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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References and Notes

1. G. J. Roth and P. W. Majerus, *J. Clin. Invest.* **56**, 624 (1975).
2. M. Hamberg, J. Svensson, B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2994 (1975).
3. S. Moncada, R. Gryglewski, S. Bunting, J. R. Vane, *Nature (London)* **263**, 663 (1976).
4. G. de Gaetano, C. Cerletti, V. Bertelé, *Lancet* **1982-II**, 974 (1982).
5. E. A. Jaffe and B. B. Weksler, *J. Clin. Invest.* **63**, 532 (1979).
6. S. Villa, M. Livio, G. de Gaetano, *Br. J. Haematol.* **42**, 425 (1979); E. Dejana, C. Cerletti, M. De Castellarnau, M. Livio, F. Galletti, R. Latini, G. de Gaetano, *J. Clin. Invest.* **68**, 1108 (1981).
7. F. E. Preston, S. Whipps, C. A. Jackson, A. J. French, P. J. Wyld, C. J. Stoddard, *N. Engl. J. Med.* **304**, 76 (1981); S. P. Hanley, J. Bevan, S. R. Cockbill, S. Heptinstall, *Lancet* **1981-I**, 969 (1981).
8. A. J. Marcus, *N. Engl. J. Med.* **297**, 1284 (1977).
9. S. Heptinstall, J. Bevan, S. R. Cockbill, S. P. Hanley, M. J. Parry, *Thromb. Res.* **20**, 219 (1980).
10. H. M. Tyler, C. A. P. D. Saxton, M. J. Parry, *Lancet* **1981-I**, 629 (1981); J. Vermylen, G. Defreyn, L. O. Carreras, S. J. Machin, J. Van Schaeren, M. Verstraete, *ibid.*, p. 1073; J. B. Smith and W. Jubiz, *Prostaglandins* **22**, 353 (1981).
11. V. Bertelé, C. Cerletti, A. Schieppati, G. Di Minno, G. de Gaetano, *Lancet* **1981-I**, 1057 (1981); L. J. Grimm, D. R. Knapp, D. Senator, P. V. Halushka, *Thromb. Res.* **24**, 307 (1981).
12. G. Di Minno, M. J. Silver, G. de Gaetano, *Br. J. Haematol.* **43**, 637 (1979).
13. M. Rowland, S. Riegelman, P. A. Harris, S. D. Sholkoff, *J. Pharm. Sci.* **61**, 379 (1972).
14. In human volunteers, aspirin concentrations in the plasma reached about 2.5 μM 15 minutes after intravenous injection of 20 mg of aspirin (M. Bonati, unpublished data).
15. These results confirm the specificity of dazoxiben as a TxA-synthetase inhibitor. Such specificity had previously been shown only with the use of PGI_2-synthetase preparations of animal origin [M. J. Randall, M. J. Parry, E. Hawkeswood, P. E. Cross, R. P. Dickinson, *Thromb. Res.* **23**, 145 (1981)].
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.
17. J. W. Aiken, R. J. Shebuski, O. V. Miller, R. R. Gorman, *J. Pharmacol. Exp. Ther.* **219**, 299 (1981); V. Bertelé, A. Falanga, M. C. Roncaglioni, C. Cerletti, G. de Gaetano, *Thromb. Haemostasis* **47**, 294 (1982).
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.
19. C. Ingerman-Wojenski, M. J. Silver, J. B. Smith, *J. Clin. Invest.* **67**, 1292 (1981).
20. H. Sors, P. Pradelles, F. Dray, M. Rigaud, J. Maclouf, P. Bernard, *Prostaglandins* **16**, 277 (1978).
21. B. Barbieri, G. Balconi, E. Dejana, M. B. Donati, *Proc. Soc. Exp. Biol. Med.* **168**, 204 (1981).
22. Supported by the Italian National Research Council (Progetto Internazionale CT 82-00138-04 and Progetto Finalizzato Medicina Preventiva, Sottoprogetto Malattie Degenerative). Dazoxiben and OKY 1581 were obtained through the courtesy of H. M. Tyler, Pfizer Research, and M. Itoh, Ono Pharmaceutical Co., respectively. The 12-HETE was a gift of J. Maclouf, Hôpital Lariboisière, Paris. J. Baggott, M. P. Bonifacino, A. Bottazzi, V. de Ceglie, F. de Ceglie, and I. Garimoldi helped prepare the manuscript. M.T. was on leave of absence from the Department of Biochemistry, Medical Academy, Bialystok, Poland.

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

examined interactions between VIP and ACh receptors under conditions in which long-term treatment with muscarinic antagonists induced supersensitivity of the muscarinic receptors. Our study shows that long-term treatment of rats with atropine induces an increase not only in the number of muscarinic receptors but also in the number of VIP receptors in the salivary gland.

