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EFFECT OF ADENINE NUCLEOTIDES ON CYCLOOXYGENASE AND LIPOXYGENASE ENZYME PRODUCTS OF ARACHIDONIC ACID IN HUMAN PLATELETS

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Abstract—Nucleotides are known to enhance cyclooxygenase product formation in several tissues and, in addition, are believed to function as cofactors for mammalian 5-lipoxygenases. Since nucleotides are released by stimulated platelets and by damaged tissue, we examined the hypothesis that nucleotides can affect the metabolism of arachidonic acid (AA) in washed human platelets. The various nucleotides were given 15 sec prior to the addition of 3 μM arachidonic acid and 1 μCi [^{3}H]AA. We found that the phosphorylated adenine derivatives (ATP, ADP, and AMP) increased the formation of 12-hydroxyeicosatetraenoic acid (12-HETE) by 2-fold without altering the formation of cyclooxygenase products. Adenosine was without effect on 12-HETE formation. ATP also stimulated 12-HETE formation in lysed platelets. This suggests that the 12-lipoxygenase enzyme of platelets can be regulated by adenine nucleotides. We next determined the portion of the nucleotide molecule responsible for the enhanced 12-lipoxygenase activity of platelets. Alteration of the nucleotide base led to a decrease in stimulation, with GTP less active than ATP, and UTP even less active than GTP. Studies with adenine nucleotides showed that the length of the phosphate chain was not important. We also found that the stable methylene isosters of ATP (α,β-methylene ATP and β,γ-methylene ATP) increased 12-HETE formation, suggesting that the conformation and hydrolysis of the phosphate chain are not responsible for the stimulatory activity. Cyclic 3′,5′AMP and 3′AMP were inactive, implying the necessity for a free phosphate at the 5′ position for nucleotide stimulation of 12-HETE synthesis. In conclusion, platelet 12-lipoxygenase was stimulated by ATP, as is true for several mammalian 5-lipoxygenases. However, cyclooxygenase product formation by platelets was not altered by nucleotide addition. These studies suggest that following *in vivo* injury or platelet aggregation, when local concentrations of nucleotides are high, platelet lipoxygenase activity may be stimulated.

The involvement of prostaglandins in the actions of ATP and ADP has been inferred by indomethacin's inhibition of the effects of these nucleotides on smooth muscle function [1–4]. In addition, perfusion of the vasculature with ATP or ADP, but not with AMP or adenosine, enhances prostaglandin formation [5–10]. A tissue-specific pattern of prostaglandin biosynthesis has been observed in rabbit heart and kidney perfused with [^{14}C]arachidonic acid ([^{14}C]AA)† and ATP, thus directly demonstrating an increase in prostaglandin biosynthesis in response to exposure to adenine nucleotides [4].

Adeninine nucleotides, which are released extracellularly by damaged tissue [11, 12], by the vasculature [13], and by platelets [14], may influence neurotransmission, vascular tone, cardiac function, and platelet aggregation [11, 15]. Certain conditions such as traumatic shock, burns, lacerations, and incisions cause cell disruption which can result in exposure of the adjacent tissue to near intracellular concentrations of nucleotides [16]. Platelet dense granule concentration of both ATP and ADP is approximately 400 mM [17, 18]. Therefore, upon stimulation of the release of ATP and ADP from degranulated platelets other nondegranulating platelets and the nearby vasculature could be exposed to millimolar concentrations of the released adenine nucleotides. Because ATP increases the formation of prostaglandins in several tissues and is released from platelets in large quantity, we have examined the effect of ATP on the metabolism of [^{3}H]AA by washed human platelets in order to examine the hypothesis that platelet ATP is involved in the regulation of platelet arachidonate metabolism.

