Continuous Measurements of ATP Secretion in Vivo

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Abstract: Blood was withdrawn continuously from femoral veins of anesthetized rabbits at a rate of 0.07 ml/min. Sodium citrate was pumped into the blood to prevent coagulation, and luciferin-luciferase reagent was added to permit the continuous detection of extracellular ATP. Subsequently, the red blood cells were lysed and the platelet count was recorded continuously. Injection of platelet activating factor or collagen into rabbit ear veins caused an almost immediate but short-lived increase in extracellular ATP with a simultaneous but more prolonged decrease in the platelet count. Although both the endoperoxide analog 9,11-azo-PGH_2 and ADP also decreased the platelet count, little extracellular ATP was detected after the azo-PGH_2 and none after ADP. These studies demonstrate that those agents that cause platelet secretion from rabbit platelets *in vitro* also cause secretion *in vivo*. The method described should be useful in evaluating the capacity of antithrombotic drugs to modify platelet secretion *in vivo*.

Introduction

Several potentially important substances are known to be secreted from blood platelets in response to agents such as thrombin and collagen *in vitro*. These include the adenine nucleotides (ATP and ADP), 5-hydroxytryptamine, calcium, fibrinogen, platelet factor 4, platelet-derived growth factor, and lysomal enzymes (1). Secretion from platelets is selective as the released substances are stored in very dense granules, alpha granules, or lysosomes and become exteriorized with minimal loss of cytoplasmic platelet constituents. The major evidence that platelet secretion occurs *in vivo* may be summarized as follows:

1. During the formation of hemostatic plugs, the platelets within an aggregate become degranulated (2). A corollary to this is that patients with a deficiency of stored platelet adenine nucleotides usually have bleeding problems (3).
2. The plasma levels of platelet-specific proteins are elevated in patients with diseases that cause high rates of platelet turnover (4–7). There also is a decrease in the density of the platelets from these patients (8).

It has been suggested that the secretion of platelet-derived growth factor is the pathologic mechanism necessary for the growth of atherosclerotic plaques (9). Thus, it is important to elucidate the factors (such as exposure of collagen, formation of thrombin, release of prostacyclin, presence of epinephrine) that may influence platelet secretion *in vivo* and to develop ways to inhibit secretion in patients with atherosclerosis.

We describe here a sensitive method for the continuous measurement of extracellular ATP in blood withdrawn at a very slow rate from animals, and demonstrate that ATP was secreted simultaneously with a fall in the platelet count when rabbits received bolus intravenous injections of platelet-activating factor, collagen, or the prostaglandin endoperoxide analog 9,11-azo-PGH_2.

Materials and Methods

To withdraw blood continuously from animals, we adopted the Smith, Holmes, and Freuler (9–11) method in which a pumping mechanism and double cannula were used both to infuse 3.8 % sodium citrate anticoagulant into the blood at the rate of 0.015 ml/min and to withdraw the anticoagulated blood at a rate of 0.085 ml/min. The construction of the cannula was such that, although the citrate never entered the animal, the blood was anticoagulated as soon as it entered the cannula. A schematic diagram of the system used to record ATP secretion and the platelet count simultaneously in the blood leaving the animal is shown in Figure 1. New Zealand white rabbits (male or female, 2.75 to 4 kg) were anesthetized intravenously with pentobarbitone sodium (30 to 40 mg/kg). The femoral vein was cannulated, and the continuous supply of anticoagulated venous blood was mixed with luciferin-luciferase reagent (Chrono-lume, Chrono-log Corp. Havertown, PA) just before it entered the cuvette well of a Lumi-aggregometer (Chrono-log Corp).

