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Phosphorylation and Dephosphorylation of Soluble Proteins in Human Eosinophils

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The effect of phorbol 12-myristate 13-acetate (PMA), calcium ionophore (A23187), opsonized zymosan (OZ), and N-formylmethionyl-leucyl-phenylalanine (f-Met-Leu-Phe) on protein phosphorylation was examined in purified eosinophils (eos) isolated from human peripheral blood. Eos were prelabeled with [^{32}P]orthophosphate, stimulated with several activating agents for varying periods of time. The soluble proteins were then analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In resting eos, there was phosphorylation of endogenous soluble proteins with molecular weights of 12, 16, 21, 40, and 66 kilodaltons (kDa). PMA, a potent activator of oxidative metabolism, induced phosphorylation of 19-, 40-, and 67-kDa proteins. A23187, a strong degranulating stimulus, caused phosphorylation of 40-, 53-, and 67-kDa proteins. OZ, a relatively weak stimulus for eos function, caused phosphorylation of 30–34-, 59-, 67-, and 93-kDa proteins. In addition, all the above stimuli caused a time-dependent dephosphorylation of 21-kDa protein. In contrast, f-Met-Leu-Phe caused neither phosphorylation of new proteins nor dephosphorylation of preexisting eos proteins. These findings demonstrate that selected stimuli affect phosphorylation of soluble eos protein. These results also suggest that phosphorylation of specific proteins in eos is an intermediary step in external stimulus-induced cell activation, which may involve many different cell functions.

Key words: eosinophils, protein phosphorylation, cell activation, zymosan

Activation of human eos by PMA, A23187 (calcium ionophore), f-Met-Leu-Phe, particulate stimuli opsonized zymosan (OZ), and serum- and IgG-coated Sephar-

Abbreviations used: DMSO, dimethyl sulfoxide; eos, eosinophils; f-Met-Leu-Phe, formyl-methionyl-leucyl-phenylalanine; kDa, kilodalton; Mr, relative molecular mass; Oz, opsonized zymosan; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate.

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ose beads is accompanied by extensive changes in intracellular metabolism. Changes associated with in vitro activation of eos include (1) generation of reactive oxygen products [1–3] and 5-lipoxygenase leukotriene metabolites [4], (2) degranulation with release of enzymes and granular proteins [5,6], and (3) an increase in the expression of surface receptors for immunoglobulin G (IgG), IgE, and C3 proteins [7,8]. In addition, we have recently shown that eos activation with OZ leads to synthesis and secretion of new proteins [9] that may possess important biological functions [10]. Protein function and cellular metabolism may be controlled by posttranslational modifications [11].

Protein phosphorylation is an important posttranslational modification associated with activation of cells and plays a critical role in signal transduction [12,13]. Phosphorylated proteins have been implicated in initiation of proliferation when lymphocytes are activated by mitogens, lectins, phorbol esters, and interleukin 2 (IL-2) [14–17] and in activation of neutrophil degranulation and respiratory burst [18–20].

Little is known about the occurrence of phosphorylated proteins in eos and their role in cell activation and function. In this report, we examine the effects of soluble agents such as PMA, A23187, f-Met-Leu-Phe and particulate stimuli such as OZ on the phosphorylation and dephosphorylation of soluble proteins in human eos. Identification of such phosphorylated proteins may provide insight into the mechanisms governing eosinophil activation.

