylinositol 3-kinase (PI 3-kinase) catalyses the enzymatic conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate. In neutrophils, this reaction is apparently pre-requisite for the activation of the NADPH oxidase since selective inhibitors of PI 3-kinase, such as wortmannin and LY294002, effectively suppress the generation of O_2^- in response to fMLP (Ding et al. 1995, Vlahos et al. 1995). Furthermore, the use of these inhibitors has facilitated the identification and characterisation of PI 3-kinase activated protein kinases that are able to phosphorylate peptides derived from $p47^{phox}$ (Ding et al. 1995, 1996).

Currently, little is known of the role of PI 3-kinase during activation of the eosinophil NADPH oxidase. While wortmannin attenuates eotaxin-induced NADPH oxidase activation in human eosinophils (Elsner et al. 1996), it has no affect upon LTB_4-induced H_2O_2 generation in guinea-pig eosinophils at concentrations that abolish the fMLP evoked respiratory burst in neutrophils (Perkins et al. 1995).

ROLE OF PHOSPHOLIPASE A_2 AND ARACHIDONIC ACID

It has been proposed that arachidonic acid (AA), cleaved from membrane phospholipids by PLA_2, may play an important role in the activation of the human neutrophils (Badwey et al. 1984, Curnette et al. 1984, Aebischer et al. 1993,

Henderson et al. 1993). The mechanism underlying these responses is still unknown although AA has been demonstrated to have a number of intracellular actions in other cell types. These include the inhibition of ras GTPase activating protein (Homayoun & Stacey, 1993, Sermon et al. 1996), activation of PKC (Khan et al. 1995) and MAP kinases (Rao et al. 1994, Hii et al. 1995), increasing intracellular Ca^{2+} concentration (Hardy et al. 1995) and to synergise with GTPγS to cause rac p21 translocation to membrane fractions and the subsequent activation of the NADPH oxidase in cell-free systems (Sawai et al. 1993). We have found that addition of exogenous AA to guinea-pig eosinophils stimulates H_2O_2 generation in a concentration-dependent manner (Lindsay et al. 1995a). This response was unaffected by inhibitors of cyclo-oxygenase and lipoxygenase indicating that is not mediated by its metabolism to prostaglandins, thromboxane or leukotrienes and may reflect a direct action of AA. However, the role of PLA_2 activation and the release of AA during receptor mediated NADPH oxidase activation in eosinophils is virtually unknown. Studies with fMLP- (White et al. 1993) and opsonized zymosan-stimulated (Shute et al. 1990) eosinophils have implied a possible role for endogenous PLA_2 in the mechanism of O_2^- generation. However, these conclusions were derived pharmacologically using the non-selective PLA_2 inhibitors, mepacrine and 4-bromophenacyl bromide and did not attempt to measure the AA release. In recent experiments, using the release of [^{3}H]AA from pre-loaded cells as a marker of PLA_2 activation, we have investigated the role of PLA_2 during LTB_4-induced NADPH oxidase activation. We have found that the liberation of [^{3}H]AA from eosinophils occurs with a time- and concentration-dependence consistent with a causal role in the generation of H_2O_2 (Fig. 3). However, since the non-selective PLA_2 inhibitor, mepacrine caused only a small inhibition of H_2O_2 generation at a concentration (50mM) that completely attenuated [^{3}H]AA release, this suggests that PLA_2 activation is not central to the mechanism of LTB_4-induced NADPH oxidase activation (Fig. 3).

ROLE OF MAP KINASES AND TYROSINE KINASES

MAP kinases is the generic term used to describe an ever increasing family of serine/threonine kinases. At present, the three most characterised MAP kinases families are the extra-

