E. coli carrying pDT101 or pDT201 was due to fragment A of diphtheria toxin was obtained by allowing cell extracts to react with polyvalent antiserum to fragment A and monoclonal antibody to diphtheria toxin before determining enzymatic activity. Both types of antibody specifically inhibited the transferase activity (Table 1).

Two earlier reports had suggested that diphtheria toxin–related molecules would be secreted in *E. coli*. The first demonstrated that toxin is synthesized exclusively on membrane-bound polysomes in *C. diphtheriae* (*10*). This process involves the cotranslational secretion of the polypeptide across the cytoplasmic membrane, cleavage of an NH_2-terminal signal sequence, and release of the toxin into the culture medium. The second report demonstrated that translation of *tox* messenger RNA in vitro in the presence of membrane vesicles resulted in the vectorial transport of the polypeptide into those vesicles (*17*).

Escherichia coli SM529, a $PhoR^-$ derivative of strain MC1000 that is constitutive for alkaline phosphatase, was transformed with pDT201. The localization of ADPR-transferase activity in these strains was compared to that of glucose-6-phosphate dehydrogenase and alkaline phosphatase, which were used as markers for the cytoplasmic and periplasmic fractions, respectively (Table 2). The transferase activity clearly partitioned with alkaline phosphatase in the periplasmic fraction. These experiments strongly support the hypothesis that the signal sequence for diphtheria toxin is recognized by the *E. coli* secretory system and allows fragment A of diphtheria toxin to be translocated across the inner membrane and to be localized as a soluble protein in the periplasmic compartment of *E. coli*.

The study of the structural and functional domains of diphtheria toxin has relied largely on the isolation and characterization of polypeptides produced from mutated *tox* genes (*1*). These studies have been hampered by the inability to isolate a broad spectrum of different types of *tox* mutations. The capability of cloning the NH_2-terminal portion of the diphtheria toxin structural gene in *E. coli* and the resulting expression and secretion of toxin-related polypeptides will allow molecular genetic methods to be applied to this problem. In addition, hybrid toxin genes can be developed in which the enzymatically active fragment A and hydrophobic domain of fragment B are fused to various eukaryotic cell receptor–specific ligands. The expression and secretion of these defined chimeric toxins will advance the study of toxin entry into eukaryotic cells and the development of targeted chimeric toxins for the potential treatment of particular malignancies.

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10 February 1983

Platelet Thromboxane Synthetase Inhibitors with Low Doses of Aspirin: Possible Resolution of the "Aspirin Dilemma"

Abstract. *Selective pharmacological inhibition of thromboxane A_2 synthesis did not prevent arachidonate-induced aggregation of human platelets in vitro. Prevention was instead achieved by a combination of thromboxane A_2 inhibitors with low concentrations of aspirin. The latter partially reduced the proaggregatory cyclooxygenase products that accumulated when thromboxane A_2 synthesis was blocked. The aspirin concentrations did not affect per se either platelet aggregation or prostacyclin synthesis in cultured human endothelial cells. The combination of thromboxane synthetase inhibitors with low doses of aspirin may offer greater antithrombotic potential than either drug alone.*

Aspirin, by inhibiting cyclooxygenase (*1*), prevents the formation of two arachidonic acid metabolites that have opposite biological effects, namely, the aggregating thromboxane (Tx) A_2 in platelets (*2*) and the antiaggregatory prostaglandin (PG) I_2 or prostacyclin in vascular cells (*3*). Simultaneous inhibition of TxA_2 and PGI_2 synthesis might be the reason for the disappointing results of clinical trials of the antithrombotic effect of aspirin (*4*). Studies with cultured endothelial cells

Table 1. Effect of aspirin (2.5 μM), dazoxiben (40 μM), and a combination of the two drugs on PGI_2 generation by human endothelial cells stimulated by sodium arachidonate. Human endothelial cells derived from umbilical veins were cultured in 2-mm^2 wells (NUNC-Libco) as described (*21*) and used when confluent. At the beginning of the experiment the cells were washed once with phosphate saline buffer (free of Ca^{2+} and Mg^{2+}) and incubated with the drugs or the vehicle in 0.3 ml of buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 4 percent bovine albumin (Sigma) for 10 minutes at 37°C. Sodium arachidonate (200 μM) was then added for 5 minutes at 37°C, and the buffer was removed and stored at −20°C until assayed for 6-keto-$PGF_{1\alpha}$ (the stable derivative of PGI_2) by radioimmunoassay (*19*). At the end of the experiment the cells were detached by 0.05 percent trypsin and 0.2 percent EDTA and counted in a hemacytometer. Each well contained approximately 10^5 cells. The results are expressed as means (± standard error of the mean) of four different replicates. No statistical differences were detected by Duncan's new multiple range test.