MATERIALS AND METHODS

Materials

Reagents and supplies were obtained from the following sources: PMA, DMSO, A23187, f-Met-Leu-Phe, sodium fluoride (NaF), and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO); phenylmethylsufonyl fluoride (PMSF) was from Boehringer Mannheim Gmbh (Mannheim, FRG); zymosan was from NBCO Biochemicals, Inc. (Cleveland, OH); phosphate-free Minimal Essential Medium (MEM) was from Irvine Scientific (Santa Ana, CA); [^{32}P]orthophosphoric acid [$^{32}PO_4$] in HCl-free and carrier-free water was from New England Nuclear (Boston, MA); electrophoresis reagents including molecuar weight standards were from Bio-Rad laboratories (Richmond, CA); metrizamide was from Accurate Chemical and Scientific Corporation (Westbury, NY). PMA, A23187, and f-Met-Leu-Phe were dissolved and stored in DMSO at −20°C and used at a final DMSO concentration of 0.1% (v/v). At this concentration, DMSO by itself had no noticeable effect on [$^{32}PO_4$] labeling of proteins in eos.

Eosinophil Isolation

Eos were isolated from the blood of normal and mildly atopic donors having 3–8% eosinophils. Informed consent was obtained, and the study was performed in accordance with the procedures of the Human Investigation Review Committee of the New England Medical Center Hospital. Eos were purified by means of discontinuous (18–25%) metrizamide density gradient centrifugation, as previously described [21]. Only those preparations that contained greater than 90% eos were employed. Contaminating cells were exclusively neutrophils.

[$^{32}PO_4$] Labeling of Eos Proteins

Eos were washed three times with phosphate-free MEM at pH 7.2 and resuspended in the same buffer at a concentration of 3×10^7 cells/ml. [$^{32}PO_4$] 5 mCi/ml (1 Ci = 37 GBq) was added, and the cells were incubated for 1 hr at 37°C with gentle agitation in order to label intracellular ATP pools. The cells were then washed three times with cold Tris-buffered saline (pH 7.2). The cells were then resuspended in phosphate-free medium at 3×10^7 cells/ml, and the indicated stimulus was added. Stimuli included PMA, A23187, f-Met-Leu-Phe, and OZ. Over 90% of the cells remained viable at the end of the activation period with these stimuli as indicated by trypan blue dye exclusion.

Cell Disruption and Sample Preparation

The phosphorylation reaction was terminated at the indicated time (5–60 min) by instant freezing of the cells in a dry ice-acetone mixture. The cells now suspended in Tris-buffered saline (pH 7.2) containing 0.1 mM PMSF, 0.4 mM EDTA, and 10 mM NaF were disrupted by repeated freeze-thawing (at least five times), and then the broken cell suspension was centrifuged at 15,000g. The supernatant containing soluble proteins was separated. Proteins contained in the supernatant were precipitated by cold acetone (−20°C) and the precipitates were washed in acetone and dried.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis SDS-PAGE

The above sample proteins were electrophoresed on 12% acrylamide gels at pH 8.3, stained with Coomassie blue, destained, dried, and then subjected to autoradiography, as previously described [9]. Protein concentrations of samples ranged from 40 to 60 μg. The Mr of phosphorylated proteins was determined by using a mixture of nonradioactive standard proteins, which were loaded as a mixture in a separate well on the same gel along with sample proteins. The subunit Mr of proteins was calculated by an extrapolation method using a plot of relative electrophoretic mobility versus log Mr of standard proteins. The Mr of phosphorylated proteins was a mean of three determinations. Standard deviations were less than ±5%.

RESULTS

In this study, phosphorylation of soluble proteins of resting and stimulated eos was investigated by one-dimensional PAGE followed by autoradiographic analysis. [$^{32}PO_4$]-labeled proteins present in the soluble extracts obtained from resting eos are shown in Figure 1. Five to six phosphoproteins were identified consistently when resting cells were incubated with [$^{32}PO_4$] for 60 min. Prominent among these proteins was a 20-kDa species, which incorporated the greatest amount of the label. PMA, a soluble stimulus, enhances oxidative metabolism in eos without causing appreciable degranulation [21]. Treatment of [$^{32}PO_4$]-loaded eos with PMA (1 μM) for 5, 30, and 60 min caused time-dependent alterations in the phosphorylation of proteins (Fig. 1). There was a marked increase in phosphorylation of endogenous proteins with approximate Mr 67, 40, and 19-kDa. In contrast, there was a time-dependent dephosphorylation of a 21-kDa protein. These PMA-induced changes in eos protein phosphorylation and dephosphorylation were concentration dependent and occurred over a range of 0.01–1.0 μM (data not shown).