Because the red cells in the blood greatly quench the amount of light emitted during the reaction of extracellular ATP with luciferin-luciferase, attempts were made to maximize the input for the photomultiplier tube. The cuvette area of the Lumi-aggregometer was filled with a continuous length of polyethylene tubing (0.58 mm ID, 0.965 mm OD) wrapped in a tight coil around a core coated with a light reflecting substance. The cuvette area was sealed from external light and blood entered one end of the coil and left by the other. The dwell time of the blood in this area was 2 min. Blood leaving the Lumi-aggregometer was mixed with a solution of 1 % ammonium oxalate and 0.002 % saponin to lyse the red cells, and the number of platelets was continuously recorded using a Technicon Autocounter as described elsewhere (9–11). The platelet counter was calibrated by previously published procedures (10). The luminescence response was calibrated by introducing known amounts of ATP into the rabbit blood as described below.

In eight rabbits, immediately after the cannulation of a femoral vein, blood entering the Lumi-aggregometer produced a strong luminescence signal. However, this signal decreased continuously during the first 15 to 30 min until a steady baseline was reached. This baseline was recorded with the Lumi-aggregometer set at maximum gain indicating that once stable conditions

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On-Line Platelet Counting and ATP Recording

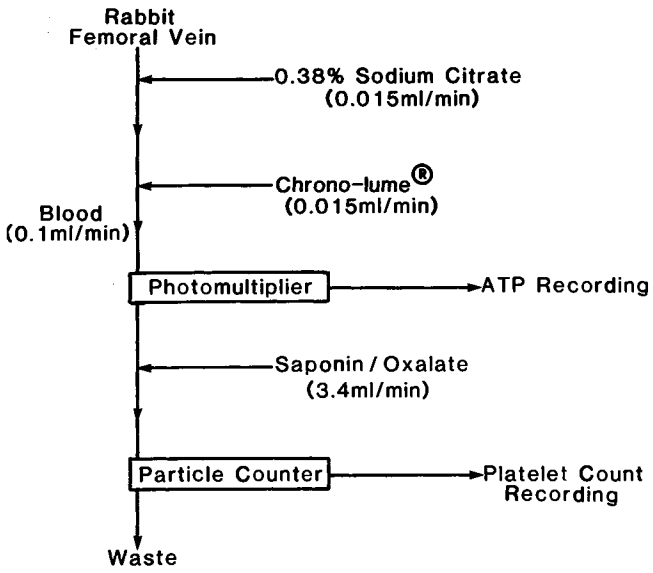

Fig. 1 Schematic representation of the system used to continuously record ATP secretion and circulating platelet counts.

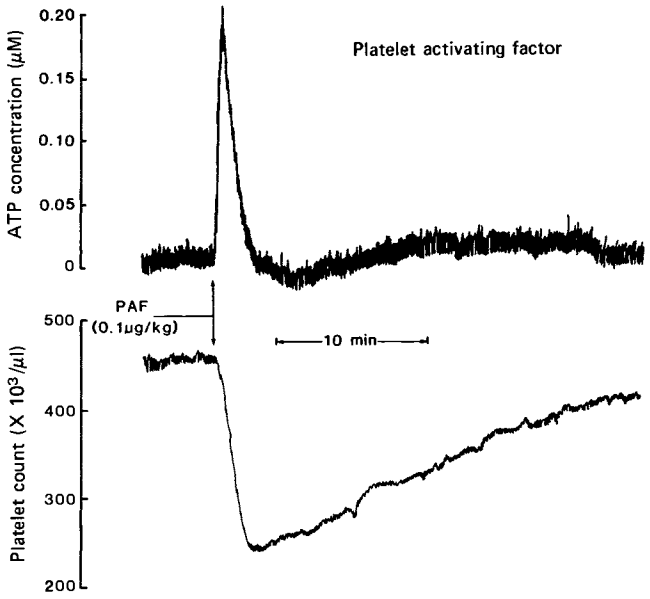

Fig. 2 Platelet activating factor-induced ATP secretion and decrease in circulating platelet counts. The concentration of ATP was determined by a calibration based on the introduction of a known amount of ATP into the citrate with which the blood was being anticoagulated.

Table I. Correlation between ATP Infusion into Rabbit Blood and Luminescence Signals.