Adult male Sprague-Dawley rats were injected subcutaneously with atropine (20 mg/kg) for 14 days. This treatment was shown previously to increase the number of muscarinic receptor sites in the dorsal hippocampus, striatum, and cerebral cortex (*4*) and in the heart (*5*) of rats. In the present experiments the treatment led to a 101 ± 7 percent (mean ± standard deviation) (N = 18) increase in the number of muscarinic receptor sites (Fig. 1A). The number of VIP binding sites was also increased by 104 ± 5 percent (N = 18) (Fig. 1B). The affinity of muscarinic receptors for the high-affinity antagonist *N*-methyl-4-piperidinyl benzilate (NMPB) was unaltered, whereas the affinity for VIP was almost doubled in membranes from atropine-treated rats compared with controls (Fig. 1B).

Table 1. Changes in VIP content of the rat salivary gland induced by long-term treatment with atropine. The rats were treated as described in Fig. 1. The radioimmunoassay for VIP was as described (*6*). The results are expressed as means (± standard deviation).

Treatment	VIP (picomoles per milligram of protein)	Samples (No.)
Control	2.28 ± 1.24	19
Atropine	0.89 ± 0.77*	18
Denervated	0.64 ± 0.47*	6

*P < .001, by Student's t-test.

The functional correlate of the muscarinic and VIP supersensitivity is increased salivary secretion in the presence of ACh and VIP after removal of atropine from the tissue. The atropine dose used was somewhat high (*5*) and one cannot exclude the possibility that atropine affected the VIP binding sites indirectly. Experiments with tritiated atropine indicate that the dose we used leads to a concentration in the tissue of 2 to 4 nM (*4*). Since this atropine concentration did not affect VIP-evoked vasodilation (*2*), it is unlikely that the VIP receptors were affected by atropine indirectly.

Long-term treatment with atropine led to depletion of VIP from the tissue (Table 1), indicating that release of VIP is subject to negative feedback control by ACh acting at muscarinic receptors (*1*). Such treatment should yield a disinhibition of VIP release. Disinhibition of the VIP release and subsequent depletion of VIP in the tissue caused the observed supersensitivity of VIP receptors (Fig. 1B). However, this mechanism may not be the only explanation of VIP supersensitivity, since we also observed that 14 days after denervation (sectioning) of the postganglionic nerves that innervate the salivary gland there was a 61 ± 5 percent (N = 5) increase in the number of muscarinic receptors, but no statistically significant increase [4 ± 7 percent (N = 6)] in the number of VIP receptors, although VIP concentrations (Table 1) were markedly reduced under these conditions too.

That long-term blockade of muscarinic receptors with atropine increased both the muscarinic and VIP receptors is of additional interest because the peptidergic supersensitivity was induced toward a peptide (VIP) that is costored and released from the same nerves as the classical neurotransmitter (ACh) whose receptors were blocked. A similar situation may occur with other coexisting neurotransmitter-peptide pairs, for example, dopamine and cholecystokinin (CCK) and serotonin and substance P. It is therefore important to examine any alterations in sensitivity to costored neurotransmitters that may develop upon treatment with various types of drugs, since such effects may influence the actions of these drugs. As one example, antipsychotic drugs, which are potent dopamine antagonists, may alter the sensitivity of the costored peptide CCK and contribute to the therapeutic or side effects of these substances.

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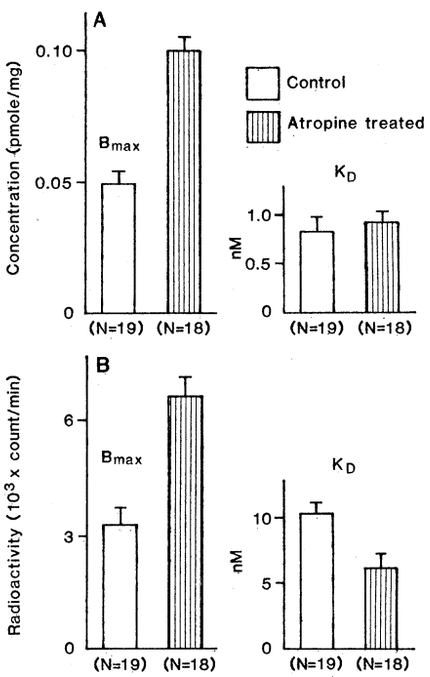

