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Production of inositol phosphates and reactive oxygen metabolites in quartz-dust-stimulated human polymorphonuclear leukocytes

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The present paper explores phosphoinositide turnover in quartz-stimulated human polymorphonuclear leukocytes. Separation of inositol phosphates was carried out with a new ion-pair, reverse-phase high performance liquid chromatographic method applying a gentle tetrabutyl ammonium phosphate buffer gradient. The method separates inositol monophosphates, inositol 1,4-bisphosphate, inositol trisphosphates and inositol 1,3,4,5-tetrakisphosphate. Reactive oxygen metabolites, indices for leukocyte activation, were measured with a luminometric assay. Quartz increased the production of reactive oxygen metabolites, preceded by facilitated inositol phosphate turnover. This finding provides evidence that inositol phosphate second messengers may be involved in quartz-induced leukocyte activation and subsequent production of reactive oxygen metabolites.

Quartz; Inositol phosphate; Reactive oxygen metabolite; Polymorphonuclear leukocyte

1. INTRODUCTION

Phosphoinositide signalling plays an important role in cell activation. Stimulation of cell surface receptors coupled to the hydrolysis of PIP_2 results in the formation of 2 second messengers, diacylglycerol (DAG) and inositol triphosphates ($InsP_3$). $InsP_3$ releases calcium from non-mitochondrial intracellular stores, and DAG stimulates protein kinase C (PKC) [1]. $InsP_3$ is either dephosphorylated to inositol 1,4-bisphosphate ($InsP_2$), which is subsequently degraded to free inositol, or phosphorylated to inositol 1,3,4,5-tetraphosphate ($InsP_4$), which may be involved in calcium metabolism [2,3]. Ultimately, also $InsP_4$ is hydrolyzed to inositol [2].

The most commonly used method to measure the production of inositol phosphates is the labelling of membrane phosphoinositides with [^{3}H]inositol, and to measure the water-soluble hydrolysis products, inositol phosphates [4]. A wide variety of methods have been used to separate inositol phosphates in stimulated cells [5–10]. Ion-exchange chromatography [11,12] and HPLC [13,14] have been intensively used. An anion-exchange technique on Dowex resin, and on HPLC, have been used extensively as well [15]. This approach has proved especially successful in a number of cell and tissue preparations [2,3,16,17]. We have developed an ion-pair chromatographic HPLC method that separates inositol phosphates up to $InsP_4$ by a gentle linear tetrabutyl ammonium phosphate (TBAP) gradient. Previously, another modification of an ion-pair chromatographic system for the analysis of water-soluble inositol phosphates had only been used by Shayman and Be-Ment [18].

The ability of inorganic dusts, such as quartz, to induce production of reactive oxygen metabolites in phagocytic cells has been implicated in the pathophysiology of lung inflammation fibrosis and cancer [19,20]. The exact mechanisms by which quartz-dust induces its early biological effects have not, however, been clearly defined. There are data to indicate that the production of reactive oxygen metabolites subsequent to chrysotile-induced macrophage activation may be linked to receptor-mediated facilitation of phosphoinositide signalling [16]. In fact, the release of reactive oxygen metabolites by phagocytic cells may be mediated by the hydrolysis of PIP_2 to DAG and $InsP_3$ [16,17]. This study was carried out to explore the involvement of inositol phosphates in quartz-induced activation, and subsequent production, of reactive oxygen metabolites in human polymorphonuclear leukocytes (PMNL).

Abbreviations: HPLC, high performance liquid chromatography; PIP_2, phosphatidyl-inositol 4,5-bisphosphate; DAG, diacylglycerol; $InsP_1$, inositol monophosphates; $InsP_2$, inositol 1,4-bisphosphate; $InsP_3$, inositol trisphosphates; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; PKC, protein kinase C; TBAP, tetrabutyl ammonium phosphate; PMNL, polymorphonuclear leukocytes; CL, chemiluminescence.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Myo-[2-^{3}H]inositol (20 Ci/mmol) and Aqueous Counting Scintillant were obtained from Amersham (Arlington Hts., IL, USA). myo-[2-^{3}H]Inositol 1-phosphate (5.4 Ci/mmol), myo-[2-^{3}H]inositol 4-phosphate (4.5 Ci/mmol), myo-[2-^{3}H]inositol 1,4-bisphosphate (4.5 Ci/mmol), myo-[2-^{3}H]inositol 1,3,4-trisphosphate (20 Ci/mmol), myo-[2-