Concentration of ATP Infused (μM)	Concentration of ATP in Blood Leaving via Double Cannula (μM)	Deflection on Chart Recorder (mm)
0.1	0.0177	19,17
0.2	0.0354	30,38
0.5	0.0880	120,96

Blood was collected by cardiac puncture of a New Zealand white rabbit into one tenth volume of 3.8% trisodium citrate. Heparin (10 units/ml) and Prostaglandin E_1 (1 μM) were added to reduce thrombin formation and platelet activation. The blood was placed in a plastic tube and continuously drawn into the double cannula as in the *in vivo* studies except that saline (rather than citrate) or known amounts of ATP dissolved in saline were introduced at a rate of 0.015 ml/min. Blood leaving via the double cannula was continuously mixed with luciferin-luciferase reagent and passed through the cuvette well of a Lumi-aggregometer. Each deflection noted in the Table represents a stable increase in light output lasting at least 5 min relative to basal light output determined when saline was infused. After each infusion of ATP, saline was reinfused to reestablish the baseline. Similar results were obtained when known amounts of ATP were introduced into the citrate used to anticoagulate blood as it was continuously withdrawn from the femoral veins of rabbits.

had been achieved, the levels of extracellular ATP were extremely low. In one experiment, blood was withdrawn through the pumping apparatus before entering the Lumi-aggregometer. This produced much stronger luminscence signals, even one hour after cannulation, indicating that the pumping mechanism damaged red cells or platelets and released ATP. In three other experiments, attempts were made to cannulate femoral arteries. However, the basal luminescence signal detected was higher than that in blood from femoral veins and was more variable. Because these higher signals may have been due to ATP released as the pulsatile arterial blood entered the cannula, we restricted further experiments to examining blood withdrawn from the femoral vein.

To calibrate the output of the Lumi-aggregometer in terms of extracellular ATP reacting with luciferin-luciferase in presence of red cells, we continuously drew citrated rabbit blood into the double cannula used for the *in vivo* studies and introduced known amounts of ATP via the tube normally used to introduce citrate (Fig. 1). These experiments indicated that the luminescence signal was directly proportional to the amount of extracellular ATP in whole blood (Table I) and that the limit of detection of this ATP was about 10^{-8} M.

A total of six rabbits received a bolus injection of platelet activating factor (Avanti, Birmingham, Alabama) at 100 ng/kg via an ear vein once basal luminescence conditions had been achieved. Within a minute of the injection, there was an increase in the level of extracellular ATP and a fall in the platelet count in all animals (Fig. 2). The increase in free ATP was transient, lasting only a minute or two, whereas the fall in the platelet count lasted for 15 minutes or more. However, the platelet count usually returned to a value close to that prior to challenge given sufficient time. In three of the rabbits studied the increases in extracellular ATP were quite large (130–600 nM ATP), whereas in the other three the increases were modest (10–60 nM ATP). These differences were not associated with differences in the platelet count (average 4.4 ± 0.3

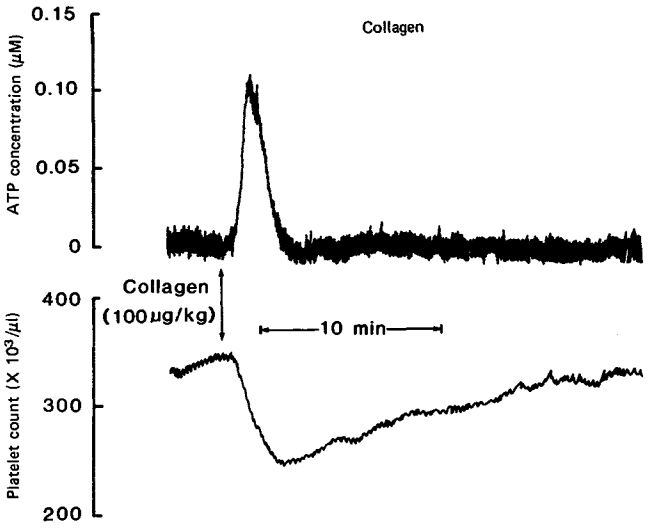

Fig. 3 Collagen-induced ATP secretion and decrease in circulating platelet counts. (Other details as in Fig. 2.)

