EFFECTS OF DAZOXIBEN AND LOW-DOSE ASPIRIN ON PLATELET BEHAVIOUR IN MAN

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1 We have studied the effects on platelet behaviour of ingestion of the thromboxane synthetase inhibitor dazoxiben (UK 37248), by healthy subjects, and compared the results with the effects of a low dose of aspirin (a cyclo-oxygenase inhibitor), and of a combination of dazoxiben and a low dose of aspirin.

2 Dazoxiben ingestion prevented the release reaction induced by sodium arachidonate (NaAA) in platelet-rich plasma (PRP) from some individuals ("responders") but not in PRP from others ("non-responders"). *In vitro* testing of PRP from the same subjects, incubated with 10^{-4} M dazoxiben, correlated with the effect of dazoxiben ingestion on NaAA-induced release. Platelets from "non-responders" tended to undergo a more extensive release reaction than platelets from "responders" even in the absence of any drug although there was some overlap between the results in the two groups. Platelets from "non-responders" required significantly lower concentrations of NaAA to induce release reaction than platelets from "responders". Platelets from "responders" and "non-responders" did not differ in the amount of malondialdehyde (MDA) produced or in the effectiveness with which dazoxiben ingestion inhibited MDA production.

3 Low dose aspirin had comparable effects on NaAA-induced release to dazoxiben, but in contrast to dazoxiben, the effectiveness of low-dose aspirin in inhibiting NaAA induced release reaction was related to its effectiveness in inhibiting MDA generation.

4 Neither dazoxiben nor low-dose aspirin significantly affected the release reaction induced by adenosine diphosphate (ADP), although both drugs significantly inhibited adrenaline-induced release.

5 A combination of dazoxiben and low dose aspirin had a greater effect on platelet behaviour in response to NaAA, ADP, and adrenaline than either drug alone.

Introduction

INTRAVASCULAR platelet deposition may be important in thrombogenesis. Thromboxane A_2 (TXA_2) is a potent platelet aggregating agent, and is the main product of arachidonic acid (AA) metabolism in platelets (Figure 1), so agents which prevent the production of TXA_2 by platelets may have a role in preventing thrombosis. This has recently been reviewed by Mitchell (1981). Cyclo-oxygenase inhibitors such as aspirin prevent the synthesis of TXA_2 from arachidonic acid and have been shown to have a profound effect on platelet behaviour (O'Brien, 1968; Weiss *et al.*, 1968; Zucker & Peterson, 1968).

The conventional dose of aspirin 300 mg results in the total abolition of platelet aggregation and release reaction induced by sodium arachidonate (NaAA), and abolishes the second phase of aggregation and release reaction induced by adenosine diphosphate (ADP) and adrenaline. However, the main metabolite of AA in the vessel wall is prostacyclin (PGI_2), a potent inhibitor of platelet aggregation (Moncada *et al.*, 1977). By inhibiting cyclo-oxygenase, aspirin may not only prevent the formation of the pro-aggregatory TXA_2 by platelets, but also prevent the formation of the anti-aggregatory PGI_2 by the vessel wall. A better strategy against thrombosis might be inhibition of platelet TXA_2 production with preservation of vessel wall PGI_2 .

PROSTAGLANDIN PATHWAYS IN VESSEL WALL AND PLATELETS

Figure 1 Prostaglandin pathways in vessel wall and platelets.

One approach through which this might be possible is to use a reduced dose of aspirin. Hanley *et al.* (1981) have shown that aspirin 40 mg markedly reduces platelet TXA₂ production for up to 96 h, but has little effect on vascular PGI₂ production. Another approach would be to use a selective inhibitor of thromboxane synthetase such as dazoxiben (UK 37248) Vermynen *et al.*, 1981). A disadvantage of this approach could be that the prostaglandin endoperoxides (PGG₂ and PGH₂) that could still be formed (Figure 1), and which are also platelet aggregating agents, would replace TXA₂, in influencing platelet behaviour. However, a possible advantage would be that these prostaglandin endoperoxides could be made available to the vessel wall, and redirected via prostacyclin synthetase to increase the production of vascular PGI₂. In this study, we have compared the effects of a single dose of aspirin 40 mg, of dazoxiben 200 mg, and of a combination of the two drugs, on platelet behaviour in man.

Methods

Five women and seven men voluntarily took part in the study (ages ranged from 22–39 y). All were healthy and denied taking aspirin or any similar drug in the 2 weeks before testing. Blood was taken from an antecubital vein by clean venepuncture on four occasions from each subject. Twenty-four hours after the first (control) sample had been taken, the subject swallowed dazoxiben 200 mg. The second sample was taken 1 h later. The subject then swallowed aspirin 40 mg, and 24 h later, the third sample was taken. A further 200 mg of dazoxiben was then taken, and the fourth blood sample obtained 1 h later. Thus we studied the effect of dazoxiben 200 mg alone, aspirin 40 mg alone, and the combined effects of aspirin 40 mg and dazoxiben 200 mg. At the same time as these platelet studies were being carried out, studies were being made of forearm vasoconstriction in response to cold challenge. The results of these studies will be reported separately.

In each case, 9 ml aliquots of the blood were dispensed into polystyrene tubes containing 1 ml 3.8% (w/v) trisodium citrate dihydrate, and 6 µl solution of [¹⁴C]-5-hydroxytryptamine (5-HT) containing 50 µCi [¹⁴C]-5-HT/ml (specific activity=58 mCi/mmol). Platelet-rich plasma (PRP) was obtained by centrifuging blood at 300 g for 10 min, and platelet-poor plasma (PPP) by recentrifuging blood, after the PRP had been removed, at 1200 g for 20 minutes. the PPP was used to dilute the PRP to give a final count of 3×10^8 platelets/ml. In all the preparations of PRP, the uptake of radioactivity by the platelets was greater than 90%.

