Effects of Organometals on Cellular Signaling. II. Inhibition of Reincorporation of Free Arachidonic Acid and Influence on paf-acether Synthesis by Triethyllead

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Organometal compounds affect many enzymes, especially those containing SH-groups as acyl- and acetyltransferases involved in lysophospholipid reacylation. In HL-60 cells, organotin and -lead compounds stimulate phospholipase A2 activity, contributing thus to increase the level of lysophospholipids. In the present study, we have tested whether paf-acether (paf) biosynthesis was affected by treatment with triethyllead (Et3PbCl) in HL-60 cells. Et3PbCl inhibits the incorporation of exogenous arachidonic acid in the presence of high (≥50 µM) but not low concentrations (≤1 µM). High concentrations of the lead compound are unable to induce pafoformation by itself, however, lower concentrations (<10 µM) acted synergistically with TPA or fMLP to stimulate pafoformation. Whereas unstimulated cells produced 0.4 pmole paf/2 x 106 cells, the stimulation with low fMLP (0.1 µM) resulted in the synthesis of 1.7 pmole and with low TPA (2 ng/ml) in 0.5 pmole paf. Preincubation of the cells with 10 µM Et3PbCl for 20 to 30 min increased the amount of paf formed by these cells to 3.3 pmole after treatment with 0.1 µM fMLP and 1.5 pmole after TPA. Furthermore, the results showed an inhibition of acetyltransferase (the key enzyme of paf synthesis) by the high and not by low concentrations of the lead compound. We conclude that low concentrations of Et3PbCl (≤10 µM) may act as a synergistic inducer of paf synthesis initiated via a receptor-coupled stimulation. — Environ Health Perspect 102(Suppl 3):331-334 (1994).

Key words: organolead, synergism, lipid metabolism, arachidonic acid, acetyltransferase, paf-acether, HL-60 cells

Introduction

The balance of decylation and reacylation of cellular membrane phospholipids plays an important role in many cell types as well as in multiple physiological and pathological processes. More and more investigations have shown that environmental toxicants as heavy metal compounds affect this sensitive mechanism within human blood platelets (1-6), macrophages (5), granulocytes (6-8), as well as in fibroblasts (9). These xenobiotics enhance the decylation either via the activation of phospholipase A2 (2,7,8) or the inhibition of fatty acid-CoA synthetase or lysophospholipid acyltransferase (4-6) and all lead to an increased amount of free fatty acids, predominantly of arachidonic acid. This precursory of the biologically active eicosanoids acts in concert with other lipid mediators. Another important path-way coupled to this decylation-reacylation cycle is involved in paf production, a very potent mediator of anaphylaxis and inflammation (10): The acetylation of lysopaf-acether (lysopaf, 1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine) by the activity of the key enzyme lysopaf:acyt-CoA acyltransferase mediates the biosynthesis of paf (11-13).

As mentioned above, the release of arachidonic acid from phospholipids is stimulated by organometals and its reacylation into lysophospholipids by acyltransf erases is inhibited. It is well documented that these effects induce an increase of arachidonic acid as well as of its metabolites, the eicosanoids, but little is known about the destiny of the increased amount of lysophospholipids. To assess the possible role of these lysophospholipids in the biosynthesis of paf we examined the activity of acetyltransferase and the amount of paf produced after treatment with triethyllead (Et3PbCl). Our results suggest that low concentrations of the organolead compound may act synergistically together with other cell agonists in the augmentation of paf synthesis in HL-60 cells.

Materials and Methods

Chemicals

RPMI medium, fetal calf serum and other medium additives were obtained from Gibco (Eggenstein, Germany). The calcium ionophore A 23187, 12-0-tetradecanoylphorbol 13-acetate (TPA), lysopaf, acetyl-CoA, and fMet-Leu-Ph (fMLP) were from Sigma (Munich, Germany). The [1-14C]arachidonic acid (2.07 GBq/mmol) and the [N-methyl-14C]platelet activating factor (2.04 GBq/mmol) were purchased from Amersham (Braunschweig, Germany) and the [acetyl-1H]coenzyme A (115.07 GBq/mmol) was from New England Nuclear (Dreieich, Germany). Triethyllead chloride was obtained from Vention Alpha Products (Karlsruhe, Germany) and was further purified prior to use. All other chemicals were of analytical grade.