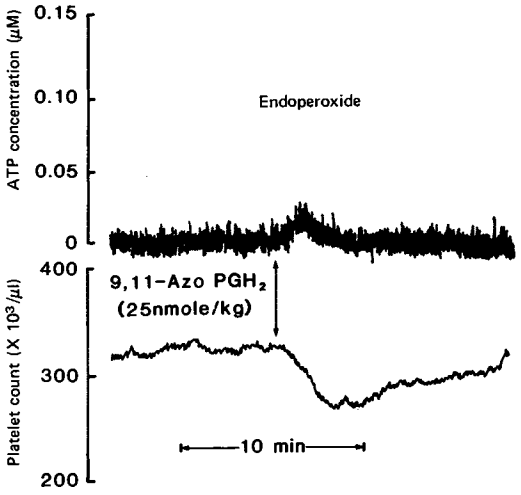

Fig. 4 ATP secretion and decrease in circulating platelet counts induced by the stable prostaglandin analog 9,11-azo-PGH_2. (Other details as in Fig. 2.)

SE × 10^8 platelets/ml) or in the fall in the platelet count after challenge with platelet activating factor (average 30 ± 5% SE) between the individual rabbits. The reason for the differences is presently unknown, but they may reflect differences in the amounts of ATP secreted or in the rates that ATP was metabolized *in vivo*.

An increase in the amount of extracellular ATP and a decrease in the platelet count were also detected in three rabbits challenged with collagen (Hormonchemie, Munich, W. Germany) at 100 μg/kg. The increases in the extracellular concentrations of ATP detected ranged from 50 to 125 nM and were slightly more prolonged than observed with platelet activating factor (Fig. 3). Furthermore, the platelet count, after falling by 10 to 30%, failed to completely return to the prechallenge values as was observed with platelet activating factor. Two rabbits were repeatedly challenged with collagen at a constant dosage, and the amount of extracellular ATP determined. In both animals, the amount of extracellular ATP detected on subsequent challenge was about half that detected during the previous challenge; for example, in one experiment the values obtained on repeated challange were 125 nM, 50 nM, and 25 nM ATP respectively. This reduction in ATP secretion was not detected during repeated challenge with platelet-activating factor (two experiments).

Only small increases in extracellular ATP (from 20–45 nM) were detected in three rabbits challenged with 9,11-azo-PGH_2 (25–40 nmol/kg, gift of Dr. Don Harris, Squibb Institute, Princetown, NJ). These increases were associated with falls in the platelet count of from 16 to 33% (Fig. 4). At doses above 40 nmol/kg azo-PGH_2 the animals died (3 experiments). In three rabbits challenged with ADP (100 μg/kg), a fall in the platelet count of 13% was consistently observed without any detectable increase in extracellular ATP.

The use of the firefly luminescence method to study the secretion of ATP on a continuous basis was first introduced by Detwiler and Feinman (14). They studied the secretion of ATP from washed human platelets induced by thrombin *in vitro*. Subsequent studies showed the firefly luminescence method was also applicable to the *in vitro* studies of ATP secretion from platelets in citrated plasma (15) or in whole blood (16).