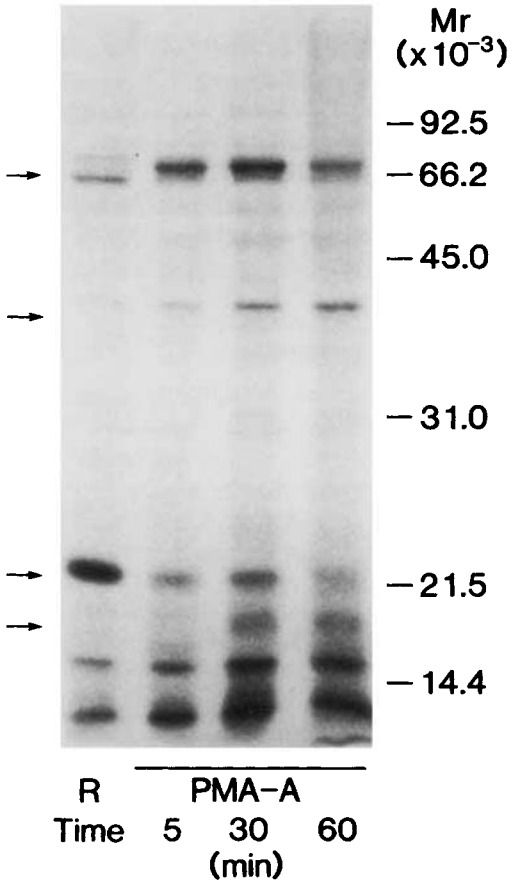

Fig. 1. Effect of PMA on phosphorylation of soluble proteins using autoradiographic analysis. Purified eos (3×10^6/0.1 ml of PO_4-free medium) were incubated with 5 mCi of [$^{32}PO_4$] for 1 hr. Cells were washed three times with cold washing buffer to remove unincorporated radioactivity. Eos were incubated for 0 min (R = resting) without PMA and for 5, 30, or 60 min with 1 μM PMA at 37°C. After the indicated time, the cells were disrupted by freeze-thawing (5 times), centrifuged, and the supernatant was removed. Soluble supernatant proteins were precipitated by cold (−20°C) acetone. The protein precipitate was dissolved in SDS-buffer (50μl) containing 5% (v/v) 2-mercaptoethanol and heated (90°C for 5 min). Samples were then electrophoresed on 12% gels (SDS-PAGE). The gels were stained with Coomassie blue to locate the marker proteins, dried, and then subjected to autoradiography. Numbers on the right are Mr of marker proteins (phosphorylase b 92,500; bovine serum albumin 66,200; ovalbumin 45,000; carbonic anhydrase 31,000; soybean trypin inhibitor 21,500; lysozyme 14,400). Arrows on the left indicate the location of proteins that undergo phosphorylation or dephosphorylation.

A23187, a nonphysiological agent that circumvents membrane calcium-gating mechanisms, stimulates eos degranulation and leukotriene generation without affecting oxidative metabolism [21]. Figure 2 shows the effect of A23187 (1 μM) on eos protein phosphorylation. Like PMA, A23187 also causes a time-dependent phosphorylation of proteins with Mr 67, 53, and 40, and dephosphorylation of 21-kDa protein. Thus, although PMA and A23187 enhance different functions, they have similar effects on phosphorylation and dephosphorylation.

