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EFFECT OF 4-HYDROXYALKENALS ON HEPATIC PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE-PHOSPHOLIPASE C

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Abstract—The effects of some 4-hydroxyalkenals, carbonylic products of lipid peroxidation, on hepatic phosphatidylinositol-4,5-bisphosphate (PIP_2)-phospholipase C (PL-C) activity were investigated. The enzymatic activity was assayed *in vitro* by measuring the hydrolysis of [^{3}H]PIP_2 added as exogenous substrate to liver membranes. 4-Hydroxyhexenal (HEE), 4-hydroxyoctenal (HOE) and 4-hydroxynonenal (HNE) were able to stimulate both the basal and the GTPgammaS induced PL-C activity, whereas 4-hydroxyundecenal was inactive. HOE was the most active compound, being able to accelerate PIP_2 breakdown at concentrations between 10^{-12} and 10^{-6} M, while in the case of HEE the effective doses ranged from 10^{-11} to 10^{-7} M and from 10^{-9} to 10^{-6} M in the case of HNE. 4-Hydroxynonenal was able to increase also bombesin stimulated PL-C activity. As these aldehydes accelerated PIP_2 breakdown at doses which can be actually reached in tissues, the effects shown *in vitro* are likely to occur *in vivo*.

The peroxidative decomposition of polyunsaturated fatty acids of membrane phospholipids leads ultimately to the production of several different carbonyls [1, 2]; in 1960 Benedetti *et al.* [3] found that 4-hydroxy-2,3-trans-nonenal (HNE) was the major diffusible toxic compound generated by peroxidizing liver microsomes.

Subsequent researches demonstrated that HNE and other 4-hydroxyalkenals could modify several cell functions [4, 5]; most toxic effects of HNE and related aldehydes could be ascribed to the block of -SH groups and the consequent inhibition of -SH enzymes, such as hexokinase, lactate dehydrogenase, glucose-6-phosphatase, alpha and beta DNA polymerases, 5′ nucleotidase (for review, see Refs 2 and 6).

These enzymatic inhibitions, however, occur *in vitro*, at concentrations ranging from 10^{-5} to 10^{-3} M; more recently it has been found that low doses of HNE, micromolar or less, are able to stimulate *in vitro* liver adenylate cyclase [7] and phosphatidylinositol-4,5-bisphosphate (PIP_2)-phospholipase C (PL-C) [8, 9] activities.

Both adenylate cyclase and PL-C are effector enzymes coupled, through regulatory G proteins, to specific membrane receptors for hormones and growth factors. The hormone binding to these receptors leads to the generation of second messenger molecules, respectively cyclic AMP (cAMP) and the products of PIP_2 breakdown: diacylglycerol (DG) and inositol-triphosphate (IP_3).

cAMP activates cAMP-dependent protein kinases [10], while DG activates protein kinase C [11] and IP_3 promotes the mobilization of Ca^{2+} from intracellular stores [12].

In the present paper, we compare the activity of some 4-hydroxyalkenals, 4-hydroxyhexenal (HEE), 4-hydroxyoctenal (HOE), and 4-hydroxyundecenal (HUE), to HNE effect on hepatic PL-C and investigate the possible interference of HNE with the activity of bombesin, which is known to activate PL-C [13].

MATERIALS AND METHODS

Chemicals. [^{3}H]PIP_2 was obtained from Amersham International (Amersham, Bucks, U.K.). The universal liquid scintillation solution Pico-Fluor TM-30 was purchased from Packard Inst. SpA (Milano, Italy). Bombesin and all the other chemicals were from the Sigma Chemical Co. (St Louis, MO).

Animals. Male Wistar rats (Nossan, Correnzana, Italy), weighing 150–200 g, were used throughout the experiments. They were fed a semisynthetic diet (Dottori Piccioni, Brescia, Italy).

Methods. Liver homogenates were prepared in 20 mM Tris–maleate (pH 7.4), containing 0.5 mM EGTA and 0.5 mM EDTA, and centrifuged at 120 *g* for 7 min to remove nuclei. The supernatant was centrifuged at 25,000 *g* for 15 min. The pellet was resuspended in 20 mM Tris–maleate (pH 7.4) at a protein concentration of 2–3 mg/mL to obtain a crude membrane preparation which was used for the assays.

Membrane-bound PL-C activity was determined according to Magnaldo *et al.* [14] by measuring the amount of inositol-phosphates produced from [^{3}H]PIP_2 added as exogenous substrate to the isolated membranes. Each reaction tube contained membrane protein (40–50 μg), 20 mM Tris–maleate (pH 7.4), 100 nM free Ca^{2+} (adjusted with Ca^{2+}/EGTA).