MATERIALS AND METHODS

Materials. ATP, ADP, AMP, GTP, GDP, cAMP, ribose, ribose phosphate, pyrophosphate, α,β-methylene ATP, β,γ-methylene ATP, and adenosine were obtained from the Sigma Chemical Co. (St Louis, MO). Glass distilled methanol and ethyl acetate were purchased from Burdick & Jackson (Muskegon, MI). Water was deionized and purified with a Milli-R/Q Water Purification System (Millipore, Bedford, MA). Authentic tritiated AA and AA metabolite standards were obtained from New England Nuclear (Boston, MA).

Platelet preparation. Blood (50 ml) was drawn from the antecubital vein of healthy adult human

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† Abbreviations: AA, arachidonic acid; TxB_2, thromboxane B_2; HHT, 12-hydroxyheptadecatrienoic acid; and 12-HETE, 12-hydroxyeicosatetraenoic acid.

volunteers who had not ingested any medications in the preceding 10 days. The blood was drawn into a syringe containing 5 ml of 3.8% trisodium citrate, pH 7.4, and washed platelets were prepared by the method of Hamberg *et al.* [19]. The final resuspension was in pH 7.4 Krebs–Henseleit buffer which was made without calcium.

Incubation and extraction procedure. Aliquots of washed platelets (0.5 ml, 1×10^8 platelets) were equilibrated in siliconized glass cuvettes in a dual channel Payton aggregometer (Payton Electronics, Buffalo, NY) at 37° for 3 min prior to drug addition. Lysed platelets were prepared by three cycles of freezing and thawing.

The nucleotide to be tested was added to the platelet suspension 15 sec before addition of the AA substrate. Unlabeled arachidonic acid (NuCheck Prep, Elysian, MN) at a final concentration of 3 μM AA and 1 μCi[^{3}H]AA (240 Ci/mmol) were combined and prepared as the sodium salt. Our previous, unpublished experiments showed that at this AA concentration the cyclooxygenase enzyme is not saturated and, therefore, increases or decreases in both cyclooxygenase (CO) and lipoxygenase (LO) metabolism could be detected [20]. Aggregation was monitored throughout the experiment, and secondary aggregation was not observed for any of the agents tested.

The AA substrate (10 μl) was added to the platelet suspension, and the incubation terminated after 5 min by the addition of 0.4 ml of acetone. The platelet suspension was then placed on ice and the pH lowered to 3 with formic acid. Next, the reaction mixture was extracted twice with 3 ml of ethyl acetate and the extracts were pooled and dried under nitrogen. The residue was dissolved in 2 ml of methanol and filtered through a 0.45 μm filter (Millipore, Bedford, MA).

HPLC analysis. The sample (0.5 ml) was injected by a WISP 710B autosampler onto a 30 cm × 3.9 mm μBondapak C_{18} 10 μM particle size reverse-phase column with a phenyl Corasil packed guard column (Waters Associates, Milford, MA). Elution was accomplished in less than 1 hr with two solvents which were pumped at a total flow rate of 2 ml/min. Two model 6000A Waters pumps (Waters Associates) were employed. Solvent A was distilled water containing 0.1% glacial acetic acid and 0.15% ammonium hydroxide, pH 6.2. Solvent B was methanol.

Initial conditions were 45% methanol and 55% solvent A. The elution program consisted of a 10-min isocratic elution of initial conditions, followed by a 20-min linear gradient from 45 to 85% methanol. The column was next eluted with 100% methanol for 10 min, followed by a 10-min re-equilibration at initial conditions before the next sample was injected. The gradient elution was controlled by a Waters 720 System Controller (Waters Associates). The column eluate was directed into an on-line radiometric detector (Ramona LS from IN/US, Fairfield, NJ). Within the detector the eluant was split and half mixed with scintillation fluid (Budget Solv, RPI, Mt. Prospect, IL) in a 3:1 ratio of scintillant to eluant. Peak identity was established by comparison with the chromatographic mobility of authentic radiolabeled standards. The retention time of the standards was: TxB_2, 8 min; HHT, 22 min; 12-HETE, 26.5 min; and AA, 31 min. HHT was identified by comparison with a standard that had been identified as HHT by gas chromatography/mass spectrometry.