Fig. 1. Supersensitivity of muscarinic and VIP receptors in the rat salivary gland induced by long-term atropine treatment. (A) Number and affinity of muscarinic receptors. (B) Number and affinity of VIP receptors. Atropine (20 mg/kg) was injected subcutaneously once daily for 14 days into male Sprague-Dawley rats (150 to 160 g). The rats were killed by decapitation 48 hours after the last injection. The salivary glands were removed and homogenized on ice in 0.32M sucrose in a glass/Teflon homogenizer at 695 rev/min, 15 up-and-down strokes. The homogenate was centrifuged (and kept cool) at 1000g for 3 minutes. The supernatant was subjected to further centrifugation at 10,000g for 40 minutes. The pellet obtained was resuspended in Krebs-Ringer buffer (137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 5 mM Hepes, 10 μM phenyl methyl sulfonyl fluoride, 1 g of glucose per liter, pH 7.4). This membrane preparation was used, without further treatment, for the receptor binding assays. The number of muscarinic receptor sites in the membranes was determined by measuring the specific binding of tritiated NMPB (33 Ci/mmole). Nonspecific binding was determined by incubating membranes with tritiated NMPB in a total volume of 1 ml in the absence and presence of scopolamine (10 μM) for 15 minutes at 37°C, which gave the mixture sufficient time to reach equilibrium. The mixture was filtered (Whatman GF/C) and washed (with 10 ml of ice-cold normal saline) and the radioactivity counted as described (*4*). [The K_D value for tritiated NMPB was corrected for the residual atropine (estimated to be 3 nM) in the tissue (*4*).] The number of VIP receptor sites in the membranes was determined by use of ^{125}I-labeled VIP (*6*). The VIP was iodinated as described (*6*) and had a specific activity of approximately 15 Ci/mmole. Membranes were incubated together with ^{125}I-labeled VIP (15 nM) and 1 percent bovine serum albumin, in the absence and presence of unlabeled VIP (100 nM) in a total volume of 1 ml in 1.5-ml plastic centrifuge tubes for 15 minutes at 37°C. The tubes were then placed in an ice bath for 1 to 2 minutes before being centrifuged for 30 seconds in a Beckman Microfuge B. The supernatant was removed and the bottom of the tubes, containing the membrane pellet, punched off in a glass tube and counted for radioactivity in a Packard gamma counter.

References and Notes

1. J. M. Lundberg, *Acta Physiol. Scand. Suppl.* **496**, 1 (1981).
2. ———, A. Anggård, J. Fahrenkrug, T. Hökfelt, V. Mutt, *Proc. Natl. Acad. Sci. U.S.A.* 77, 1651 (1980).
3. J. M. Lundberg, B. Hedlund, T. Bartfai, *Nature (London)* **295**, 147 (1982).

4. A. Westlind, M. Grynfarb, B. Hedlund, T. Bartfai, K. Fuxe, *Brain Res.* **225**, 131 (1981); Z. Herman and J. Slominska-Zurek, *Psychopharmacology* **64**, 337 (1979).
5. B. C. Wise, M. Shoji, J. F. Kuo, *Biochem. Biophys. Res. Commun.* **92**, 1136 (1980).
6. J. Fahrenkrug and O. B. Schaffalitzky de Muckadell, *J. Lab. Clin. Med.* **89**, 1379 (1977).
7. This study was supported by grants from the National Institute of Mental Health and the Swedish Medical Research Council. We thank M. Sokolovsky for the tritiated NMPB and V. Mutt for the VIP.
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Stimulation of Prostaglandin Biosynthesis by Urine of the Human Fetus May Serve as a Trigger for Parturition

Abstract. *Urine of the human fetus stimulated prostaglandin biosynthesis in vitro by increasing the conversion of arachidonic acid into prostaglandins. The stimulatory activity in urine from fetuses delivered at term after labor of spontaneous onset was greater than that in urine from fetuses delivered by cesarean section at term before the onset of labor. Such stimulation of prostaglandin biosynthesis by the fetal membranes, by way of a substance released into the urine and thence into amniotic fluid, could serve as a signal for the initiation of parturition.*

That prostaglandins are involved in the biochemical processes of human parturition is well established (*1*), and there is evidence that the fetal membranes serve a central role in the molecular events that either signal or accompany the onset of labor (*2*). Since the putative signal for the onset of labor may be of fetal origin (*3*), it is also possible that the amniotic fluid serves as a route of communication between the fetus and the fetal membranes. A substance originating in the fetus could be excreted directly into the amniotic fluid through the kidneys, lungs, skin, or umbilical cord and, once there, could trigger parturition.

We recently found that an inhibitor of prostaglandin synthase is present in human amniotic fluid (*4*). The potency of the inhibitory activity in amniotic fluid obtained at term during labor of spontaneous onset was significantly lower than that in amniotic fluid obtained at term before the onset of labor.