[3H]inositol 1,4,5-trisphosphate (20 Ci/mmol) and myo-[2-3H]inositol 1,3,4,5-tetrakisphosphate (20 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). Tetrabutyl ammonium phosphate was from Fluka Chemika (Buchs, Switzerland). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). Quartz-dust min-u-sil standard sample was from Pennsylvania Sand and Glass Corporation (Pittsburgh, PA, USA). Luminol and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. *Isolation of human polymorphonuclear leukocytes*

PMNL were isolated from human buffer coat blood (at most 12-h-old, obtained from Finnish Red Cross) by Ficoll density centrifugation as has been described earlier [21]. Erythrocytes were lysed with an isotonic NH_4Cl solution containing 0.155 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (pH 7.4 at 0°C) [22]. Isolated PMNL were suspended in a base buffer containing 124 mM NaCl, 4.0 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 0.2 mM $MgCl_2 \times 6\ H_2O$, 0.5 mM $CaCl_2 \times 2\ H_2O$, 5.6 mM glucose and 10 mM HEPES (pH 7.4) [17].

2.3. *Labelling of human polymorphonuclear leukocytes*

Inositol phospholipids were labelled with tritiated myo-inositol. The suspension of PMNL, 2×10^8/ml, in a base buffer containing 0.025% bovine serum albumin was pre-incubated for 30 min at 37°C in a water bath and then 100 μCi/ml of myo-[2-3H]inositol was added. Cells were labelled in a water bath for 90 min at 37°C under a carbogen (O_2/CO_2;95%/5%) atmosphere. After the labelling, the cells were washed 3-times with a base buffer without albumin, and suspended in a buffer containing 10 mM of LiCl [17]. The cells (5×10^7 cells/ml) were activated by the addition of 200 μg of quartz. The stimulation was terminated at indicated times by adding 20% ice-cold trichloroacetic acid on the cells. Supernatant was extracted with water-saturated diethylether and the solution containing the water-soluble inositol phosphates was lyophilized. After the lyophilization, the samples were diluted in 100 μl of water and analyzed by HPLC (injection volume 20 μl). The results are expressed as dpm of myo-[3H]inositol incorporation/5×10^7 PMNL.

2.4. *Chromatographic analysis*

Inositol phosphates were separated with a Hewlett-Packard Model 1090 high pressure liquid chromatographic system (Hewlett-Packard, Palo-Alto, CA, USA) consisting of 2 gradient pumps. Separation was carried out with a Waters μBondapak C_{18} column (0.39 × 30 cm) (Milford, MA, USA). In addition, a Waters C_{18} Guard-Pak precolumn was used to increase the life of the column.

The 2 mobile phases were water (distilled and de-ionized) and acetonitrile:water 19:81 (v/v), KH_2PO_4 0.04 M and TBAP 0.05 M, pH 2.5 adjusted with phosphoric acid [18]. The gradient used for the assay was as follows: 0–10 min, isocratic elution with water, flow rate 0.75 ml/min; 10–35 min linear increase to 100% TBAP buffer, flow rate 0.75 ml/min; 35–45 min, linear increase from a flow rate of 0.75 ml/min to 1.5 ml/min, same buffer; 45–70 min isocratic elution with 100% TBAP buffer, flow rate 1.5 ml/min; 70–80 min, linear decrease to water and to 0.75 ml/min flow rate. The column was re-equilibrated for 10 min before the next injection. Fractions were collected every minute with a LKB 2112 Redirac fraction collector (Bromma, Sweden) and mixed with 5 ml of Aqueous Counting Scintillant. The radioactivity was determined by liquid scintillation counting (LKB Wallac, Turku, Finland).