During platelet aggregation induced by collagen, platelet activating factor and ADP *in vivo*, platelets are known to accumulate in the pulmonary microvasculature (17) explaining the fall in platelet counts observed in our studies. It was previously shown that infusion of platelet activating factor into rabbits causes the secretion of platelet factor 4, as determined by discontinous assay of plasma samples (18). We show here that the secretion of ATP can be continuously followed *in vivo*. Platelet-activating factor and collagen caused relatively large increases in the extracellular ATP content of femoral venous blood, whereas only small increases were detected with the endoperoxide analog azo-PGH_2 and no increases in extracellular ATP were induced by ADP. The extracellular concentrations of ATP detected in femoral vein blood may be much lower than may be present elsewhere in the body after platelet activation as the actual secretion of ATP is likely to take place either immediately at the site of injection of the agent or in the lungs, the arterial circulation, or the peripheral capillaries. Furthermore, ATP is rapidly metabolized *in vivo*. Indeed, the bolus injection of ATP into a rabbit ear vein failed to produce a response in the femoral venous blood passing through the Lumi-aggregometer. Nevertheless, the *in vivo* luminescence method should be useful in elucidating the factors which regulate platelet secretion *in vivo* and for the development of drugs which inhibit platelet secretion.

Acknowledgements

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High Pressure Liquid Chromatographic Analysis of Metoclopramide in Serum

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Abstract: Metoclopramide has recently been approved at dose levels of 1 to 2 mg/kg for the treatment of nausea and vomiting resulting from cancer chemotherapeutic agents. A rapid, sensitive reverse phase HPLC quantitative procedure for metoclopramide in serum is described. The method involves a single-step extraction of metoclopramide and disopyramide (internal standard) from alkalinized serum into benzene and utilizes a reverse-phase C-8 system with a mobile phase of 11 : 22 : 66, methanol : acetonitrile: pH 3.7 acetate buffer, and UV detection at 268 nm. The method is highly reproducible and has a limit of sensitivity of 2.5 ng/ml from a 2.0 ml serum sample. The method has been successfully applied to clinical pharmacokinetic studies involving administration of IV oral metoclopramide to cancer patients receiving highly emetogenic cis-diamminedichloroplatinum.

Introduction

Metoclopramide is structurally related to procainamide and has been utilized clinically for more than 10 years as an antiemetic and for disorders of gastrointestinal motility. It has been administered parenterally as an aid to GI diagnostic procedures and orally for the symptomatic relief of diabetic gastroparesis (1). Typical dosage levels are in the 0.1 to 0.3 mg/kg range, and information on its pharmacokinetics following single doses has appeared in the literature (2–6). Recently, metoclopramide has received FDA approval for the control of nausea and vomiting associated with cancer chemotherapeutic drug administration. The drug has been reported to be highly effective at 2.0 mg/kg dosage levels, repeated at 2 h intervals for up to 8 h (7). Since CNS toxicity is apparent at these dosages (8), but about 25 to 30% of patients do not respond (9), pharmacokinetic and clinical pharmacologic studies are indicated in cancer patients receiving multiple doses of metoclopramide to allow dose optimization.

Although several quantitative analytical methods for metoclopramide in serum have appeared, none meets all of the requirements in terms of sensitivity, reproducibility, cost, routines, and the turn-around time for clinical studies. Published assays include thin-layer chromatography (9), gas chromatography with electron capture (10) and mass spectrometric detection (2), normal phase HPLC (3, 4), and reverse phase HPLC (12). In this paper, we report the details of a sensitive, yet rapid, reverse phase HPLC procedure that has enabled us to initiate pharmacokinetic studies of metoclopramide in patients receiving multiple dose intravenous and oral therapy.

Materials and Methods

Chemicals and Reagents

Purified, unformulated metoclopramide and disopyramide phosphate, the internal standard, were obtained from A. H. Robbins Co. (Richmond, VA) and G. D. Searle and Co. (Chicago, Ill), respectively, and were used as analytical standards as received. Benzene, methanol, and acetonitrile were all chromatography grade (Omnisolve, Matheson Coleman and Bell, Cincinnati, OH), and all other chemicals and solvents were analytical reagent grade. Distilled water was purified by passing through a reverse osmosis four filter system (Millipore, Bedford, MA). A stock standard solution of metoclopramide was prepared in methanol at a concentration of 0.1 mg/ml. An internal standard stock solution of disopyramide phosphate was prepared in purified water at a concentration of 5.18 mg/100 ml. These were refrigerated at 4° C and found to be stable over several months.

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