J. Pharm. Pharmacol. 1994, 46: 1032–1034
Received November 26, 1993
Accepted April 18, 1994

Upregulation of antithrombotic ectonucleotidases by aspirin in human endothelial cells in-vitro

PO KAM CHEUNG, JAN VISSER*, WINSTON W. BAKKER, *Department of Pathology, and *Department of Pharmacology and Therapeutics, University of Groningen, Groningen, The Netherlands*

Abstract—Ecto ATP-diphosphohydrolase (apyrase) activity of human endothelial cells following aspirin treatment has been studied in-vitro. It was shown by HPLC analysis of supernatant samples that pre-incubation of the cultures with aspirin resulted in a significantly increased turnover of supplemented ATP into its degradation products (ADP and AMP). Enhanced expression of ectoenzyme after aspirin treatment could be observed as demonstrated by immunofluorescence-staining with monoclonal anti-apyrase antibodies. This suggests enhancement of endothelial ATP-diphosphohydrolase activity induced by aspirin. The present data obtained in human vascular cells in-vitro are in line with results from previous animal studies in-vivo, suggesting a novel cyclo-oxygenase-independent antithrombotic activity of aspirin.

Aspirin, (acetylsalicylic acid) is an analgetic, anti-inflammatory and antipyretic drug which is widely used in human medicine. Recently, potent antithrombotic activity of aspirin has been recognized, giving rise to various clinical studies dealing with prevention of thrombotic events in cardiovascular diseases (Farrell et al 1991).

The major antithrombotic action of this anti-cyclo-oxygenase drug is ascribed to its ability to inhibit synthesis of the prothrombotic agent thromboxane A_2 by activated platelets (Moncada et al 1976). Although the production of prostacyclin is also affected by aspirin, restoration of prostacyclin synthesis by nucleated endothelial cells may readily occur in contrast to the non-nucleated platelets following exposure to aspirin. This may explain the net effect of this drug in-vivo being antithrombotic rather than prothrombotic (Clarke et al 1991).

Other potent vessel wall-associated antithrombotic mechanisms have been identified. These include endothelial-derived relaxing factor (Furchgott & Zawadski 1980), and ectonucleotidase activity such as ATP-diphosphohydrolase (Lieberman et al 1977). The latter enzyme, also called apyrase, is able to hydrolyse the pro-inflammatory adenosine triphosphate (ATP) as well as the pro-aggregatory nucleotide adenosine diphosphate (ADP) (Poelstra et al 1991). The localization of this protective ectoenzyme is not confined to the plasma membrane (Wilson et al 1982), but is also detected in extracellular structures such as the glomerular basement membrane of the kidney (Hardonk et al 1985). In experimental conditions vascular ATP-diphosphohydrolase, for instance in kidney, aorta or placenta, appears to exert potent anti-inflammatory and antithrombotic actions (Bakker et al 1993), whereas the activity of this enzyme can be influenced by aspirin (Cheung et al 1991). In the present communication we demonstrate that incubation of aspirin induces upregulation of ATP-diphosphohydrolase activity in human endothelial cells in-vitro, suggesting a cyclo-oxygenase-independent action of aspirin upon the antithrombotic potential of these human vascular cells.

Materials and methods

Cell cultures. Human umbilical vein endothelial cells from eight individuals were isolated by trypsin digestion according to standard procedures with minor modifications (Jaffe et al 1973). Culture medium contained RPMI-1640, supplemented with 20% (pooled) human serum from healthy donors, 150 $\mu g\,mL^{-1}$ endothelial cell growth factor (kindly provided by Dr V. W. M. van Hinsbergh, Leiden), 100 units mL^{-1} penicillin, 100 $\mu g\,mL^{-1}$ streptomycin and 2 mM L-glutamine.

Endothelial cells derived from each donor were cultured on fibronectin-coated culture flasks, and subsequently subcultured on chamber slides (1 cm^2 per chamber) or 24-well plates (2 cm^2 per well). Isolated cultures were screened for morphology, and for the presence of von Willebrand factor by using standard immunofluorescence-staining procedures (as described below). Viability of the cells was tested by dye exclusion using trypan blue.