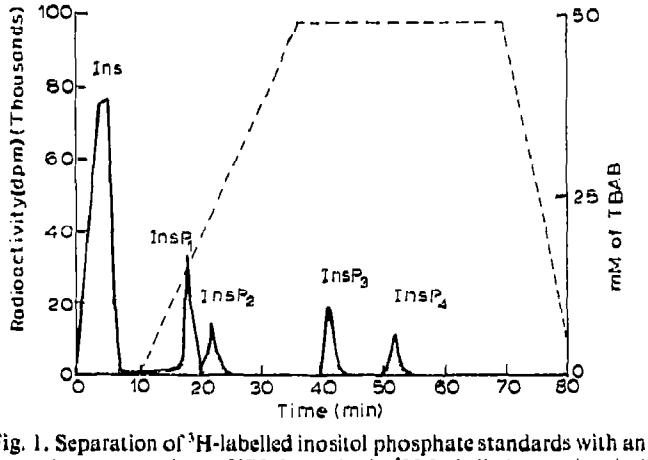

Fig. 1. Separation of 3H-labelled inositol phosphate standards with an ion-pair, reverse-phase HPLC method. 3H-Labelled myo-inositol (Ins), inositol monophosphates ($InsP_1$), inositol 1,4-bisphosphate ($InsP_2$), inositol trisphosphates ($InsP_3$) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) were separated on a μBondapak C_{18} column by a linear gradient from 0–0.05 M of tetrabutyl ammonium phosphate buffer (– –). $InsP_1$ indicates inositol 1- and 4-monophosphate; $InsP_3$ indicates inositol 1,4,5- and 1,3,4-trisphosphate. For details, see Materials and Methods.

2.5. *Chemiluminescence assay*

CL of human PMNL was measured using a Bio-Orbit 1251 luminometer (Bio-Orbit, Turku, Finland). The reaction mixture consisted of 700 μl of 10^{-4} M luminol in the buffer, 100 μl of the cell suspension (5×10^6/ml), and 200 μl of the quartz-dust suspension (0.5 mg/ml); i.e. the dose of quartz was 100 μg/5×10^5 cells. The CL response was followed for 40 min at 37°C and the CL of each sample was recorded at intervals of 2 min [20].

3. RESULTS AND DISCUSSION

To our knowledge this is the first report which provides evidence on the involvement of phosphoinositide signalling in quartz-induced leukocyte stimulation. Inositol phosphates were separated with a new HPLC method in quartz-stimulated human PMNL after different stimulation times (0, 15, 30, 60, 120, 240 and 480 s). A standard sample chromatogram of inositol monophosphate ($InsP_1$), $InsP_2$, $InsP_3$ and $InsP_4$ is shown in Fig. 1. The HPLC system uses an isocratic water solution to elute myo-inositol, and then a gradient of TBAP from 0–0.05 M at pH 2.5 to elute the inositol phosphates from the column. The dose of quartz used in these experiments was 200 μg/5×10^7 PMNL. The chromatographic profile of one representative quartz-stimulated sample is displayed in Fig. 2. The peaks have been identified by retention times as free myo-inositol, $InsP_1$, $InsP_2$ and $InsP_3$. The integrated areas of the peaks were 7240 dpm for $InsP_1$, 896 dpm for $InsP_2$ and 103 dpm for $InsP_3$ at 120 s. Background radioactivity has always been subtracted from the values. After the stimulation, 1 unknown compound, indicated with a question mark in Fig. 2, eluted at 25 min. This peak may be an isomer of $InsP_2$, but it has not been identified because other standards of $InsP_2$ isomers were not available.

The new HPLC method proved suitable to separate and quantify 3H-labelled inositol phosphates, and to assess quartz-induced phosphoinositide signalling in quartz-stimulated human PMNL. Shayman and Be-Ment [18] have also used TBAP successfully to elute inositol phosphates in stimulated renal papillary collecting tubule cells. In their study, TBAP proved to be the most suitable of the 5 quaternary amines used to sepa-

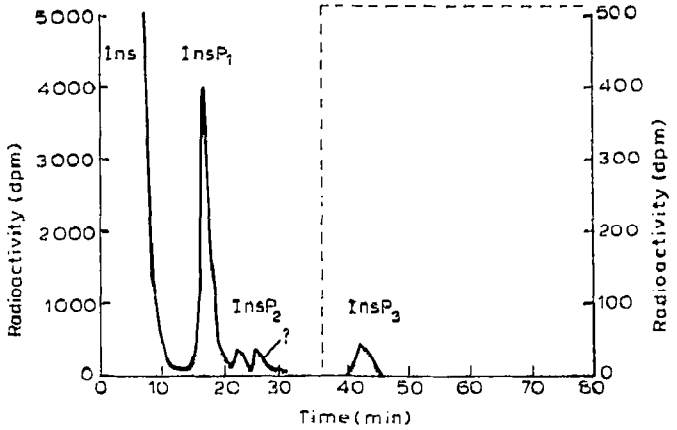

Fig. 2. Inositol phosphate metabolism in quartz-stimulated human polymorphonuclear leukocytes (PMNL). Human PMNL labelled with myo-[^{3}H]inositol were stimulated with quartz (200 μg/5 × 10^7 PMNL) for 120 s, and the water-soluble inositol phosphates were analyzed with a HPLC. The background radioactivity has been subtracted from the values. The figure shows one representative chromatogram of quartz-stimulated PMNL with myo-inositol, $InsP_1$, $InsP_2$ and $InsP_3$ peaks. Note that the scale for radioactivity of the $InsP_3$ peak is different from the scale for $InsP_1$ and $InsP_2$. An unidentified inositol phosphate peak has been indicated by a question mark. Abbreviations are as indicated in Fig. 1.