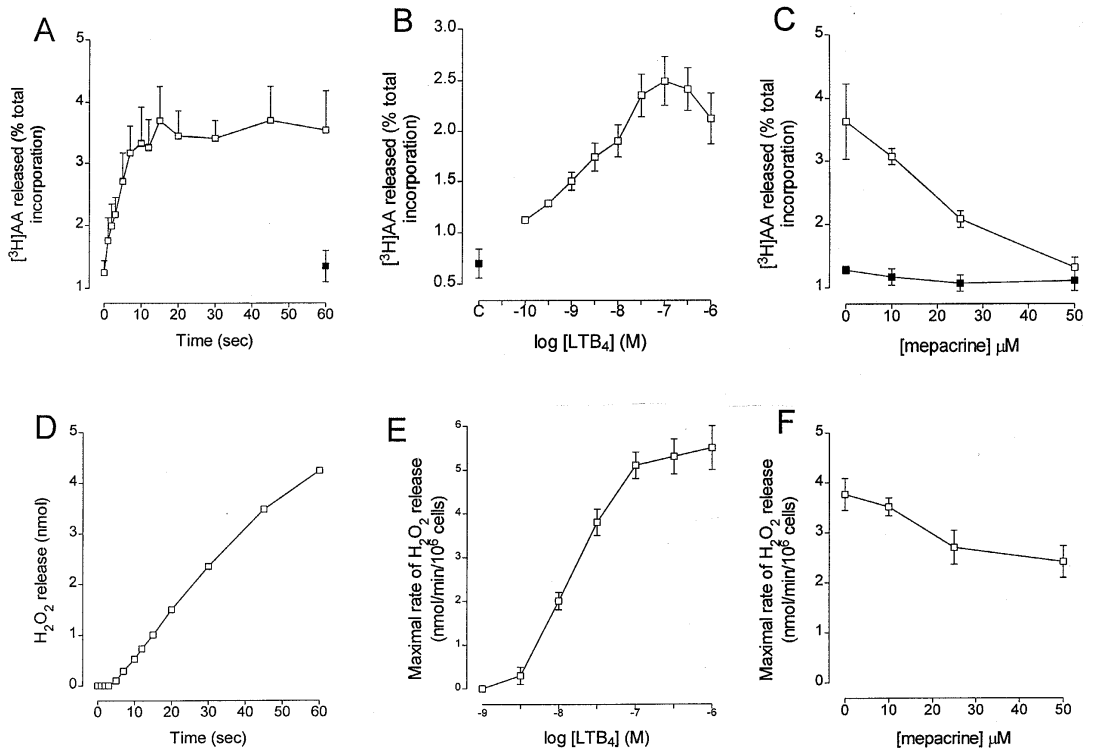

Fig. 3: LTB_4-induced phospholipase A_2 and NADPH oxidase activation in guinea-pig eosinophils. The time (A,D) and dose-dependent (B,E) release of [^{3}H]AA and maximal rate of H_2O_2 generation and the affect of the PLA_2 inhibitor, mepacrine upon the these two responses (C,F), was measured in control (- -) and LTB_4-stimulated (1μM) (- -) guinea-pig eosinophils. Control H_2O_2 release was essentially zero.

cellular regulated kinases 1 and 2 (ERK1/2), the c-jun N-terminal kinases 46 and 54 (JNK46/JNK54) and the p38 kinases. The upstream mechanisms that regulate the activation of the MAP kinases are presently an area of intense investigation.

The LTB_4-, C5a- and fMLP-stimulated responses are thought to activate eosinophils via intercalation with receptors linked to the pertussis toxin sensitive G-protein, G_i (Kita et al. 1991, Miyamasu et al. 1995, Wymann et al. 1995, Lindsay et al. 1995b). Recent studies in both neutrophils and transfected cell lines, have identified some salient aspects of the mechanism of G_i-linked MAP kinase activation (for reviews see Bokoch, 1995, 1996, Denhardt 1996). In the case of ERK1/2 activation, the release of the $\beta\gamma$ subunit of G_i results in the phosphorylation of Shc and the subsequent engagement of Grb2-Sos by a mechanism involving phosphatidylinositol 3-kinase (Downey et al. 1996) and the a Src-like tyrosine kinase (Wan et al. 1996). The guanine nucleotide exchanger, Sos stimulates GDP/GTP exchange and activation of $p21^{ras}$. Activated $p21^{ras}$ recruits the serine/threonine kinase Raf-1 to the plasma membrane where it is stimulated by an as yet unidentified mechanism. Raf-1 then catalyses the phosphorylation and activation of MAP kinase kinase 1/2 (MEK1/2) which can subsequently phosphorylate and activate the ERK1/2 MAP kinase. At present, much less is known of the pathway responsible for G_i-linked activation of the JNK and p38 MAP kinases. Once again the mechanism is thought to involve the $\beta\gamma$ subunit which acts through members of the Rho family of small GTP-binding proteins (rac1 and cdc42). These GTP-binding proteins are believed to stimulate PAK, a p21-activated kinase, which in turn phosphorylates and activates a sequence containing MEK kinases, then MEKs and finally the JNK and p38 MAP kinases. Since the cytosolic component $p47^{phox}$ has been demonstrated to contain possible MAP kinase phosphorylation sites whilst another cytosolic component, rac1 is involved in the mechanism of MAP kinase activation, this pathway is potentially important in the mechanism of NADPH oxidase activation.