Experimental design. The effect of aspirin upon ATP degradation by endothelial cells was evaluated by comparison of cell cultures after aspirin treatment with their control-treated parallel cultures from the same donor.

Chromatography. After washing of the cell cultures (grown on 24-well plates; approx. 4×10^4 cells/well) with serum-free medium (RPMI-1640 supplemented with 2 $mg\,mL^{-1}$ bovine serum albumin, 37°C), confluent cultures were pretreated in 1 mL serum-free medium with or without aspirin in various concentrations (0·1, 0·5 and 1·0 mM) for 30 min at 37°C. Upon removal of the drug by washing with medium, the cultures were incubated in 1 mL fresh serum-free medium containing 250 μM ATP and 250 μM $MgCl_2$ (60 min, 37°C). Subsequently, samples of supernatants were analysed for ATP, ADP, and AMP using reversed-phase high-performance liquid chromatography (HPLC) as described by Molema et al (1992).

For each individual batch of cells the most effective dose of aspirin was evaluated. Ecto ATP-diphosphohydrolase activity of cells, reflected by the conversion of the added ATP, is expressed as relative change of ATP in supernatants of cultures with or without aspirin pretreatment.

Statistical analysis. Each experiment was performed in triplicate. Data are expressed as means ± s.d. Statistical significance was determined by Student's *t*-test (two-tailed) with $P < 0.05$ considered as statistically significant.

Immunofluorescence microscopy. The presence of ATP-diphosphohydrolase along the membranes of cultured endothelial cells was demonstrated by indirect immunofluorescence microscopy according to standard methods. Confluent cultures grown on chamberslides treated with or without aspirin were fixed with methanol at −20°C for 15 min, air-dried, and subsequently washed with phosphate-buffered saline (pH 7·4). As a first step, mouse monoclonal antibody raised against apyrase was used (Bakker et al 1993), followed by fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse antibody as a second step. Staining for von Willebrand factor was done using monoclonal

Correspondence: P. K. Cheung, Department of Pathology, University of Groningen, Oostersingel 63, 9713 EZ Groningen, The Netherlands.

Table 1. Results from a typical experiment showing nucleotides detected in supernatants of confluent cultures of human umbilical vein endothelial cells (in triplicate) after incubation with aspirin. The amount of nucleotides was measured in supernatant samples 1 h after supplementation of substrate (250 μM ATP). It can be seen that while ATP concentrations gradually decrease in a dose-dependent manner after aspirin treatment, concomitant increase of ADP and AMP occurs.

Aspirin (mM)	ATP (mM)	ADP (mM)	AMP (mM)
0	126·40 ± 14	16·40 ± 2·0	47·86 ± 2·8
0·1	113·60 ± 3·6	23·23 ± 2·4	49·74 ± 1·9
0·5	106·80 ± 6·0	24·44 ± 2·4	48·20 ± 2·9
1·0	93.60 ± 2·6	33.60 ± 1·6	52·47 ± 2·9

Values are mean ± s.d.

mouse-anti-human von Willebrand factor antibody followed by FITC-conjugated goat-anti-mouse antibody. Incubation steps with antibodies and washing procedures were carried out at room temperature (21°C).

Results

All cultures used in this study irrespective of their treatment showed positive staining for von Willebrand factor and were able to exclude trypan blue indicating intact plasma membrane of endothelial cells (results not shown). Table 1 depicts results of a typical experiment. Aspirin-treated cultures vs control-treated cultures show dose-dependent decrease of the added substrate (ATP) in culture supernatants after 1-h incubation. Simultaneously, a gradual increase of ATP degradation products (ADP and AMP) occur. Although the extent of ATP turnover in cultures from different donors appeared to vary considerably, in seven out of eight cultures tested, a decrease of ATP following aspirin treatment was observed (Fig. 1). In these individual cultures, concurrent increase of ADP and AMP in the culture supernatants occurred (results not shown). Comparison of immunofluorescence-staining patterns of confluent cultures with or without aspirin treatment revealed, in all cultures tested (n = 4), a more obvious cell membrane staining after incubation with aspirin (Fig. 2).