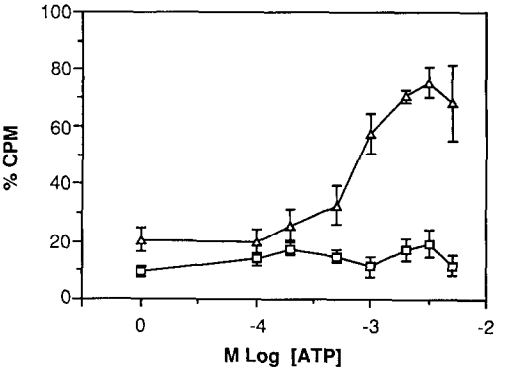

Fig. 1. Effect of ATP on arachidonic acid metabolism in washed human platelets. Platelets (0.5 ml, 1×10^8 platelets) were incubated for 5 min with 3 μM AA and 1 μCi [^{3}H]AA in the absence or presence of increasing concentrations of ATP. ATP had no effect on cyclooxygenase metabolites [thromboxane B_2 and 12-hydroxyheptadecatrienoic acid (□)] but caused a significant ($P < 0.05$) elevation in lipoxygenase metabolites (12-hydroxyeicosatetraenoic acid (△). For all figures, values are means ± SE for 3–4 different platelet preparations. Each preparation was conducted in duplicate.

Statistical analyses. An ANOVA was performed on the various treatment groups. When a significant F ratio was obtained, a Duncan's procedure was employed; otherwise a Student's *t*- or a modified Student's *t*-test was performed. In all cases, the null hypothesis was rejected at P values greater than 0.05. Data presented for each experiment are given as the mean ± the standard error of 3–4 platelet preparations. Each preparation was performed in duplicate.

RESULTS

We first examined the effect of ATP pretreatment on platelet metabolism of [^{3}H]AA by the cyclooxygenase and lipoxygenase pathway. As shown in Fig. 1, a significant elevation ($P < 0.05$) of 12-HETE formation was observed when ATP was included in the incubation medium. At the highest concentration of ATP, a 3-fold increase in 12-HETE formation was observed. ATP did not alter cyclooxygenase product (TxB_2 plus HHT) formation over the same 0.1 to 5 mM ATP concentration range.

Platelet lysates were stimulated with various concentrations of ATP to determine whether the activity of a kinase found on the platelet surface or a direct action of ATP on the lipoxygenase enzyme might be involved in the ATP-induced stimulation of 12-HETE formation. Figure 2 shows that lipoxygenase products were greater in lysed platelets, even in the absence of exogeneous ATP ($P < 0.05$). Figure 2 also shows that ATP stimulated 12-HETE formation in both whole and lysed platelet preparations. With

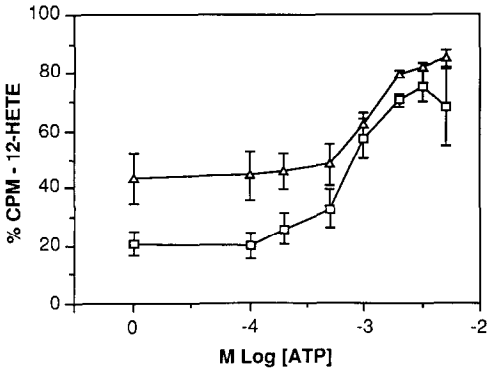

Fig. 2. Effect of ATP on arachidonic acid metabolism in whole (□) and lysed (△) washed human platelets. Lysed platelets were produced by three cycles of freezing and thawing. In the absence of ATP, lysed platelets made more 12-HETE than intact platelets ($P < 0.05$). Both lysed and whole platelets were stimulated significantly by ATP ($P < 0.05$).