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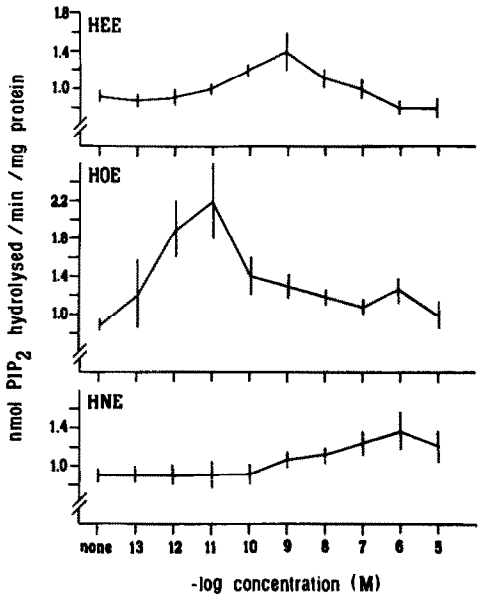

Fig. 1. Effect of increasing concentrations of 4-hydroxyhexenal (HEE), 4-hydroxyoctenal (HOE) and 4-hydroxynonenal (HNE) on the basal activity of membrane-associated PIP_2-phospholipase C of rat liver. The results are expressed as nmol PIP_2 hydrolysed/min/mg protein and are the means ± SD of six different experiments.

The reaction was initiated by the addition of 40 μl of the stock [^{3}H]PIP_2:PE (phosphatidylethanolamine) (1:10) vesicle preparation in a final volume of 0.1 mL.

The mixtures were incubated at 37° for 2 min and the reaction terminated with 0.75 mL ice-cold chloroform/methanol (1:2 v/v).

The radioactivity present in the aqueous phase of the samples was measured and the amount of PIP_2 hydrolysed was calculated. Blanks were prepared by stopping the reaction immediately after the addition of samples.

Protein concentration was estimated by the procedure of Lowry *et al.* [15].

Statistical analysis was carried out with Student's *t*-test.

RESULTS

Figure 1 compares the effects of HOE and HHE to HNE action on hepatic PL-C. The aldehydes were added to the reaction mixture just before starting the reaction and the samples were incubated for 2 min at 37°.

All these aldehydes induced a significant stimulation of the enzymatic activity. The most active compound was HOE which was able to accelerate PIP_2 breakdown at concentrations ranging from 10^{-12} to 10^{-6} M; the highest degree of PL-C stimulation was given by 10^{-11} M HOE. HEE induced a small, but significant increase of the enzymatic activity at doses ranging from 10^{-11} to 10^{-7} M.

As previously reported [9], the stimulation

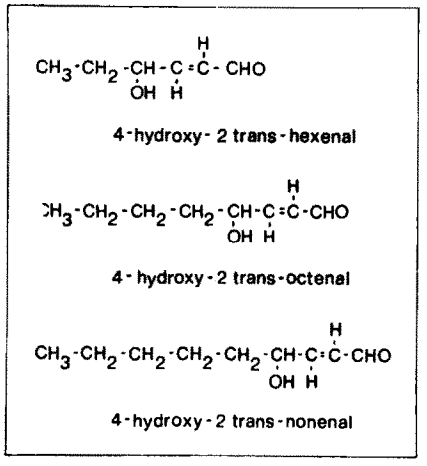

Fig. 2. Structure of the 4-hydroxyalkenals used.

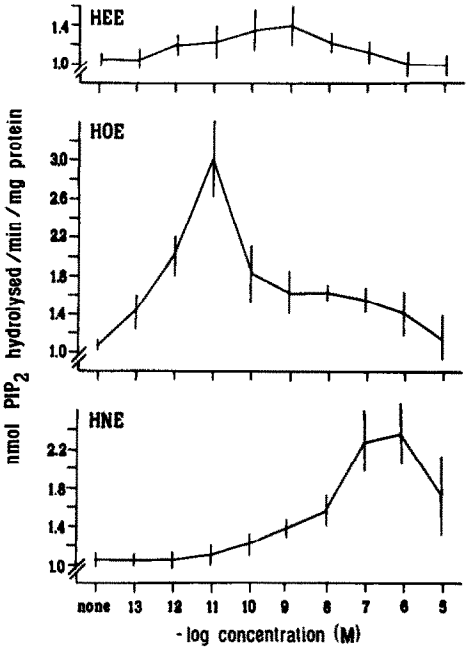

Fig. 3. Effect of increasing concentrations of 4-hydroxyhexenal (HEE), 4-hydroxyoctenal (HOE) and 4-hydroxynonenal (HNE) on membrane-associated PIP_2-phospholipase C activity measured in the presence of 10 μM GTPgammaS. The results are expressed as nmol PIP_2 hydrolysed/min/mg protein and are the means ± SD of six different experiments.

induced by HNE occurred at concentrations ranging from 10^{-9} to 10^{-6} M.