Discussion

In this study, the enhanced hydrolysis of supplemented ATP by endothelial cell cultures following aspirin treatment has been demonstrated.

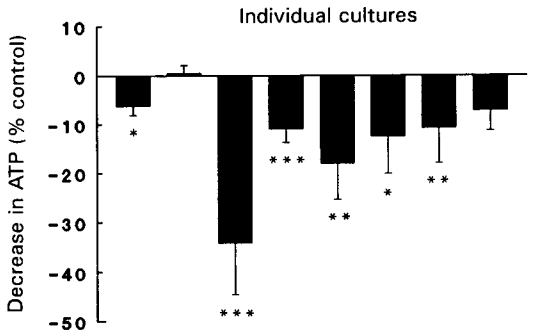

FIG. 1. Effect of aspirin treatment upon ATP-diphosphohydrolase activity of eight individual human endothelial cell cultures. Columns represent relative decrease of supplemented ATP, detected in culture supernatants of aspirin-treated cultures (n = 3) and expressed as percentage (± s.d.) of untreated (control) cultures (n = 3). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}025$, $^{***}P < 0{\cdot}005$.

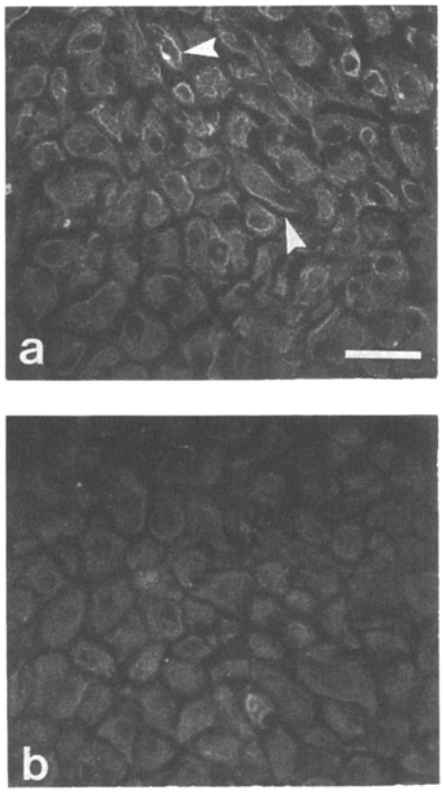

FIG. 2. Representative immunofluorescent micrographs of confluent endothelial cell cultures stained for ATP-diphosphohydrolase using mouse monoclonal antibody, followed by FITC-conjugated goat-anti-mouse antibody as a second step. Endothelial cells after treatment with aspirin (0·5 mM) show a more pronounced linear cell membrane staining (a) compared with medium-treated cells (b), illustrating enhanced expression of ectonucleotidase molecules following aspirin treatment.

The finding of decreased amounts of ATP in the culture supernatants of pre-incubation of the cells with aspirin raises the question whether this alteration reflects increased ATP-diphosphohydrolase activity, or a nonspecific exchange of intracellular nucleotides. The latter possibility is unlikely for several reasons: firstly, nucleotides do not easily cross intact cell membranes; secondly, no nucleotides could be detected in culture supernatants without substrate addition; and finally, using [^{3}H]ATP instead of the native substrate, complete recovery of the radiolabel in the nucleotides can be detected in the supernatants of both aspirin-treated and untreated cultures (results not shown). Therefore, enhancement of endothelial ecto ATP-diphosphohydrolase by aspirin seems the most likely explanation. Since the immunofluorescence staining for membrane nucleotidase shows more clear staining after aspirin treatment of the cultures, it is suggested that increased enzyme activity is supported by increased expression of ectonucleotidase molecules.

