Regulation of the biosynthesis of acyl analogs of platelet-activating factor by purinergic agonist in endothelial cells

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Abstract We have previously shown that platelet-activating factor (PAF)-dependent transacetylase (TA) contains three catalytic activities, namely PAF: lysophospholipid TA (TA_L), PAF: sphingosine TA (TA_S) and PAF acetylhydrolase. It serves as a modifier of PAF actions by producing different lipid signal molecules. The TA_L activity is involved in the biosynthesis of acyl analogs of PAF (acyl-PAF, 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine, acylacetyl-GPC) in agonist-stimulated endothelial cells. In the present investigation, we have studied the mechanism(s) by which the TA activity is regulated in ATP-treated endothelial cells. We have demonstrated that ATP, and thiol-modifying agents with ATP, specifically regulate only the TA_L part of the TA activities.

Key words: Platelet-activating factor; Transacetylase; Acetylhydrolase; Acyl analog of PAF; Translocation; Endothelial cell

1. Introduction

We have identified two enzyme activities, namely, platelet-activating factor (PAF): lysophospholipid transacetylase (TA) (TA_L) and PAF: sphingosine TA (TA_S), that can modify PAF functions by converting PAF to analogs of PAF and N-acetylsphingosine (C_2-ceramide), respectively [1,2]. Analogs of PAF, such as acyl analogs of PAF, are the predominant components in activated endothelial cells, mast cells and basophils [3], and in two B-lymphoblastoid cell lines [4]. The generation of acyl analogs of PAF is also detected in stimulated primary cultures of human keratinocytes [5]. They act as the competitor of PAF and have their own unique biological property [6]. C_2-ceramide, used widely by many investigators as a permeable analog of long-chain acyl ceramide, is involved in apoptosis, cell cycle arrest and senescence [7–9]. We have shown that C_2-ceramide is a naturally occurring compound and that TA_S is responsible for its biosynthesis [2]. The cellular concentration of C_2-ceramide (micromolar) is in the similar concentration range that elicits its biological effects [2].

To establish the structural and functional relationships between TA_L and TA_S, we have purified TA_L to apparent homogeneity from rat kidney membrane and cytosol with a molecular mass of 40 kDa [10]. The amino acid sequences of peptides isolated from tryptic digest of the purified TA_L from both membranes and cytosol share total homologues with those of deduced amino acid sequences obtained from the cDNA of rat intracellular cytosolic PAF acetylhydrolase (AH) II [11,12]. In addition, purified TA_L from rat kidney membranes cross-reacts with a mouse monoclonal antibody against human PAF AH II [13]. The most intriguing of the findings is that the purified TA contains all three enzymatic activities, namely, TA_L, TA_S and AH [10].

We have elucidated that TA_L is activated through direct/indirect phosphorylation by several agonists, including ATP, in endothelial cells [6]. The induction of this enzyme is partly responsible for the increased incorporation of [^{3}H]acetate into acyl analogs of PAF. The purpose of the present investigation is to use this cellular system to determine whether the TA_L portion of the TA activities is specifically enhanced and the mechanism(s) responsible for regulating this specificity. We examined the effects of ATP on these three separate activities of TA and their intracellular movements. In addition, the effects of thiol-modifying agents on the biosynthesis of acyl analogs of PAF were investigated.

2. Materials and methods

2.1. Materials

1-*O*-Hexadecyl-2-acetyl-GPC, ATP, BSO (L-buthionine-(*SR*)-sulfoximine), *N*-acetylcysteine (NAC), diamide (DA) and N-acetylsphingosine (C_2-ceramide) were obtained from Sigma. 1-*O*-Alkyl-2-acetyl-GPC was from Avanti. Alkenyllyso-*sn*-glycero-3-phosphoethanolamine was a product from Serdary Research Lab. 1-*O*-Hexadecyl-2-[^{3}H]acetyl-GPC (13.5 Ci/mmol) and 1-*O*-[1′,2′-^{3}H]hexadecyl-2-acetyl-GPC (60 Ci/mmol) were purchased from NEN Life Science Products. Sphingosine was bought from Matreya, Inc. All culture reagents were from Life Technologies, Inc.