Group	6-keto-$PGF_{1\alpha}$ (pmole/10^5 cells)
Control	11.53 ± 1.27
Aspirin	7.72 ± 1.62
Dazoxiben	10.81 ± 1.10
Aspirin plus dazoxiben	7.91 ± 1.12

(5), laboratory animals (6), and, more recently, humans (7) have failed to dissociate the effects of single doses of aspirin on platelets and vascular cells. Endothelial cell cyclooxygenase is as sensitive to aspirin as the enzyme in platelets (5).

The most logical way to resolve the "aspirin dilemma" (8) is to induce a selective pharmacological blockade of platelet TxA_2-synthetase. This apparently simple approach has attracted great interest, in part because of the recent availability of highly specific inhibitors of this enzyme (9, 10). However, there is evidence that TxA_2 may not be necessary for platelet aggregation to occur. Indeed, several compounds completely prevent TxA_2 synthesis but do not inhibit platelet aggregation (9, 11). Formation of PG-endoperoxides or other cyclooxygenase products may be sufficient to mediate aggregation induced by arachidonic acid when TxA_2 generation is pharmacologically inhibited.

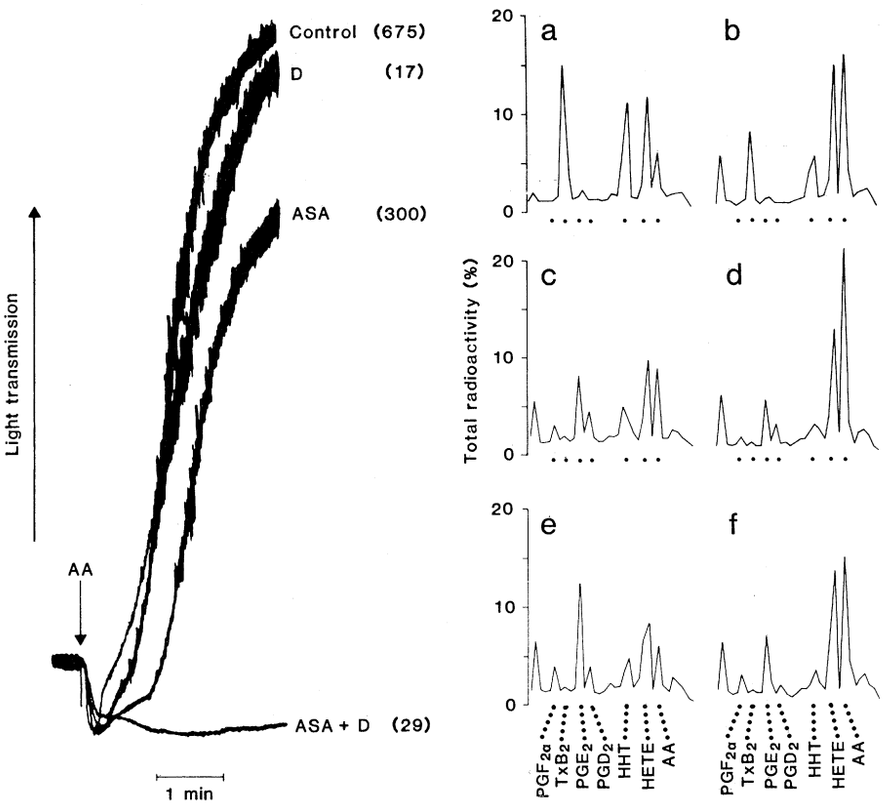

Fig. 1 (left). Representative tracings of human platelet aggregation induced by sodium arachidonate. Aggregation was monitored on a Born aggregometer (Elvi 840, Elvi Logos, Milan) and sodium arachidonate was prepared from arachidonic acid (> 99 percent pure, Sigma) (12). For each individual PRP sample, the threshold concentration of the stimulus that induced a 70 percent increase in light transmission within 3 minutes was selected (12) (in this experiment, 0.4 m*M*). Samples of PRP (250 μl) were incubated for 10 minutes at 37°C with aspirin (*ASA*) and either dazoxiben, 4-(2-[IH-imidazol-1-yl]-ethoxy)benzoic acid–HCl (*D*) or OKY 1581, sodium-(E)-3-[4-(3-pyridylmethyl)phenyl]-2-methylacrylate. Aspirin was the water-soluble lysine salt of acetylsalicylic acid (Flectadol, Maggioni). Both of the TxA_2-synthetase inhibitors were dissolved in 0.15*M* tris buffer, *p*H 7.4. Aspirin concentrations ranged from 2.5 to 10 μ*M*; in this experiment the lowest concentration was used. The minimum concentrations of dazoxiben that almost completely suppressed TxA_2 synthesis ranged from 10 to 60 μ*M* (in this experiment, 40 μ*M*). The generation of TxA_2 was measured by radioimmunoassay (19) of TxB_2, its stable derivative, and is expressed as picomoles per 4.10^8 platelets, in parentheses. Similar results were obtained when OKY 1581 was used instead of dazoxiben, at the minimum concentrations (0.4 to 4 μ*M*) almost completely preventing TxB_2 generation. Fig. 2 (right). Representative thin-layer chromatograms of platelets labeled with ^{14}C-labeled sodium arachidonate (*AA*) and stimulated with thrombin (5 NIH U/ml) (20). Briefly, PRP was incubated with aspirin or buffer for 10 minutes at 37°C; platelets were then washed and incubated with 0.2 μCi of ^{14}C-labeled sodium arachidonate (Amersham; specific activity, 58.6 mCi/mmole) for 1 hour at 37°C. After removal of the excess labeled sodium arachidonate, platelets were incubated with dazoxiben or OKY 1581 or their solvent for 10 minutes and then stimulated by thrombin. After 3 minutes the reaction was stopped by formic acid and samples were extracted with ethyl acetate and dried. Then they were dissolved in a mixture of chloroform and methanol (2:1 by volume) and spotted on silica gel plates (Merck) for thin-layer chromatography. The plates were developed with chloroform, methanol, acetic acid, and water (90:8:1:0.8 by volume). Peaks were identified by their comigration with authentic standards of $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2 (Upjohn), 12-HETE, and sodium arachidonate (Sigma). (a) TxB_2, PGE_2, HHT, 