Enhanced activity of human vascular ectonucleotidase induced by aspirin is in agreement with previous observations in animal studies, also showing increased vessel wall-associated nucleotidase activity, as detected by biochemical and histochemical techniques (Cheung et al 1991). Thus, our data and those of others (Lewis et al 1977) suggest a dual antithrombotic mechanism of aspirin: on the one hand inhibition of cyclo-oxygenase leading to down regulation of platelet thromboxane A_2 and on the other hand, upregulation of vessel wall-associated ATP-diphosphohydrolase.

The mechanism of ectonuncleotidase upregulation by aspirin is unknown. Since previous results using isolated renal glomeruli or aorta fragments in-vitro show similar nucleotidase enhancement induced by other prostacyclin-inhibiting drugs (e.g. minoxidil) as well as by cAMP-increasing drugs (e.g. caffeine, isoprenaline), we feel that this effect may be mediated by the adenylyl cyclase system (Cheung et al 1991). Interestingly, both in-vitro as well as following aspirin administration in-vivo, these effects of aspirin could be observed already after a 30-min incubation period, or intraperitoneal injection, respectively.

Although Crutchley et al (1980) studied ADPase and aspirin in bovine lung endothelial cells in-vitro, their data do not contradict our present results obtained in human endothelial cell cultures. Those authors concluded that 1 mM aspirin had no inhibiting effect on the metabolism of ADP by endothelial cells. In contrast, instead of a reduction of the ectoenzyme activity, a slight enhancement of nucleotide conversion was shown, even after a short incubation period (15 min) with aspirin. Thus, from their data it also appears that aspirin is able to induce increased conversion of the added nucleotides suggesting upregulation of ectoenzyme activity.

From the present results we conclude that one of the antithrombotic mechanisms of aspirin may involve enhancement of ecto ATP-diphosphohydrolase activity of cultured human endothelial cells. This novel aspect of aspirin action may increase our understanding of this intriguing drug.

This study was supported by the J. K. de Cock Foundation, Groningen, The Netherlands. We wish to thank Dr J. J. Pratt and Mr W. Bosman for their help with the detection of radiolabelled nucleotides, and Mr H. Wierenga for performing the microphotography.

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J. Pharm. Pharmacol. 1994, 46: 1034–1035

Book Review

Ophthalmic Drug Delivery Systems
(Drugs and the Pharmaceutical Sciences/58)
Edited by Ashim K. Mitra
Published 1993 Marcel Dekker, Inc., New York
536 pages
ISBN 0 8247 8806 0 $165.00

This is volume 58 of the very successful series, under the general editorship of James Swarbrick, which has covered every conceivable area of the pharmaceutical sciences and yet still expands. This volume deals with delivery of drugs to the eye, and is a timely volume given the increasing interest in routes of delivery which offer an alternative to the oral route for the systemic delivery of biological agents, particularly those compounds which have properties which disallow oral absorption. The ocular route has been explored for the delivery of insulin, among other proteins and peptides (dealt with in two chapters), and thus requires serious consideration as a route of drug entry, which is achieved mainly via the capillary blood supply of the conjunctival sac. However, the eye is an important organ with other functions and the first chapter deals with both a review of ocular drug delivery and iatrogenic ocular states arising from topical delivery of drugs and vehicles. The remaining 20 chapters take the reader logically through ocular anatomy and physiology, precorneal, corneal and post-corneal factors in drug delivery in a section entitled Fundamental Considerations. Transport Models are dealt with in five chapters in part II, dealing with ocular pharmacokinetics and pharmacodynamics, cell culture systems, and animal studies inter alia. Disease states such as bacterial and viral keratitis, conjunctivitis and corneal trauma obviously impact on drug delivery. The chapter on disease state models deals with animal models and not mathematical models of these conditions. Traditional delivery systems are dealt with in a lone chapter in Part III. Part IV introduces advanced delivery and attacks such approaches as mucoadhesion, ocular inserts, nanoparticles and liposomes as vehicles. Chemical delivery systems derived from drugs with modified structures to enhance delivery has a separate chapter in Part V.