Although there are no studies demonstrating NADPH oxidase activation by interleukin-5 (IL-5), this cytokine has been reported to cause activation of the lyn-ras-raf1-MEK-ERK pathway in human eosinophils (Pazdrak et al. 1995, Bates et al. 1996). Furthermore, 5-oxo-eicosatetraenoate (5-oxoETE) has been shown to phosphorylate the p42 and p44 MAP kinase (probably ERK1/2) in human eosinophils (O'Flaherty et al. 1996) whilst Araki et al. (1995) have demonstrated PKC-independent activation of raf1 and ERK following LTB_4-activation of guinea-pig eosinophils. We have extended the later study and shown LTB_4-induced phosphorylation of the p38 MAP kinases although we were unable to demonstrated activation of JNKs (Fig. 4). However, since the selective inhibitors of ERK and p38 MAP kinases, PD098059 (Alessi et al. 1995, Dudley et al. 1995) and SK203580 (Lee et al. 1994) respectively, failed to significantly attenuate H_2O_2 generation (Fig. 5), this suggested that MAP kinases do not mediate LTB_4-induced NADPH oxidase activation.

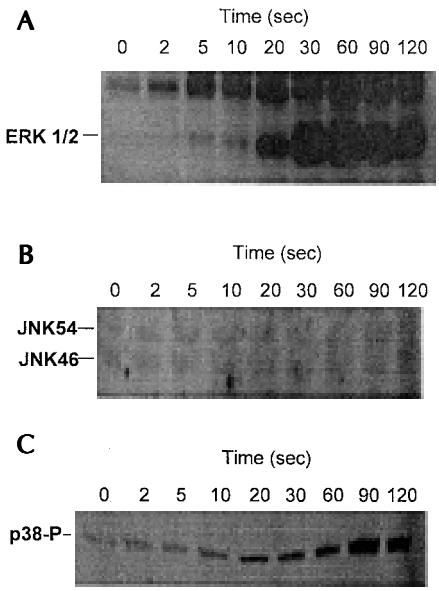

Fig. 4: LTB_4-induced MAP kinase activation in guinea-pig eosinophils. Time dependent effect of LTB_4 stimulation (1μM) upon ERK1/2 (A) and JNK46/54 (B) activation and p38 MAP kinase phosphorylation (C) in guinea-pig eosinophils. ERK1/2 and JNK46/54 activity were measured using an in-gel renaturation assay employing myelin basic protein and GST-c-jun, respectively, as the substrates whilst p38 phosphorylation was determined by western blotting with an anti-phospho-p38 specific antibody (p38-P).

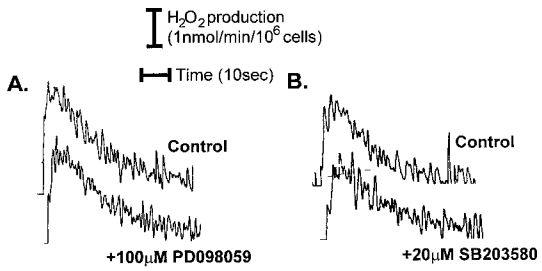

Fig. 5: effect of MAP kinase inhibitors upon LTB_4-induced NADPH oxidase activation in guinea-pig eosinophils. Eosinophils were pre-incubated for 10 min and 30 min with PD098059 (A) and SB203580 (B), respectively, stimulated with 1μM LTB_4 and the maximum rate of H_2O_2 generation determined. Control H_2O_2 release was essentially zero.