12-HETE, and unconverted sodium arachidonate were apparent in control samples. (b) Aspirin (2.5 μ*M*) reduced by about 50 percent the peaks corresponding to the cyclooxygenase derivatives and increased the peaks for 12-HETE and unconverted sodium arachidonate (c) Dazoxiben almost completely suppressed TxB_2 but increased the peaks corresponding to $PGF_{2\alpha}$, PGE_2, and PGD_2; HHT was partially reduced and 12-HETE was unchanged. (d) Aspirin and dazoxiben combined reduced by about 50 percent the cyclooxygenase products formed in excess in concomitance with TxA_2-synthesis inhibition. (e and f) Similar results were obtained when OKY 1581 (0.4 μ*M*) was used instead of dazoxiben (40 μ*M*).

In the study reported here, two selective inhibitors of TxA_2-synthetase, dazoxiben (UK 37,248-01) and OKY 1581, did not prevent arachidonic acid–induced aggregation of human platelets. Prevention was instead obtained by combining TxA_2-synthetase inhibitors with low concentrations of aspirin. The latter did not affect per se either platelet aggregation or PGI_2 synthesis in cultured human endothelial cells. These results imply that a low dose of aspirin combined with a TxA_2-synthetase inhibitor might result in vivo in suppression of platelet aggregation while sparing vascular PGI_2 generation.

Venous blood was collected from healthy volunteers and platelet aggregation was monitored on platelet-rich plasma (PRP) as described (12). Platelet aggregation induced by sodium arachidonate was not prevented by either aspirin or dazoxiben when the PRP was incubated with these drugs at the concentrations indicated (Fig. 1). However, when both compounds were used aggregation was completely inhibited. Similar results were obtained when OKY 1581 was used instead of dazoxiben.

Analysis of platelet arachidonic acid metabolism by thin-layer chromatography (Fig. 2) revealed that both dazoxiben and OKY 1581 almost totally blocked generation of TxB_2 (the stable derivative of TxA_2) and induced a concomitant marked increase in $PGF_{2\alpha}$, PGE_2, and PGD_2. Aspirin (2.5 μ*M*) reduced synthesis of TxB_2 by about 50 percent. When combined with either of the TxA_2-synthetase inhibitors, aspirin caused a 50 percent reduction of the cyclooxygenase products formed in excess concomitantly with the inhibition of TxA_2 synthesis.

These results indicate that suppression of TxA_2 synthesis does not modify platelet aggregation but makes platelets susceptible to the inhibitory effect of very low concentrations of aspirin. Studies in man have shown that 650 mg of aspirin given orally will yield plasma aspirin levels of 128 μ*M* at 10 minutes, 56 μ*M* at 25 minutes, and 18 μ*M* at 1 hour (13). These figures are many times higher than

the concentrations of aspirin used in this study (2.5 to 10 μM) and reveal the amount of "excess" aspirin that would not have to be administered if aspirin and TxA_2-synthetase inhibitors were used in combination (*14*). When TxA_2 synthesis is selectively inhibited, PG endoperoxides and other cyclooxygenase products are generated in sufficient amounts to stimulate platelet aggregation. Partial inhibition of cyclooxygenase activity by aspirin in these conditions may be enough to reduce the amount of PG endoperoxides and proaggregatory PG's such as PGE_2 to a level below that needed to produce platelet aggregation in the absence of TxA_2 generation. The combination of very low doses of aspirin and a TxA_2-synthetase inhibitor thus seems to offer greater antithrombotic potential than the use of either drug alone. Such treatment could result in complete suppression of both TxA_2 synthesis and platelet aggregation without significantly affecting vascular prostacyclin synthesis.