The structure of these 4-hydroxy-alkenals is shown in Fig. 2.

PL-C activity was examined also in the presence of doses of HUE ranging from 10^{-12} to 10^{-6} M (data not shown in the figure), but three different experiments failed to demonstrate any modification of PIP_2 cleavage by this alkenal.

Figure 3 shows the effects of HEE, HOE and

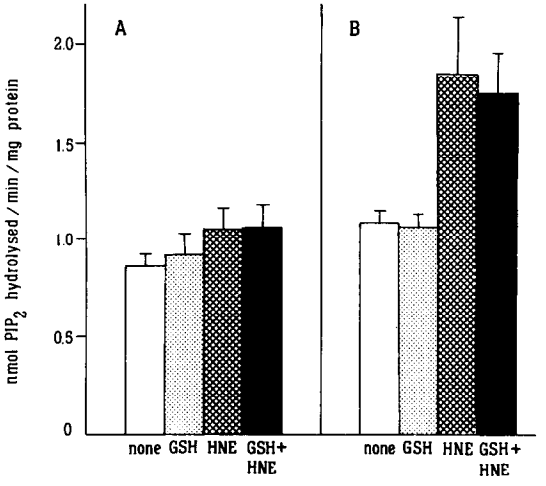

Fig. 4. Influence of reduced glutathione (GSH) on basal and GTPgammaS stimulated PIP_2-phospholipase C activity in the presence and in the absence of 4-hydroxynonenal (HNE). The data shown in panel A represent the effects of 1 mM GSH, 0.1 μM HNE and 1 mM GSH + 0.1 μM HNE on the basal activity (open column); the results shown in panel B have been obtained in the presence of 10 μM GTPgammaS. The results are expressed as nmol PIP_2 hydrolysed/min/mg protein and are the means ± SD of four different experiments.

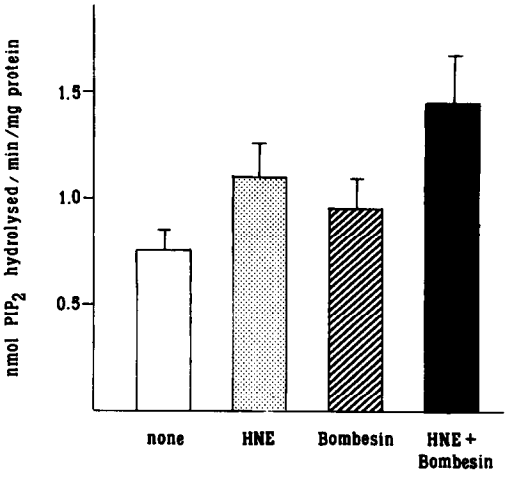

Fig. 5. Effect of 0.1 μM 4-hydroxynonenal (HNE) on the stimulation of PIP_2-phospholipase C induced by 1 μM bombesin. The results are expressed as nmol PIP_2 hydrolysed/min/mg protein and represent the means ± SD of five different experiments.

HNE on guanosine-5'-O-(3-thio)triphosphate (GTPgammaS) induced -PL-C activity.

The ability of GTP or nonhydrolysable GTP analogues, such as GTPgammaS, to stimulate PL-C activity has been demonstrated in liver and in other tissues by several authors [16–18]. These nucleotides also play an important role in the acceleration of PI turnover induced by hormones [19].

All the aldehydes able to stimulate the basal activity of the enzyme could increase also the activity induced by 10 μM GTPgammaS. As far as HNE and HOE are concerned, the highest degree of synergism with the nucleotide occurred at aldehyde concentrations identical to those producing the strongest stimulation of the basal activity when used alone. HEE effect on PL-C was only slightly enhanced by GTPgammaS.

It is well known that aldehydes easily react with -SH groups [1, 2]; it appeared therefore interesting to check if their action could be modified in the presence of reduced glutathione (GSH).