A number of inhibitor studies have implicated a possible role for protein tyrosine kinases during NADPH oxidase activation in eosinophils (Nagata et al. 1995, Elsner et al. 1996). Since these inhibitors may exert their action through inhibition of the src-related tyrosine kinases, their affects maybe secondary to inhibition of the MAP kinases cascade. However, our observation that the tyrosine kinase inhibitors, herbimycin A and lavendustin A, can dose dependently inhibit the MAP kinase-independent LTB_4 response in guinea-pig eosinophil (Fig. 6), suggests the existence of an additional tyrosine kinase dependent pathway(s) responsible for NADPH oxidase activation.

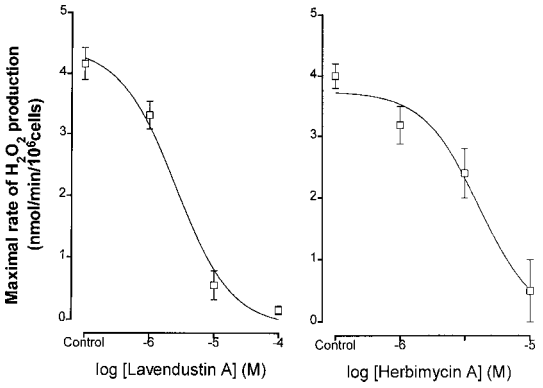

Fig. 6: Effect of tyrosine kinase inhibitors upon LTB_4-induced NADPH oxidase activation in guinea-pig eosinophils. Eosinophils were pre-incubated for 5min with the stated concentration of lavendustin A and herbimycin A. Following 1μM LTB_4 stimulated, the maximal rate of H_2O_2 generation was determined. Control H_2O_2 release was essentially zero.

INHIBITION OF THE NADPH OXIDASE BY CYCLIC AMP

A number of cyclic AMP-elevating drugs inhibit agonist-induced activation of the NADPH oxidase in eosinophils. Pre-treatment of eosinophils with β_2-adrenoceptor agonists such as salbutamol, partially suppress this response but short periods of pre-incubation are necessary if inhibition is to be seen (Yukawa et al. 1990, Rabe et al. 1993). This phenomenon is believed to be due to the rapid development of tachyphylaxis, and may be due to uncoupling of β-adrenoceptors since receptor down-regulation is not observed. Paradoxically, the long-acting β_2-agonists salmeterol is inactive on guinea-pig eosinophils and actually behaves as a competitive antagonist. However, this might relate to the very poor efficacy of salmeterol coupling, with a low density of β-adrenoceptors on eosinophils.

Lipophilic cyclic AMP analogues (Dent et al. 1991) and selective inhibitors of the phosphodiesterase (PDE) 4 isoenzymes family also effectively prevent activation of the respiratory burst oxidase (Dent et al. 1991, 1994, Souness et al. 1991, Barnette et al. 1995, Hatzelmann et al. 1995).

CONCLUSION

In comparison to neutrophils, little is known of the mechanism of NADPH oxidase activation in eosinophils. As a consequence of the difficulties in obtaining sufficient numbers of cells for biochemical studies, the majority of the detailed biochemical studies have been performed using guinea-pig peritoneal eosinophils. However, where detailed studies have been performed, these results suggest there maybe fundamental difference between the mechanism of NADPH oxidase in eosinophils and neutrophils. Thus, increases in intracellular Ca^{2+} concentration and protein kinase C activation are not required for NADPH oxidase activation in either human or guinea-pig eosinophils. Furthermore, in contrast to fMLP stimulation of neutrophils, LTB_4-stimulated NADPH oxidase activation in guinea-pig eosinophils appears to be mediated via a tyrosine kinase dependent mechanism that is esssentially independent of PLD, PI 3-kinase, PLA_2 and MAP kinases. These disparities probably derive from the both the differences in the stimuli and/or the functional roles of these two cell types.

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