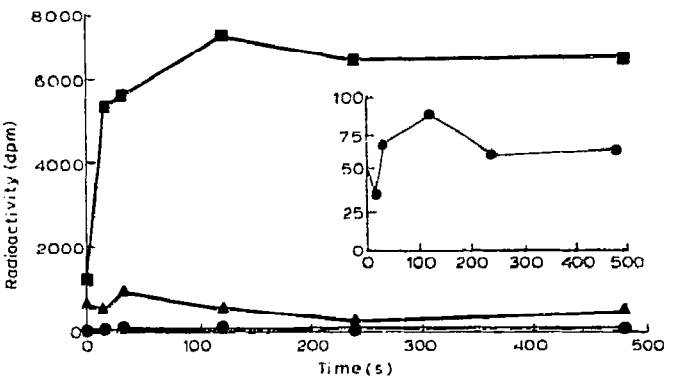

Fig. 3. Time-course of inositol phosphate formation in quartz-stimulated human polymorphonuclear leukocytes (dose 200 μg/5 × 10^7 PMNL). The data are from one experiment done in triplicate (the mean of a triplicate measurement is shown for each of different inositol phosphates). $InsP_1$ (■); $InsP_2$ (▲); $InsP_3$ (●). Increase of $InsP_3$ subsequent to stimulation of human PMNL by quartz has been depicted in the insert. Abbreviations are as indicated in Fig. 1.

rate inositol phosphates. They [18] also observed that solvent pH impacts on the capacity ratio, and affects the resolution and the retention of inositol phosphates. In the present study, the solvent pH was 2.5 with a subsequent good separation and short retention time of inositol phosphates.

The separation of $InsP_1$ and $InsP_2$ from inositol in human PMNL may be problematical because the retention times of all of the 3 compounds are close to each other. However, uncharged inositol is not retained in the column. Therefore inositol in the sample could be washed out with water without significantly affecting the separation of inositol phosphates. Higher inositol phosphates, notably $InsP_3$ and $InsP_4$, were eluted with a good peak sharpness with 0.05 M TBAP from the column at 40 and 50 min in the present chromatographic conditions.

Table 1

Quartz-induced production of reactive oxygen metabolites in human polymorphonuclear leukocytes

Time (min)	Control	Quartz
2	8 ± 2	29 ± 13*
4	6 ± 2	99 ± 29*
6	4 ± 1	176 ± 17*
8	2 ± 0	108 ± 5*

The measurements were carried out at 2, 4, 6 and 8 min after the beginning of the stimulation. Dose of quartz was 100 μg/5 × 10^5 PMNL. Mean (mV) ± SD are shown. Three duplicate measurements were done. The data were statistically analyzed with Student's *t*-test; *$P<0.05$.

The present results show that quartz facilitates the production of inositol phosphates in PMNL (Fig. 3). Stimulation of PMNL with quartz also induces the production of reactive oxygen metabolites (Table I). Facilitation of phosphoinositide signalling precedes the increased production of reactive oxygen metabolites. The peak of $InsP_3$ production was at 2 min after the beginning of the stimulation, with an increase already at 30 s, whereas the first peak of the production of reactive oxygen metabolites was at 6 min, with an increase at 2 min, after the addition of quartz. This observation provides evidence that quartz may cause an increased production of reactive oxygen metabolites in PMNL by facilitating phosphoinositide turnover. Roney and Holian [16] have also presented data to indicate that a similar mechanism may be operating in chrysotile-induced activation of guinea pig alveolar macrophages. These results indicate that the role of G-proteins and PKC in quartz-induced leukocyte activation also requires attention.

Quartz-induced production of reactive oxygen metabolites exhibits 2 peaks. The maximum of the first peak was at 6 min whereas the maximum of the latter peak was at 20 min after the addition of quartz (data not shown). The relationship between the latter peak and the facilitation of phosphoinositide signalling in PMNL remains to be explored. There are, however, data to indicate that PKC may be involved in the sustained production of reactive oxygen metabolites after various external stimuli [23].

In conclusion, facilitated inositol phosphate metabolism preceded the production of reactive oxygen metabolites in PMNL. Thus, quartz-induced PMNL activation may be mediated via recognition sites on the cell surface coupled to phosphoinositide second messengers.

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