2.2. Cell culture and isolation of membrane and cytosolic fractions

Calf pulmonary artery endothelial cells (CPAE, CCL-209) between passages 19 and 25 were used for experiments as described [6]. Homogenates were prepared from confluent cells treated with or without 1 mM ATP for 5 min [6]. Postnuclear fraction ($500\times g$ supernatant) was centrifuged at $100\,000\times g$ for 60 min to isolate the pellet as the membrane fraction and the supernatant as the cytosolic fraction. The protein of the isolated fractions was determined by the method of Lowry et al. [14].

2.3. Determination of the rate of transfer of [^{3}H]acetyl group from alkyl[^{3}H]acetyl-GPC into acyl[^{3}H]acetyl-GPC

Monolayers of cells twice washed with 10 ml of Hanks' balanced salt solution–10 mM HEPES (pH 7.2) were incubated with 2.0 or 2.5

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Abbreviations: PAF, platelet-activating factor; TA_L, PAF: lysophospholipid transacetylase; TA_S, PAF: sphingosine transacetylase; AH, acetylhydrolase; Acylacetyl-GPC, 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine; BSO, L-buthionine-(*SR*)-sulfoximine; DA, diamide; NAC, *N*-acetylcysteine

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μCi of hexadecyl[^{3}H]acetyl-GPC in the presence or absence of 1 mM ATP for 10 min. Isolation and purification of radyl[^{3}H]acetyl-GPC from the lipid extracts of the control and treated cells and determination of the distribution of the subclasses (alkyl-, acyl- and alkenyl-) of radyl[^{3}H]acetyl-GPC were as previously described [6].

2.4. Determination of the rate of incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC

Monolayers of CPAE cells in complete culture media were incubated with or without 250 μM BSO for 24 h, and then washed monolayers of cells were incubated with 25 μCi of [^{3}H]acetate in the presence or absence of 1 mM ATP for 10 min as described [6]. The CPAE cells were treated similarly with NAC (10 mM) and DA (0.5 mM) except the incubation time was 1 h in serum-free media. The amounts of [^{3}H]acetate incorporated into radyl[^{3}H]acetyl-GPC and subclasses of radyl[^{3}H]acetyl-GPC were measured in these cell samples with different treatments according to the methods outlined [6].

2.5. Determination of degradation of PAF to lyso-PAF

Treatment of CPAE cells with ATP and DA was identical to the conditions described in Section 2.4 except that [1′,2′-^{3}H]hexadecyl-acetyl-GPC (2.5 μCi) instead of [^{3}H]acetate was incubated with the cells for 10 min. Various cell samples were then extracted for lipids [15]. The amounts of [1′,2′-^{3}H]PAF converted to [1′,2′-^{3}H]lyso-PAF in the lipids were determined by thin-layer chromatography and liquid scintillation counting [6].

2.6. TA assays and PAF AH assay

TA_L and TA_S activities were determined as established before [1,2,6,10]. PAF AH was determined according to our previously described method [16].

3. Results

3.1. Effects of ATP on the TA_L, TA_S or AH activity

We have reported [6] that the specific activity of PAF: acyllyso-GPC TA (TA_L) in endothelial cells is rapidly stimulated 4-fold by ATP through direct/indirect phosphorylation. The activation of this enzyme may contribute to the increased incorporation of [^{3}H]acetate into acyl analogs of PAF in these cells. On the other hand, the ATP had a minimal effect on the PAF AH activity. In the present study, we show that the TA_S activity of the TA is also not influenced by ATP treatment of the endothelial cells. The specific activity of TA_S is 0.20 ± 0.01 nmol/min/mg protein (means ± S.E.M., $n = 3$) in the untreated cells and 0.22 ± 0.02 nmol/min/mg protein (means ± S.E.M., $n = 3$) in ATP-treated cells.