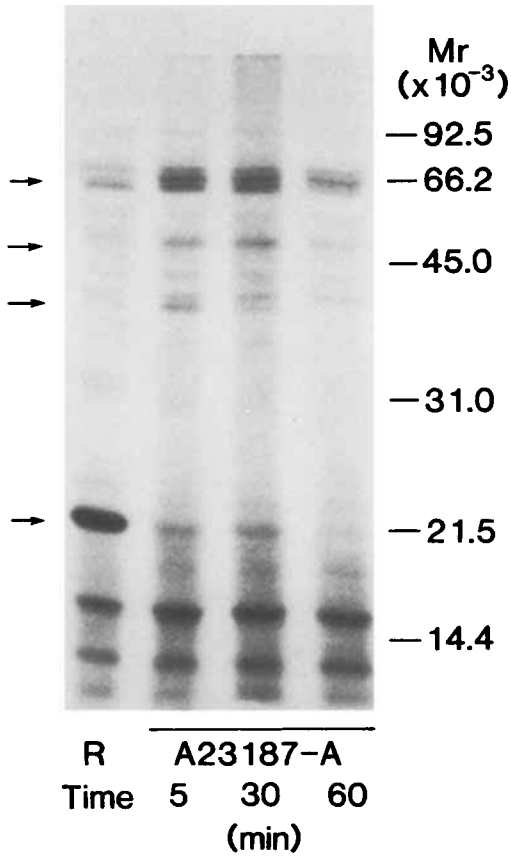

Fig. 2. Effect of ionophore A23187 on phosphorylation. Cells were loaded with [$^{32}PO_4$] as described in the legend to Figure 1. [$^{32}PO_4$]-Incorporated eos were incubated without A23187 (0 min resting [R], eos control) and with 1 μM A23187 for 5, 30, or 60 min at 37°C. Sample preparation, SDS-PAGE, and autoradiographic procedures were performed as described in "Materials and Methods." Numbers on the right are Mr of marker proteins. Arrows on the left indicate the location of proteins that undergo changes in phosphorylation/dephosphorylation in response to A23187.

In addition to its established effects on neutrophil metabolism and phosphorylation, f-Met-Leu-Phe enhances eos chemotaxis and oxidative metabolism [1,2]. Thus, we examined the effects of f-Met-Leu-Phe on eos phosphorylation. Figure 3 shows the effect of f-Met-Leu-Phe (10^{-6} M) on protein phosphorylation in eos. Unlike A23187 and PMA, this tetrapeptide affects neither phosphorylation nor dephosphorylation of soluble proteins. Concentrations from 10^{-4} to 10^{-7} M of f-Met-Leu-Phe were found to have no detectable effect on eos protein phosphorylation (data not shown).

OZ is a particulate eos activator and induces leukotriene generation, degranulation, and synthesis of new proteins. Figure 4 shows the effect of OZ on eos-soluble protein phosphorylation. After the addition of OZ, a set of proteins with Mr 93, 67, and 59-kDa was phosphorylated within 5 min. In addition, a group of proteins in the Mr range of 30–34-kDa was also phosphorylated. These newly phosphorylated proteins were dephosphorylated when [$^{32}PO_4$]-loaded cells were incubated with OZ