Figure 4 shows the influence of 1 mM GSH on the action of 10^{-7} M HNE, the major 4-hydroxyalkenal produced during lipid peroxidation [3, 20]; the assays were performed both in the presence and in the absence of 10 μM GTPgammaS. This HNE concentration was chosen because, as shown in Figs 1 and 3, it was the lowest dose able to induce a strong increase of the enzymatic activity both in the presence and in the absence of the nucleotide.

GSH induced a slight, not significant increase of PL-C basal activity, while it had no effect on GTPgammaS stimulated activity. GSH was unable to produce any change in the stimulatory effect induced by HNE.

Figure 5 shows the influence of 10^{-7} M HNE on the stimulation of PL-C activity by 1 μM bombesin. The hormone induced a small, but significant increase in PIP_2 breakdown ($P < 0.05$); the degree of its stimulation was about 30%, while HNE alone stimulated by 55%. The simultaneous presence of both compounds induced about a two-fold increase of PL-C basal activity.

DISCUSSION

This study demonstrates that HNE is neither the only nor the most active lipid peroxidation product able to stimulate PL-C activity; in fact HOE strongly increased both the basal and the GTPgammaS stimulated activity at much lower concentrations.

The well known modulation of PL-C activity by guanine nucleotides led most authors to the hypothesis that the hormones able to induce PIP_2 breakdown bound to membrane receptors coupled to PL-C through a G protein, although this protein has not been identified as yet [16–18].

The ability of HEE, HOE and HNE to increase the GTPgammaS induced PL-C activity suggests that also the action of these compounds might be mediated by a regulatory G protein.

There are several possible explanations for the mechanism of action of these aldehydes: first of all, HNE, HOE and HEE could act as agonists of a receptor coupled to PL-C; however there are other possibilities: the aldehydes might act on the enzyme itself or on the G protein which is thought to regulate PL-C activity.

Moreover, on the basis of their chemical structure, another possible mechanism of action has to be considered. 4-Hydroxyalkenals have both a lipophilic and a hydrophilic portion; since the method we used for PL-C measurement employs exogenous PIP_2 added as substrate to isolated membranes, these

aldehydes might enhance the access of the substrate to the enzyme.

The results described in Fig. 1 do not favour the last interpretation; in fact there is no correlation between the chain length of the aldehydes studied, their degree of lipophilia, and the degree of PL-C stimulation. The most lipophilic compound, HUE, was completely inactive and HNE, which displays a high degree of lipophilia, was completely inactive at doses lower than 10^{-9} M, whereas the less lipophilic HOE was able to induce a strong stimulation of PL-C at much lower doses.

Furthermore, HNE effects on PL-C cannot be related to a change in membrane fluidity since HNE concentrations lower than 10^{-5} M fail to alter membrane fluidity as measured by an ESR technique [5].

The inability of GSH to prevent HNE induced acceleration of PI turnover, even if added at a concentration 10,000-fold higher than HNE, seems to indicate that the aldehyde effect is not due, at least in this case, to its reaction with -SH groups.

The differences seen in the activity of the aldehydes used are unlikely to depend upon differences in their metabolism by liver membranes. In fact the aldehydes are rapidly metabolized by intact cells, but the degree of their catabolism is much lower in isolated membranes [20].

It is noteworthy that the doses of HNE effective on PL-C activity can be actually found in some tissues and body fluids [21, 22]; therefore the possibility exists that HNE and related aldehydes might stimulate *in vivo* this enzymatic activity. Some evidence supporting this hypothesis come from studies on the role of aldehydes in inflammation [23, 24]: in fact several hydroxyalkenals increase the oriented migration of rat polymorphonuclear leucocytes and it is well known that chemotactic agents, such as leukotriene B_4 and formyl-methionyl-leucyl-phenylalanine can accerate PI turnover [25, 26].

Further investigations are needed to elucidate the mechanism by which 4-hydroxyalkenals influence PL-C and adenylate cyclase activities, but their ability to act at very low concentrations which can actually be produced by tissues, taken together with their high diffusibility both inside and outside the cells [20] seems to make possible the perspective of defining them as a new class of autocoids.

The ability of liver membranes to respond to bombesin appears to be important at the light of the recent discovery that this growth factor can be produced by preneoplastic nodules and hepatomas induced in rats by a combination of diethylnitrosamine and 2-acetylaminofluorene [27].

The presence of an additive synergism, at least *in vitro*, between bombesin and HNE suggests that the rate of PI turnover in tissues responsive to this growth factor might depend both on the degree of bombesin stimulation and on the endogenous production of 4-hydroxyalkenals and, consequently, on cellular lipid peroxidation rate.

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