3.2. Effects of ATP on the transfer of acetyl group from hexadecyl[^{3}H]acetyl-GPC to acyl[^{3}H]acetyl-GPC

The activity of lyso-PAF: acetyl-CoA acetyltransferase, which can be responsible for the biosynthesis of both PAF and acyl analogs of PAF, is likewise stimulated by ATP [6]. Therefore, it is possible that the increased incorporation of [^{3}H]acetate into acyl analogs by ATP may be due to the activation of lyso-PAF: acetyl-CoA acetyltransferase alone. In order to rule out this possibility, we determined the effect of ATP on the rate of transfer of the [^{3}H]acetyl group from hexadecyl[^{3}H]acetyl-GPC into acyl[^{3}H]acetyl-GPC as an index for the TA_L activity in intact cells. When hexadecyl[^{3}H]acetyl-GPC was incubated with endothelial cells in the presence or absence of ATP as described in Section 2, we found that the same amounts of hexadecyl[^{3}H]acetyl-GPC were incorporated into untreated and ATP-treated cells (49 ± 2.6% (means ± S.E.M., $n = 3$) vs. 48 ± 4.9% (means ± S.E.M., $n = 3$), respectively). However, the amounts of [^{3}H]acetyl group transferring from hexadecyl[^{3}H]acetyl-GPC into acyl[^{3}H]acetyl-GPC increased from 4.1 ± 0.7% (means ± S.E.M., $n = 3$) in the control cells to 9.0 ± 0.6% (means ± S.E.M., $n = 3$) in ATP-treated cells ($P < 0.001$). These results indicate that the induction of TA_L activity is indeed reflected in the enhanced biosynthesis of acyl analogs of PAF in ATP-treated cells and ATP does not affect the AH activity.

3.3. Effects of thiol-modifying agents on the incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC and its subclasses

Several recent reports [17–19] have indicated that the thiol (i.e. glutathione) level regulates the cytokine-mediated apoptosis and the degradation of sphingomyelin to ceramide. Moreover, AH activity of the TA protects oxidant stress-induced cell death [20], and TA_S activity of TA may participate in

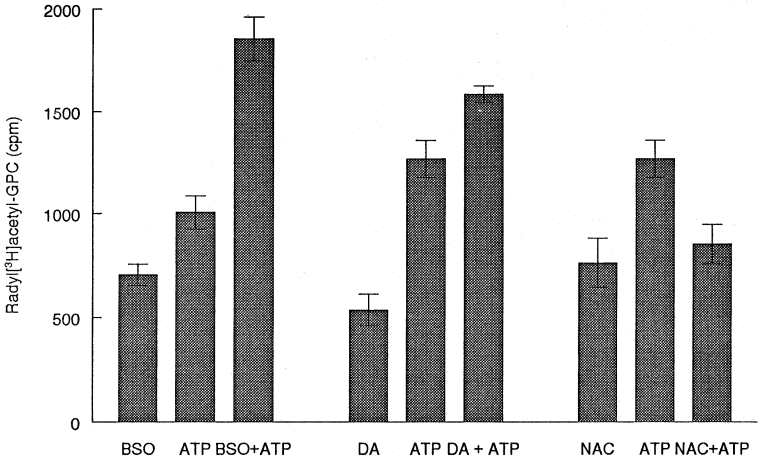

Fig. 1. Effect of BSO, DA and NAC on ATP-stimulated incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC. Monolayers of CPAE cells were incubated with 250 μM BSO in complete media for 24 h, with DA (0.5 mM) or NAC (10 mM) in serum-free media for 1 h, and then further incubated with [^{3}H]acetate in the presence or absence of 1 mM ATP for 10 min before termination of the reactions and isolation of the products as described under Section 2. Data are presented as means ± S.E.M. ($n = 3$). The P values between cells treated with ATP and cells treated with ATP and thiol-modifying agents (BSO/DA) were < 0.05. There was no difference in P values between NAC- and NAC+ATP-treated cells.

apoptosis. Therefore, we studied the effects of thiol level-modified agents on the incorporation of [^{3}H]acetate into PAF analogs to evaluate the influences of these agents on the TA_L activity. Data in Fig. 1 depict the incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC of BSO/ATP-, DA/ATP- and NAC/ATP-treated cells and their respective control cells. Both BSO and DA are thiol oxidizing reagents, while NAC is a thiol antioxidant [17,18,21]. When CPAE cells were treated with BSO and DA, the incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC increased 185% and 125%, respectively, in BSO/ATP- and DA/ATP-treated cells over that of the ATP-treated cells alone (Fig. 1). On the contrary, NAC blocked the stimulated incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC by ATP completely (Fig. 1).