Incubation of HL-60 Cells

HL-60 cells were grown in suspension culture in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1.5% glutamate (200 mM in H2O), 1% nonessential amino acid solution, 1% sodiumpyruvate (100 mM in H2O), and 0.5% of a mixture of streptomycin

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Radioactive lipids were localized by using autoradiography, cut out, and counted for radioactivity in a liquid scintillation counter.

**Results**

**Organolead-inhibited Incorporation of Exogenous Arachidonic Acid**

The incubation of HL-60 cells with exogenous \[^{14}C\]arachidonic acid resulted in 65% uptake of the fatty acid within the first 60 min, 50% of the total radioactivity was incorporated into the phospholipids (the bulk was found in PC) and 16% into the neutral lipids (Figure 1A).

As compared with vehicle-treated control cells, Et\(_3\)PbCl inhibited the incorporation of exogenous arachidonic acid into various lipid classes only when added in high concentrations (Figure 1C). One hundred times lower concentrations reduced the incorporation into the different lipid classes to only a minor not significant extent (Figure 1B). Preincubations with the same low concentration for 24 hr before exogenous arachidonic acid was added showed no detectable effect (data not shown). It is apparent from Figure 1C that the label is reduced by high amounts of Et\(_3\)PbCl mainly within phosphatidylinositol, phosphatidylethanolamine, and the neutral lipids by 83, 80, and 94%, respectively. Other lipids such as phosphatidylserine or phosphatidylinositol are unaffected during the incubation time.

**Effects of Et\(_3\)PbCl on Stimulated Activity of Acetyltransferase**

Since various heavy metal compounds inhibit the acetyltransferase, we were interested in the effect of organolead on the activity of acetyltransferase because it is the key enzyme for paf production from its lyso paf precursors. The acetyltransferase activity could be stimulated 4-fold by preincubation of HL-60 cells with calcium ionophore. Concentrations up to 10 \(\mu\)M did not inhibit notably acetyltransferase whereas 40 \(\mu\)M of Et\(_3\)PbCl reduced the enzyme activity down to basal level (Figure 2). Moreover, slightly higher concentrations (50 \(\mu\)M) blocked the enzyme below basal activity.

**Et\(_3\)PbCl and paf Formation in Differentiated HL-60 Cells**

Differentiated HL-60 cells could be stimulated with various compounds to produce paf. The calcium ionophore A 23187 induced the formation of large amounts of this lipid mediator. Smaller but significant

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**Figure 1.** Inhibition of arachidonic acid incorporation into lipids of HL-60 cells after preincubation with Et\(_3\)PbCl. Suspensions of differentiated HL-60 cells were vehicle-treated (control, A) or preincubated with Et\(_3\)PbCl (B, C) for 1 hr before radioactive labeled arachidonic acid was added to the incubation mixtures. After further incubation for 1 hr the lipids were extracted and separated by thin layer chromatography. Shown is the incorporation of arachidonic acid into the various lipid classes and calculated as percentage of total label. Values are the mean of three experiments. No significant difference between 0.5 \(\mu\)M Et\(_3\)PbCl-treated (B) and control cells (A) could be detected, whereas the values of PC, PE, NL, and free arachidonic acid of the 50 \(\mu\)M-treated cells (C) differ from the control (p < 0.005; unpaired t-test). PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; NL, neutral lipids; fAA, free arachidonic acid.

(1 mg/ml) and penicillin (1000 IU/ml). The cells were induced to differentiate to mature granulocytes by the addition of 1.3% dimethyl sulfoxide for 5 days. They were harvested by centrifugation, washed once with RPMI without any additives and finally resuspended in medium containing 1% dimethyl sulfoxide and 3.3% fetal calf serum at a concentration of 1 \(\times\) 10\(^5\) cells/ml. Experiments were started after a rest period of 30 min.

**Paf Assay**

Experiments were carried out with cell suspensions (2 \(\times\) 10\(^5\) cells/ml). After treatment of the cells with calcium ionophore (10 \(\mu\)M or 1 \(\mu\)M), fMLP (1 \(\mu\)M or 0.1 \(\mu\)M), TPA (10 ng/ml or 2 ng/ml), or Et\(_3\)PbCl (100 \(\mu\)M or 10 \(\mu\)M), the reaction was stopped by adding 4 ml ethanol. After 1 hr of stirring at room temperature the samples were centrifuged at 3,000g for 5 min. The ethanolic supernatants were dried and stored at −20°C until assay. Paf activity was measured by aggregation of aspirin-treated (0.1 mM) washed rabbit platelets in the presence of creatine phosphate (0.7 mM) and creatine phosphokinase (13.9 U/ml). Results are expressed in pmole paf per 2 \(\times\) 10\(^7\) cells, calculated over a calibration curve established with synthetic paf C 18:0 (J2). All aggregations were due to paf since the specific paf antagonist BN 52021 inhibited all samples.