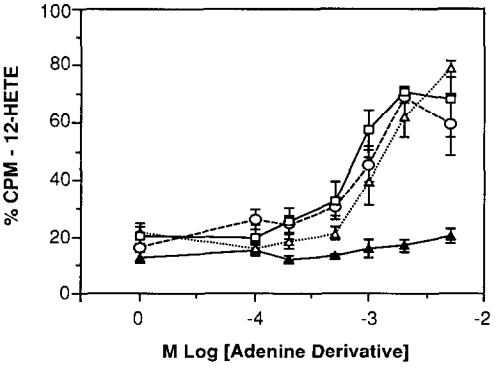

Fig. 3. Effects of adenine on derivatives on 12-HETE formation in washed human platelets. Key: (▲) adenosine, (△) AMP, (○) ADP, and (□) ATP. AMP at 1–5 mM dose-dependently enhanced 12-HETE formation above non-treated control levels ($P < 0.05$). AMP at 1–2 mM was a slightly less ($P < 0.01$) potent stimulant of 12-HETE formation compared to 1–2 mM ATP or ADP.

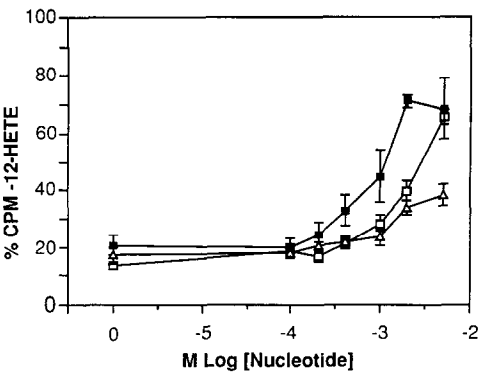

Fig. 4. Effects of nucleotide triphosphates on 12-HETE formation in washed human platelets. Key: (□) GTP, (■) ATP, and (△) UTP. Both GTP and UTP significantly stimulated 12-HETE formation ($P < 0.05$); however, most concentrations of UTP and GTP, except 5 mM GTP, had a markedly reduced effect compared to the same concentrations of ATP.

Table 1. Effects of various nucleotides and nucleotide components on the percent of radioactivity recovered as 12-HETE

Agent (5 mM)	12-HETE (%)	
	Control	Stimulated
ATP	21 ± 4	69 ± 13*
α,β-Methylene ATP	21 ± 4	56 ± 4*
β,γ-Methylene ATP	27 ± 4	51 ± 6*
Cyclic 3′,5′-AMP	26 ± 4	31 ± 5
3′,5′-AMP	28 ± 2	22 ± 2
Ribose phosphate	26 ± 4	26 ± 4
Ribose	28 ± 2	28 ± 5
Pyrophosphate	21 ± 4	17 ± 5

Washed human platelets were incubated with the various agents for 15 sec before a 5-min incubation with the 3 μM AA and 1 μCi [^{3}H]AA substrate. Values are means ± SE ($N = 3$–4).

* Significant difference in the stimulated value compared to the control ($P < 0.05$).

approximately 1 mM ATP and higher concentrations of ATP the relative stimulation of 12-HETE formation was greater in whole cells such that differences between whole and lysed cell formation of 12-HETE were no longer as readily apparent.

Figure 3 shows the effects of different adenine derivatives on 12-HETE formation in washed human platelets. ATP and ADP (0.1 to 5 mM) were not different from each other with respect to stimulation of 12-HETE formation. Since the predominant extracellular platelet metabolite of ATP is AMP, we tested AMP for a possible stimulatory effect on platelet lipoxygenase activity. Figure 3 shows that 1–5 mM AMP also dose-dependently enhanced 12-HETE formation above nontreated control levels ($P < 0.005$). AMP at 1–2 mM was a slightly less ($P < 0.001$) potent stimulant of 12-HETE formation compared to 1–2 mM ATP or ADP. At concentrations less than 1 mM, AMP was slightly less potent than ATP and ADP while adenosine was inactive at all concentrations utilized. These results show that a phosphate group is necessary for ATP's stimulation of 12-HETE formation.