The effect of thiol-modulating agents on the amounts of [^{3}H]acetate incorporated into the subclasses of radyl-[^{3}H]acetyl-GPC in control and ATP-treated cells was further analyzed. We found that BSO and DA increase the incorporation of [^{3}H]acetate into acyl[^{3}H]acetyl-GPC in ATP-treated cells, while NAC blunts the ATP effect somewhat (Fig. 2). These data demonstrate that the cellular thiol level in ATP-activated cells influences TA_L activity.

When the effect of DA on AH activity in ATP-treated endothelial cells was investigated by using the conversion of [^{3}H]PAF to [^{3}H]lyso-PAF as a parameter, we found that both DA and ATP had minimal effects on the rates of cellular uptake of [^{3}H]PAF (data not shown). Furthermore, the rate of conversion of [^{3}H]PAF to [^{3}H]lyso-PAF was not influenced by the ATP treatment or the addition of DA to ATP-treated cells (Fig. 3). Therefore, the activation of TA by ATP specifically increases the TA_L portion of the TA activity and the thiol agents only modify the induced TA activity.

3.4. Translocation of TA_L activity from cytosol to membrane in ATP-treated CPAE cells

In order to determine whether direct/indirect phosphorylation of TA_L induced by ATP [6] may cause the translocation of TA_L to the membrane, we measured the effect of ATP on the subcellular distribution of TA_L. As indicated in Fig. 4, ATP induced the translocation of TA_L from cytosol to membrane by approximately 180% (from 14.9% in the membrane fractions in untreated cells to 26.7% in the same fraction of ATP-treated cells). Concomitantly, translocation of TA_L to the membrane by ATP also caused an increase in specific activity of TA_L in membranes by 200% (89 pmol/min/mg protein in the membrane fraction of untreated cells vs. 177 pmol/min/mg protein in the membrane fraction of ATP-treated cells). The specific activity of TA_L in the cytosol remained the same in both control and treated cells (data not shown). These data indicated that induced translocation of TA_L by ATP with concomitant increase in its specific activity is a specific event, because the cellular locations of the AH activities and proteins are not affected by ATP treatment of the cells (Fig. 4).

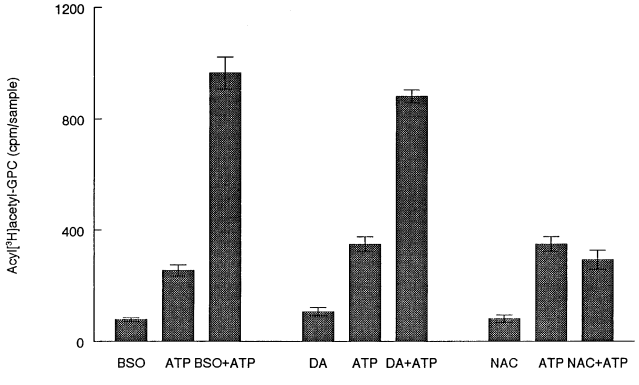

Fig. 2. Effect of BSO, DA and NAC on ATP-stimulated incorporation of [^{3}H]acetate into acyl[^{3}H]acetyl-GPC. Samples from each group in Fig. 1 were analyzed for the incorporation of [^{3}H]acetate into acyl[^{3}H]acetyl-GPC as described in Section 2.

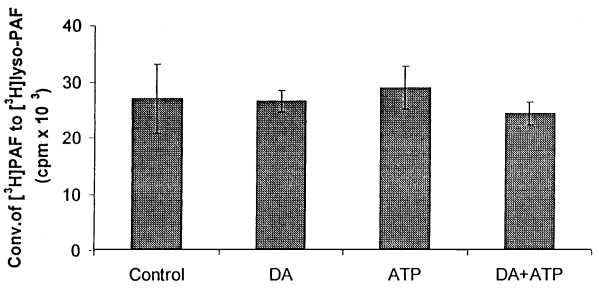

Fig. 3. The effect of DA on the conversion of [1′,2′-^{3}H]PAF to [1′,2′-^{3}H]lyso-PAF in ATP-treated endothelial cells. Monolayers of CPAE cells were treated with DA (0.5 mM) in serum-free media for 1 h before further incubation with [1′,2′-^{3}H]PAF and/or 1 mM ATP for 10 min. The methods of analyzing [^{3}H]PAF and [^{3}H]lyso-PAF were described under Section 2. Data are expressed as means ± S.E.M. ($n = 3$). Statistical analyses indicated that the differences between control vs. DA-treated group, control vs. ATP-treated group, ATP-treated vs. DA+ATP-treated group were insignificant (P values > 0.1 and higher).