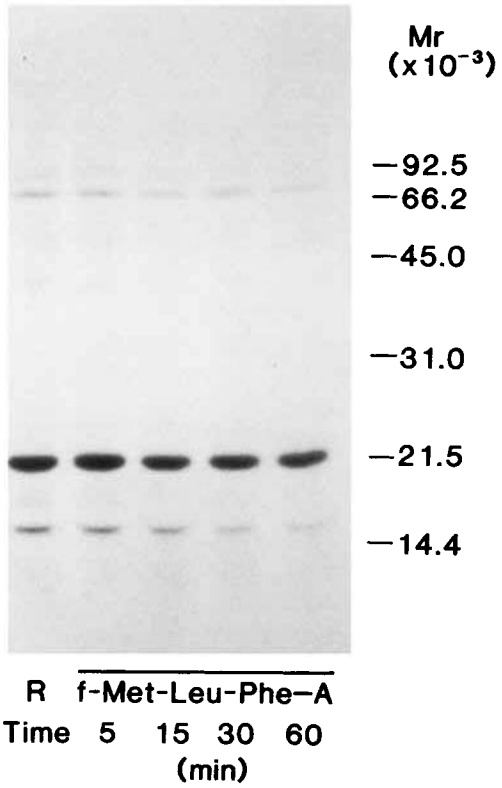

Fig. 3. Effect of f-Met-Leu-Phe on phosphorylation. Eos were prelabeled with [$^{32}PO_4$] for 1 hr and stimulated with 1 μM f-Met-Leu-Phe for 5, 15, 30, or 60 min at 37°C. R represents 0 min resting eos control with no stimulus. [$^{32}PO_4$]-Associated phosphoproteins were analyzed by SDS-PAGE and autoradiography as described in "Materials and Methods." No changes in phosphorylation patterns were seen in samples prepared from cells treated with 0.1 and 10 μM f-Met-Leu-Phe. Numbers on the right indicate the Mr of marker proteins used in calibrating the gel.

for more than 30 min. In addition to these changes, there was gradual and incomplete dephosphorylation of a 21-kDa protein and complete dephosphorylation of a 12-kDa protein.

Minor differences in the number and intensity of protein bands of resting eos were observed as shown in the four figures. This might be due to either donor-dependent variation in eos with regard to their degree of activation or to variation owing to the use of [$^{32}PO_4$] samples that underwent different degrees of radioactive decay. The relative effects of PMA, A23187, f-Met-Leu-Phe, and OZ on qualitative changes in phosphorylation/dephosphorylation of soluble proteins in eos are summarized in Table I.

DISCUSSION

In this report, we have examined the stimulus-induced changes in phosphorylation of soluble protein of human eos by using one-dimensional SDS-PAGE and autoradiographic techniques (Table I). The similarity of changes in phosphorylation of specific proteins induced by different agents suggests a common pathway of eos

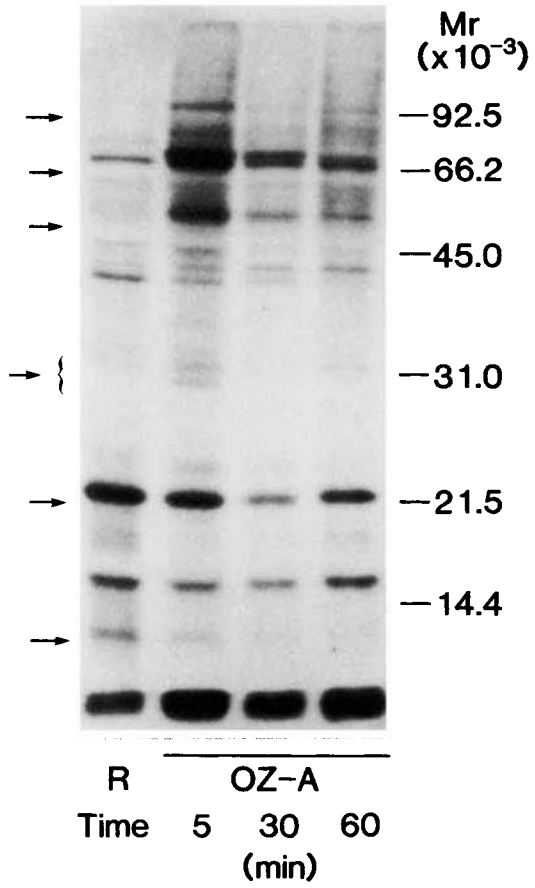

Fig. 4. OZ-induced changes in phosphorylation of eos. Three milligrams of preopsonized, washed zymosan were incubated with 3×10^6 eos (loaded with $[^{32}PO_4]$) for 5, 30, or 60 min at 37°C. R indicates unstimulated eos control. Phosphorylation reaction was stopped by instant freezing of cells. Soluble protein samples were subjected to SDS-PAGE and stained with Coomassie blue to identify the location of marker proteins. The gel was then dried and subjected to autoradiography (6–8 hr at −70°C). Mr of marker proteins are indicated on the right. Arrows on the left indicate the proteins that undergo changes in phosphorylation in response to OZ.