This assumption was tested on cultured endothelial cells from humans. Aspirin (2.5 μM), dazoxiben (40 μM), and a combination of the drugs induced statistically insignificant modifications of PGI_2 synthesis, although a 30 percent reduction occurred in the cell samples containing aspirin (Table 1) (*15, 16*). The observed moderate decrease in PGI_2 generation induced by aspirin should be counteracted by the manyfold increase in the sensitivity to PGI_2 of platelets exposed to TxA_2-synthetase inhibitors (*17, 18*).

Thus aspirin produces an overall cyclooxygenase deficiency that affects endothelial cells as well as platelets. The endothelial cells can be spared by use of inhibitors of TxA_2-synthetase that close off the production of TxA_2, the most potent proaggregatory metabolite of platelet arachidonic acid. Under these conditions, only a minute amount of aspirin is required to diminish PG endoperoxides and prevent "runoff" into other, relatively less potent, proaggregatory prostaglandins. Thus, TxA_2-synthetase inhibitors permit the use of small doses of aspirin to produce an antiplatelet effect with sparing of the endothelium.

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16. After 1 hour of incubation with aspirin, the production of PGI_2 by human cultured endothelial cells was inhibited 50 percent by 2.1 μM aspirin (*5*). The culture medium used in those experiments contained only 20 percent pooled human serum, whereas 4 percent albumin was used in the present study. This might explain the somewhat lower inhibitory effect of aspirin reported here.
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18. Studies in vitro have shown that during TxA_2-synthetase inhibition, platelet-derived PG-endoperoxides are partly converted to PGI_2 by various vascular cell preparations and by leukocytes in whole blood [A. J. Marcus, B. B. Weksler, E. A. Jaffe, M. J. Broekman, *J. Clin. Invest.* **66**, 979 (1980); G. Defreyn, H. Deckmyn, J. Vermylen, *Thromb. Res.* **26**, 389 (1982); M. J. Parry, M. J. Randall, H. M. Tyler, E. Myhre, J. Dale, E. Thaulow, *Lancet* **1982-II**, 164 (1982)]. Although the relevance of these effects in vivo has yet to be established, in a control experiment we evaluated whether aspirin would counteract the possible increase of PGI_2 synthesis occurring simultaneously with TxA_2-synthetase inhibition. Whole blood was allowed to clot in glass tubes at 37°C in the presence of aspirin (2.5 μM) or dazoxiben (40 μM), or both compounds. The serum extruded within 30 minutes was separated by centrifugation and tested by radioimmunoassays (*19*) for TxB_2 and 6-keto-$PGF_{1\alpha}$. In three normal individuals, dazoxiben completely inhibited TxB_2 generation and caused an average sixfold increase in 6-keto-$PGF_{1\alpha}$. Dazoxiben and aspirin together resulted in an average fourfold increase in 6-keto-$PGF_{1\alpha}$. Aspirin alone prevented TxB_2 generation by about 50 percent and only slightly diminished 6-keto-$PGF_{1\alpha}$. Thus, the drug combination proposed here results in a severalfold increase of PGI_2 synthesis by leukocytes in whole blood.
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2 February 1983

Vasoactive Intestinal Polypeptide and Muscarinic Receptors: Supersensitivity Induced by Long-Term Atropine Treatment

Abstract. *Long-term treatment of rats with atropine induced large increases in the numbers of muscarinic receptors and receptors for vasoactive intestinal polypeptide in the salivary glands. Since receptors for vasoactive intestinal polypeptide coexist with muscarinic receptors on the same neurons in this preparation, the results suggest that a drug that alters the sensitivity of one receptor may also affect the sensitivity of the receptor for a costored transmitter and in this way contribute to the therapeutic or side effects of the drug.*

Combined histochemical and immunohistochemical data indicate the coexistence of acetylcholine (ACh) and vasoactive intestinal polypeptide (VIP) in postganglionic nerves innervating the submandibular salivary gland in the cat and rat (*1*). Both ACh and VIP are released upon stimulation of these nerves, and VIP potentiates the ACh-induced secretion of saliva (*2*). A possible molecular mechanism for the synergistic actions of ACh and VIP is the 10,000-fold increase, induced by VIP, in the affinity of ACh for muscarinic receptors (*3*). This change in muscarinic agonist binding affinity involves a shift of a portion of receptors to the high-affinity state which mediates changes in Na^+ permeability and in secretion. The increase in the number of high-affinity muscarinic receptors in the presence of VIP (10 nM) (*3*) is commensurate with the more than 100 percent increase in ACh-induced secretion in the presence of VIP (*1, 2*). We have now