Figure 4 shows the effects of several nucleotide triphosphates on 12-HETE formation in washed human platelets. GTP, and to a lesser extent UTP, stimulated 12-HETE formation, albeit less than ATP. This suggests that the nature of the base of the triphosphate is important in the degree of stimulation of the 12-lipoxygenase by nucleotides. The importance of the base in the stimulatory activity of nucleotides was further supported by the inactivity of ribose 5′phosphate, ribose, and pyrophosphate (Table 1). The orientation and availability of the phosphate group on the sugar of the nucleoside are, however, important as AMP stimulated 12-HETE formation by over 2-fold (Fig. 3), whereas 3′AMP was inactive (Table 1). In addition, the inactivity of cyclic 3′,5′ AMP (Table 1) implies that a free 5′-phosphate is necessary for the stimulation of the 12-lipoxygenase. Although a free 5′-phosphate is necessary for increased 12-HETE formation, hydrolysis of the

nucleotides is apparently not necessary since the hydrolysis-resistant methylene isosters of ATP (α,β-methylene ATP and β,γ-methylene ATP) stimulated 12-HETE formation (Table 1).

DISCUSSION

Several mechanisms exist whereby adenine nucleotides may enhance platelet 12-HETE formation. These include action at a purinergic receptor, interaction with an ectokinase, or a direct action on the lipoxygenase enzyme. Analogous to stimulation of vascular purinergic receptors, the interaction of adenine nucleotides with platelet purinergic receptors could result in enhanced AA metabolism. Two types of purinergic receptors, P_1 and P_2, have been defined by Burnstock [21] with the primary distinction being the relative potencies of ATP, ADP, AMP, and adenosine at these receptors. P_1 receptors are those sites at which adenosine and AMP are more active than ADP and ATP, while P_2 sites are defined as those sites at which ATP and ADP are preferentially active. Based upon this classification of purinergic receptors, the stimulation of lipoxygenase metabolism could occur by stimulation of a P_2 site.

Stimulation of some P_2 receptors, including those found on the vasculature, is associated with an increased formation of cyclooxygenase products [22]. We found no effect of adenine nucleotides on cyclooxygenase product formation in platelets. However, we did observe an enhancement of 12-HETE formation, which has not been examined in other systems in which P_2 receptors are activated. In fact, our results, in which little gradation was observed between the adenine nucleotides with respect to their effects on lipoxygenase activity, suggest that the mode of adenine nucleotide stimulation of 12-HETE formation is not consistent with the activation of a classical P_2 receptor.

Brown and Burnstock [22] have examined the structural components of the ATP molecule which are required for the ATP-dependent stimulation of cyclooxygenase product formation in the vasculature. They found that changes in the conformation of the phosphate chain decrease the ability of nucleotides to stimulate prostaglandin biosynthesis. In addition, they determined that stimulation of a P_2 receptor is responsible for the enhanced prostaglandin formation, since ATP and ADP, but not AMP or adenosine, could elicit this response. In our studies, the adenine nucleotides including AMP enhanced 12-HETE formation without altering cyclooxygenase product formation, providing further evidence that stimulation of this type of P_2 receptor is not responsible for the stimulatory action of the adenine nucleotides.

Two sites on platelets are sensitive to ADP [23]. At the first site, ADP induces aggregation while ATP acts as an antagonist of ADP action. Since both ATP and ADP enhance 12-HETE formation, interaction with this ADP site does not explain the stimulation of 12-HETE formation by these nucleotides. Stimulation of the other site with ADP or ATP results in an inhibition of adenyl cyclase and AMP is much less active than either ATP or ADP at this site [6]. Since we observed very little difference between the effects of AMP, ATP or ADP on platelet 12-lipoxygenase activity, it is unlikely that the stimulatory effect of adenine nucleotides is mediated by activation of this second ADP sensitive site. Activation of the platelet sites responsive to ADP does not account for the stimulatory action of nucleotides on 12-HETE formation.

The ability of the hydrolysis-resistant methylene isosters of ATP to stimulate 12-HETE formation suggests that hydrolysis is not responsible for this action of the nucleotides. Therefore, increased ATPase activity cannot explain the increase in 12-HETE formation.