**Assay for Acetyltransferase**

The acetyltransferase activity in control cells or in cells treated for 15 min at 37°C with calcium ionophore A 23187 (1 \(\mu\)M) in the presence or absence of various concentrations of Et\(_3\)PbCl was measured as described previously (J1). Briefly, the cells (5 \(\times\) 10\(^5\) cells in 500 \(\mu\)l) were centrifuged and resuspended in 500 \(\mu\)l of 0.9% NaCl and sonicated (three pulses of 5 sec) on ice. The cell lysate (100 \(\mu\)l) was added to the reaction mixture (0.4 ml, pH 7.0), which contained 4.2 mM HEPES, 137 mM NaCl, 2.6 mM KCl, 1.25 mg bovine serum albumin, and the following substrates: 200 \(\mu\)M acetyl-CoA, 11 kBq [\(^3\)H]acetyl-CoA, and 20 \(\mu\)M 2-lyso paf. The reaction was carried out for 12 min at 37°C and was then stopped by adding 400 \(\mu\)l 5% acetic acid and a mixture of chloroform/methanol (1:1, v/v) containing 11,000 dpm of [\(^3\)H]paf as an internal standard. Extraction was performed as described previously (J3): the amount of [\(^3\)H]paf formed in the assay is expressed in % of maximal stimulation after calcium ionophore treatment.

**Lipid Extraction and Separation of Lipid Classes**

After incubation of the cell suspensions with [\(^1-14\)C]arachidonic acid for 1 hr, the lipids were extracted as reported earlier (J7). The extract was dried under nitrogen, dissolved in chloroform, spotted onto SIL G polyester plates (20 cm \(\times\) 20 cm) and separated by thin-layer chromatography by use of a sequential double system (J7). This system gives good separation of phospholipids, free fatty acids and neutral lipids. The Rf values for the lipid classes were determined by comparison of their migration with that of commercial standards.
as the dashed line indicates the activity of the enzyme in unstimulated control cells ± SEM. Results are expressed in percent related to ionophore stimulation (100%) as indicated on the ordinate. Values are the mean of three experiments with different enzyme preparations carried out in duplicate ± SEM.

amounts of paf were formed when cells were stimulated with fMLP as well as TPA (Figure 3). The paf formation was nearly unaffected in cells treated either with 10 μM or 100 μM Et₂PbCl (Figure 3).

In the next set of experiments we incubated the HL-60 cells with the low amounts of the three substances, 0.1 μM fMLP, 2 ng/ml TPA, or 10 μM Et₂PbCl. As shown in Figure 4 (open bars) only fMLP (1.7 pmolle/2 × 10⁶ cells) induced a significant production of paf.

Although Et₂PbCl alone was not able to stimulate paf formation it exhibited a synergistic action together with fMLP or TPA. Furthermore, it is apparent that together with fMLP a stimulation could be detected only when Et₂PbCl was added at first whereas the synergism with TPA was sequence-independent (Figure 4). More than the additive effect in paf stimulation by the synergistic action of Et₂PbCl and fMLP was observed. Moreover, Et₂PbCl enhanced the paf production synergistically together with low TPA to 145% of the stimulatory effect of high TPA alone (compare Figure 3).

Discussion

Human neutrophils and other cell types respond to exogenous stimuli, e.g., TPA, A 23187, or fMLP, with a rapid increase of free arachidonic acid. This is an important metabolic pathway because the enzyme that is involved, the phospholipase A₂ (PLA₂), also hydrolyses 1-O-alkyl-2-arachidonoyl-sn-glycerol-3-phosphocholine (14) to yield lyso paf (15). The lyso paf formed may be acetylated via acetyltransferase to form paf or reacylated with fatty acid residues (16). Therefore, the balance between these two transferase activities plays an important role in paf formation.