These findings suggest that, throughout gestation, substances in amniotic fluid may suppress the production of prostaglandins by the fetal membranes. At term, the fetus may excrete other substances into the amniotic fluid that stimulate prostaglandin biosynthesis and thereby initiate the events that culminate in delivery of the fetus. This hypothetical substance could be released into amniotic fluid from several fetal sources. Since, at term, the amniotic fluid is largely a product of fetal urination (*5*), the kidney seemed to be a likely source of such a stimulatory substance.

By means of established methods (*6*) we evaluated the effect of urine from human fetuses (*7*) on the production of prostaglandin E_2 (PGE_2) (*8*) by a microsome-enriched preparation of prostaglandin synthase from bovine seminal vesicles (*9*). The results with samples of urine from fetuses delivered after spontaneous labor and vaginal delivery (SVD) at term and after cesarean section scheduled at term before onset of labor (not in labor, NIL) are presented in Fig. 1. The results of similar experiments with urine specimens from four adult women and four adult men are shown for comparison.

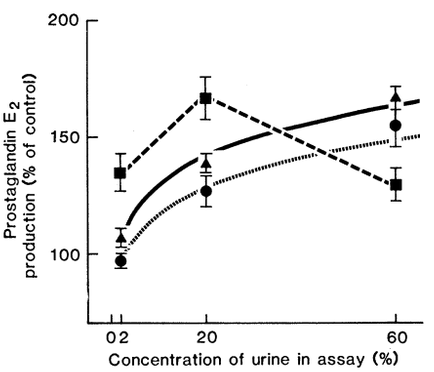

Fig. 1. The effect of human urine on the synthesis of PGE_2 by prostaglandin synthase from bovine seminal vesicle (*6*). The production of PGE_2 is expressed as a percentage of production by controls. Values shown are means (± standard error of the mean) for urine samples from (■) adults (N = 8), (▲) newborns delivered at term (38 to 42 weeks of gestation) after spontaneous labor (*SVD*, N = 10), and (●) newborns delivered after cesarean sections scheduled at term before the onset of labor (NIL, N = 10). The concentrations of urine tested in the assay were 2, 20, and 60 percent (by volume). The curved lines are indicative of best fits (by the method of least squares) to the equation $y = bx^m$. For SVD samples, b = 1.987 ± 0.0169 (± standard error), m = 0.1272 ± 0.0131, and r = .896 ($P < .001$). For NIL samples, b = 1.939 ± 0.0252, m = 0.1323 ± 0.0197, and r = .836 ($P < .001$). By analysis of variance we found that the slopes (m) were not significantly different, but the intercepts (b) were significantly different ($P < .005$).

There were no statistically significant sex-related differences in the stimulatory potency of the adult specimens; the data are therefore combined. Urine from both the fetuses and the adults significantly increased the production of PGE_2 in a concentration-dependent manner. The SVD urine samples, however, stimulated PGE_2 production significantly more than the NIL samples obtained before the onset of labor. The concentration of urine in the incubation mixture that produced a 50 percent increase in PGE_2 production, compared to control incubations (without urine), was 31 percent by volume for SVD samples and 62 percent for NIL samples.

At low concentrations, urine from adults increased PGE_2 production more than either of the SVD or NIL urine samples, but at high concentrations of urine these relations were reversed. This biphasic effect suggests that, compared with urine from fetuses, high concentrations of urine from adults may contain greater amounts of both stimulatory and inhibitory factors, and that at the 60 percent concentration the stimulatory factors were antagonized. The postulated inhibitory factors would have to be present in greater amounts (that is, > 20 percent) to exert a detectable effect. Such inhibitory effects were not apparent in experiments with urine from fetuses.

The stimulatory activity in fetal urine was not significantly altered by boiling the urine for 5 minutes (N = 4), but was not detectable in urine that had been subjected to membrane dialysis (retaining molecules greater than 12,000 to 14,000 daltons) against NaCl (0.15*M*) for 48 hours at 4°C (N = 4). Two treatments with activated charcoal, each for 15 minutes at 4°C, partially reduced the stimulatory activity in urine (40 to 80 percent; N = 4), indicating that the activity may result in part from the action of a steroid or small lipid.

Our interpretation of these results may provide an explanation for our previous finding that the prostaglandin synthase inhibitory activity in amniotic fluid obtained during labor is less than the inhibitory activity of fluid taken before labor. This decreased inhibitory activity could result from the increased production of a stimulatory substance that is released into the urine of the fetus and thence into the amniotic fluid during labor. If this were the case, such a stimulant could cause an increase in prostaglandin production by the fetal membranes and