A second possible mechanism by which ATP could act to enhance platelet 12-lipoxygenase activity is by interaction with an ectokinase. Ectokinases exist on the outer surface of cell membranes and degrade extracellular nucleotides. Several cell types including polymorphonuclear leukocytes [24], macrophages [25], hepatocytes [26], fibroblasts [27] and adipocytes [28] possess surface kinases. In fact, platelets are known to exhibit ectonucleoside diphosphokinase activity [29]. Ectokinases lack specificity for the different nucleotide triphosphates [13, 30]. We, however, found ATP to be more active than GTP or UTP in stimulating 12-HETE formation. This suggests that stimulation does not occur through an effect on ectokinase activity. In addition, we observed stimulation of 12-HETE formation at the same concentration of ATP in whole and lysed platelets. Cell lysis should dilute the ability to observe a surface event, because numerous cytosolic reactions compete for ATP [31]. Our demonstration of ATP stimulation of 12-HETE formation in platelet lysates, compared to whole platelets, should not be observed if the stimulation of lipoxygenase activity by ATP were due to the stimulation of a surface kinase [13]. Also, ectokinase activity is not known to transduce intracellular signal generation and, therefore, may not be an important mechanism by which ATP can stimulate platelet 12-HETE formation.

Finally, ATP could act directly on the 12-lipoxygenase enzyme to enhance its activity. This may be occurring in our experiments since we observed nucleotide stimulation of 12-HETE formation in a lysed platelet preparation. Precedence for this action of nucleotides exists for several 5-lipoxygenases. For example, Ochi *et al.* [32] found ATP to stimulate the calcium-dependent 5-lipoxygenase activity of guinea pig neutrophils. Similar to our results with the platelet lipoxygenase, Ochi *et al.* [32] tested GTP, UTP, AMP, and cAMP on the partially purified 5-lipoxygenase activity of polymorphonuclear leukocytes and found them to be less active than ATP. Unlike our results in which the 12-lipoxygenase was equally stimulated by ADP and ATP, Ochi *et al.* [32] found ADP to be less active than ATP at increasing 5-lipoxygenase activity. In addition, several purified 5-lipoxygenases have been found to be dependent on ATP, as well as calcium, for maximal activity [26, 33, 34].

The manner by which ATP could directly act on the cytosolic platelet lipoxygenase in our whole platelet preparation is not known, although an alteration

in membrane permeability is one possible explanation. Exogenous ATP has been shown to increase membrane permeability [35–37]. Since we observed an increase in 12-HETE formation with nucleotides other than ATP, it is unlikely that the nucleotides gain access to the cytosolic lipoxygenase enzyme by increasing membrane permeability. A second manner by which nucleotides can gain access to the cytosol is by carrier-mediated uptake [38–42] and an anion channel which takes up ATP has been demonstrated in the kidney [43]. Since it seems unlikely that platelets take up all of the nucleotides which stimulate 12-HETE formation, these nucleotides may act at a previously undescribed purinergic binding site.

We also determined the portion of the ATP molecule which may be responsible for the stimulation of 12-HETE formation. We showed that the formation of 12-HETE by nucleotides was sensitive to changes in the base of the nucleotide and to the position of the phosphate group. In addition, the length of the phosphate chain is not important since ATP, ADP, and AMP were similarly active. The availability of a free 5′-phosphate, however, is necessary for this activity as 3′-AMP and cAMP did not enhance 12-HETE formation.

In summary, we have shown that nucleotides including ATP stimulated 12-HETE formation in platelets by a mechanism which remains to be elucidated. Very recent studies by Krishnamurthi *et al.* [44] demonstrate that nucleotides may be involved in the inhibition of agonist-induced platelet activation and aggregation. Our findings suggest that this inhibition of agonist-induced aggregation may be due to the increased formation of 12-HETE which we have observed in the presence of nucleotides.

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