4. Discussion

In this study, we showed that ATP induces only the TA_L, but not TA_S or AH activity. This in vitro observation was further validated by the experiments in intact cells. We illustrated that ATP increases the incorporation of [^{3}H]acetate into radyl[^{3}H]acetyl-GPC (Fig. 1). This increase was mostly reflected as an increase in the [^{3}H]acetate incorporation into

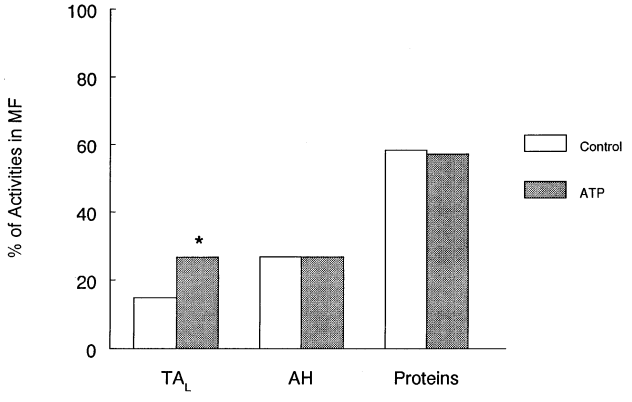

Fig. 4. Effect of ATP on the subcellular distributions of TA_L, AH and protein in CPAE cells. Cytosols and membrane fraction (MF) were isolated from postnuclear fractions of the CPAE cells that were treated with or without ATP (1 mM) for 5 min and used to determine TA_L, AH and protein as described under Section 2. Data are representative of four separate experiments that showed similar results.

acyl[^{3}H]acetyl-GPC (Fig. 2). Consistent with these data, we further demonstrated that the conversion of hexadecyl[^{3}H]acetyl-GPC into acyl[^{3}H]acetyl-GPC was also increased by the treatment of endothelial cells with ATP (see Section 3.2). Again, the hydrolysis of [1′,2′-^{3}H]hexadecylacetyl-GPC to [1′,2′-^{3}H]hexadecyllyso-GPC (a marker for AH, Fig. 3) in intact cells was not affected by ATP. These results indicate that the activation of TA by ATP causes the specific stimulation of TA_L activity in the TA.

Our data indicated that modulation of thiol levels in the cells also affects TA activities (Fig. 1). The induction of TA_L activity in ATP-treated endothelial cells was further increased by thiol oxidants (BSO and DA), and inhibited by antioxidant (NAC) (Fig. 1). On the other hand, a thiol agent such as DA had no effect on the AH activity of the TA (Fig. 3). These results likewise imply that posttranslational modification of the TA by ATP, such as phosphorylation of the enzyme, regulates the specificity of TA activities in intact cells.

Along with the TA_L activity, but not AH and TA_S activities, being stimulated by ATP, there was a corresponding translocation of TA_L from cytosol to membrane (Fig. 4). It is feasible, but as yet needs to be proven, that phosphorylation of TA_L by ATP treatment of the cells [6] induces the translocation of TA to the membranes with a concurrent increase in enzymatic activity. On the other hand, it is also feasible that modification of TA, and subcellular translocation of TA each or in combination, regulates the catalytic activities of TA.

Matsuzawa et al. [20] also reported that intracellular AH II translocated from cytosol to membranes when MDBK cells were treated with oxidants. Conversely, AH II was translocated from membranes to cytosol when cells were treated with antioxidant [20]. They suggested that intracellular AH II translocates between cytosol and membrane in response to the redox state of the cells and protects the cell against oxidative stress most probably by hydrolyzing oxidized phospholipids. However, they did not determine the effect of subcellular translocation of AH II on its enzyme activity.

Our work suggests that TA regulated by various agents, including purinergic agonists and oxidant stress, can be responsible for some of the diverse biological functions elucidated by PAF. These effects generated in the cells through TA do not need the participation of intracellular PAF receptor(s). Currently, we are studying the mechanism(s) by which a variety of agents modify the different activities of TA.

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