The incorporation of fatty acids into lysophospholipids could be prevented by the inhibition of reacylation via the acyl-CoA-synthetase and the lysophospholipid acetyltransferase. It has been shown that not only organomercury compounds such as ethylmercurithiosalicylate (3.5,6), MeHgCl (4), and p-hydroxymercurisalicylat (9) affect these enzymes, but also organolead inhibits the reincorporation of exogenous arachidonic acid (7) (Figure 1). This may lead to the assumption that the stimulated activity of the PLA₂, raises the amount of lyso paf as well as of arachidonic acid when cells were treated with the organometal compounds. We tested therefore the effect of Et₂PbCl, a severely toxic organometal compound (8), on the activity of acetyltransferase. This enzyme is located at the same cellular site as the acetyltransferase within the HL-60 cells (17). After stimulation of differentiated HL-60 cells with A 23187, the enzyme activity increased 3- to 4-fold (17) or, as shown here, 4- to 5-fold (Figure 2) over the values observed in the resting state. As demonstrated for the reacylation pathway (18) the acetylation of lyso paf is inhibited within the same concentration-range (Figure 2). In this connection it is now clear that high concentrations (50-100 μM) of Et₂PbCl that induce a substantial loss of arachidonic acid from phospholipids and prevents the reacylation of the lysophospholipids not lead to an increased paf synthesis as expected (Figure 3). This is the consequence of the concomitant inhibition of acetyltransferase by high lead concentrations, whereas low concentrations of Et₂PbCl had no effect.

On the other hand, these low concentrations (≤10 μM) have still an effect on the lipid metabolism in these cells (7). Consequently, the next question points to the different concentration-dependent effects: does stimulation of PLA₂ activity and no inhibition of both transferases at low Et₂PbCl lead to a higher sensitivity of the deacylation-reacylation cycle to other stimuli? We tested the synergistic action of organolead together with phorbol ester and the chemotactic peptide. In this case, the assumption was verified, the paf synthesis could be increased when HL-60 cells were preincubated with the lead compound. Moreover, the effect of the two stimuli added sequentially is more than additive in the case of fMLP, when the lead compound was the first admixture, as well as in the case of TPA, independent from the sequence of stimulation. A comparable effect of another organometal compound, thimerosal, was described by Haurand and Flohe (19). They find that this organomercury compound enhances the leukotriene formation in human leukocytes, but this stimulation is strictly dependent on a second triggering...
signal. Moreover, the threshold concentration of the second stimuli, e.g., fMLP, is substantially lowered after priming of the leukocytes with thimerosal as is the case in the experiments shown here for Et2PbCl together with fMLP or TPA. Since these authors found a similar concentration dependency concerning the inhibition of reacylation within the human leukocytes as we did for the differentiated HL-60 cells, they suggested a subordinate importance of this inhibitory activity compared with the induction of deacylation. The acetyltransferase in cultured mast cells, on the other hand, could be triggered by incubation with phorbol ester without accompanying paf formation (20). To trigger the full cellular response, additionally receptor-operated antigen challenge was necessary.

In HL-60 cells and many other cell types, especially those of the hematopoietic system, the deacylation-reacylation cycle plays an important role in the regulation of free arachidonic acid concentration as well as of paf formation. The eicosanoids, metabolites of the arachidonic acid, and paf are second messenger molecules with a multitude of functions, intra- as well as intercellular. Particularly neutrophilic granulocytes are able to interact with various cell types, such as macrophages, mast cells, platelets, polymorphonuclear leukocytes, and many others, e.g., via their products of the phospholipase A2 cascade (27) and the subsequently formed paf (10,22).

The results presented here demonstrate that low concentrations of the organolead compound, Et2PbCl, sensitize differentiated HL-60 cells against subsequent stimulation by other agents. These low doses do not inhibit the acetyltransferase as well as the acetyltransferase but enhance the activity of the PLA2, thereby increasing the rate of deacylation-reacylation within the remodeling cycle. As a result of such an enhanced activity within this cycle is the increased paf formation after additional stimulation. In the case of higher amounts of Et2PbCl only arachidonic acid and its metabolites could be detected because the inhibition of acetyl-transferase prevents the paf synthesis. It seems that this mechanism is not only relevant in vitro but also in vivo within occupationally lead-exposed workers (23). In this study it is well documented that in leukocytes of lead-exposed workers the amount of arachidonic acid is significantly increased and the production of LTB4 after stimulation with fMLP is significantly higher than in the control group. We described earlier comparable effects on the arachidonic acid metabolism for other organometals as well (8,18) and the assumption could be made that these compounds may lead in vivo to increase in the level of lipid mediators, eicosanoids and paf, which are considered as potent mediators of inflammatory, allergic, and pseudoallergic reactions (21,23).

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