TABLE I. Mr of Soluble Proteins in Eos Undergoing Changes in Phosphorylation/ Dephosphorylation in Response to PMA, A23187, f-Met-Leu-Phe, and OZ

Stimulus (concentration/10^6 cells)	Phosphorylated proteins (kDa)	Dephosphorylated proteins (kDa)
None	12, 16, 21, 40, 66	—
PMA (1 μM)	19, 40, 67	21
A23187 (1 μM)	40, 53, 67	21
f-Met-Leu-Phe (1 μM)	No change	No change
Opsonized zymosan (1 mg)	30–34, 59, 67, 93	21, 11.7

activation. This phosphorylation of endogenous soluble proteins may represent one of several primary biochemical events taking place during eos activation.

PMA is a versatile biologic activator with diverse actions that is believed to act via activation of its receptor, protein kinase C [22–24]. Some of our observed changes in soluble phosphoproteins are similar to those reported when human neutrophils are activated by PMA. For example, in normal neutrophils, there is phosphorylation of a 48-kDa membrane and soluble protein and dephosphorylation of a 20-kDa species after activation by PMA. The absence of such an effect in chronic granulomatous disease neutrophils suggests that phosphorylation may be involved in activation of the respiratory burst [18]. PMA stimulates oxidative metabolism, and it is possible that these PMA-induced changes in soluble protein phosphorylation may be correlated with cell activation.

Calcium ionophore (A23187) also caused phosphorylation and dephosphorylation of soluble proteins similar to that induced by PMA. A23187 induces eos degranulation [21] and leukotriene generation [4] and, to a lesser degree, activates oxidative metabolism. The correlation of A23187-induced changes in protein phosphorylation and degranulation in eos suggests that protein phosphorylation pathways and protein secretion pathways may be interdependent or similarly regulated in eos.

f-Met-Leu-Phe, a potent chemoattractant, has been shown to induce degranulation and oxidative activation in neutrophils [25]. In our present study, f-Met-Leu-Phe consistently failed to induce any significant changes in phosphorylation, suggesting that directed migration may not involve or be dependent on phosphorylation reactions of soluble proteins. Additionally, since f-Met-Leu-Phe activates eos oxidative metabolism, it is possible that, in eos, phosphorylation is not a necessary event in this cellular function.

In addition to phosphorylation of proteins similar to those induced by PMA and A23187, opsonized zymosan (OZ) specifically causes phosphorylation of high molecular weight proteins (>66 kDa). We have recently shown that eos-OZ interaction leads to synthesis of new proteins [9] that are involved in suppression of PHA-induced lymphocyte proliferation [10]. The above observations suggest that, in eos, soluble prosphoproteins may play in important role in activation by particulate stimuli such as noningestible particles.

A23187, PMA and, to a lesser extent, OZ caused a decrease in the phosphorylation of a 21-kDa protein. This phenomenon can be attributed to 1) the removal of phosphate groups on the protein by a phosphatase enzyme or 2) the translocation of soluble phosphoproteins from cytosolic to membrane fractions. Indeed such translocation or mobilization of protein kinase C has been reported during antigen-induced mast cell activation [26] and PMA-induced neutrophil activation [27]. Mobilization of cytosolic phosphoproteins to membrane fractions during eos activation is a possibility that merits further study.

The exact identity and function of the soluble proteins that are phosphorylated during cell activation are not known. Our study suggests that the external stimulus-induced activation of eos may be accompanied by changes in phosphorylation of specific proteins, which, in turn, may be participating in regulation of eos function.

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