For each blood sample, the PRP was divided into

aliquots (460 µl) which were stirred in a water bath at 37°C for 2 minutes. At this point 20 µl of a solution of the aggregating agent under test was added to each tube, and the final volume made up to 500 µl with 20 µl 150 mM NaCl. NaAA, ADP and adrenaline were diluted in 150 mM NaCl before use, and the dilutions had been adjusted to give final concentrations of NaAA ranging from 0.1 mM to 1.0 mM in incremental steps of 0.1 mM; ADP was in a final concentration of 10 µM; and adrenaline in a final concentration of 50 µM.

After addition of the aggregating agent, the tubes were stirred for a further 6 min, when 50 µl 14 mM solution of acetyl salicylic acid in 150 mM NaCl was added and the tubes were transferred to ice. After 5 min they were centrifuged (2,000 g for 7 min) and 50 µl portions of the supernatant were removed for measurement of the amount of radioactivity that was present. Control tubes containing aliquots of PRP to which 40 µl 150 mM NaCl had been added in place of the aggregating agent, were taken through the same procedure. The results that were obtained were subtracted from those obtained from the test samples to give the level of [¹⁴C]-5-HT that had been released through the effect of the aggregating agent. This was always expressed as a percentage of the amount of 5-HT that had been taken up by platelets during the labelling procedure.

After the tubes to which 1 mM NaAA had been added were analysed for released radioactivity, the residual contents of duplicate tubes were combined and analysed for malondialdehyde (MDA), a marker of thromboxane synthesis, as previously described (Heptinstall *et al.*, 1980). For these determinations, control tubes were samples of PPP to which NaAA had been added to the same final concentration of 1.0 mM. This was to allow for any non-enzymic auto-oxidation of NaAA during the procedure. These values were subtracted from those obtained for the test samples, and the results were expressed as nmol MDA/10⁹ platelets.

In addition to studying the effects of drug ingestion on platelet behaviour, we looked at the effect of *in vitro* addition of dazoxiben on NaAA-induced release. One tube of control PRP, and one tube of PRP taken after ingestion of 200 mg dazoxiben, from each subject, were each stirred with 10⁻⁴M dazoxiben for 2 min at 37°C before NaAA (in a final concentration of 1.0 mM) was added. The extent of the release reaction was then determined as before.

Materials and solutions

[¹⁴C]-5-HT was obtained from Amersham International. Dazoxiben capsules were supplied by Pfizer Ltd, Sandwich, Kent, UK. NaAA, ADP and adrenaline were purchased from the Sigma Chemical

Company. NaAA was prepared as described in the accompanying paper (Heptinstall & Fox, 1982). ADP and adrenaline were dissolved in 150 mM NaCl before use.

Results

NaAA-induced platelet behaviour

No drugs Before ingestion, the same pattern of res-

Figure 2 Results from a subject in whom dazoxiben and low-dose aspirin abolished NaAA-induced release. ●, Control; ▽, after dazoxiben 200 mg; ○, after aspirin 40 mg.

Figure 3 Results from a subject in whom dazoxiben abolished NaAA-induced release, but low-dose aspirin did not. Symbols as in Figure 2.

ponse to NaAA was observed in PRP from all 12 subjects. As the concentration of NaAA was increased, a point was reached at which an extensive release reaction occurred and large platelet aggregates were seen on inspection of the sample tubes. Further increase in NaAA concentration did not increase the extent of release. Examples of the effect of increasing NaAA concentration can be seen in Figures 2-5. For the 12 volunteers, the mean amount of NaAA that was required to induce the

Figure 4 Results from a subject in whom low-dose aspirin abolished NaAA-induced release, but dazoxiben did not. Symbols as in Figure 2.

Figure 5 Results from a subject in whom neither low-dose aspirin nor dazoxiben abolished NaAA-induced release. Symbols as in Figure 2.

platelets to aggregate and to undergo a release reaction was 0.56 mM (range 0.2–0.8); the mean percentage release reaction in response to 1 mM NaAA was 62% (range 55–74) and the mean amount of MDA that was produced in response to 1 mM NaAA was 12.1 nmol/10⁹ platelets (range 5.4–15.7).

When PRP taken from each subject before drug ingestion was stimulated with 1 mM NaAA in the presence of 10⁻⁴M dazoxiben, the effect of the dazoxiben was to completely inhibit the platelet release reaction in PRP in eight of the 12 subjects, but only partially inhibit the release reaction in the remaining four. For the eight "responders" the maximum percentage release reaction in the absence of dazoxiben was 60% (range 55–74) and this fell to 0% in the presence of dazoxiben. For the four "non-responders" the maximum percentage release reaction in the absence of dazoxiben was 65% (range 57–70) and this fell to 33% (range 39–64) in the presence of 10⁻⁴M dazoxiben. The percentage release reaction in the absence of dazoxiben was greater than 66% for three of the four "non-responders", but for only one of the eight "responders". Thus, the "non-responders" had platelets that tended to undergo a more extensive release reaction even in the absence of dazoxiben. For the eight "responders" the amount of NaAA that was required to induce a release reaction in the absence of dazoxiben was 0.65 mM (range 0.5 to 0.8), whereas for the "non-responders" it was 0.37 mM (range 0.2 to 0.5). These values are significantly different from each other ($P=0.01$). Thus, the "non-responders" required less NaAA to induce release reaction than did the "responders".

After ingestion of dazoxiben 200 mg One hour after dazoxiben 200 mg had been taken, two patterns of response to NaAA were observed. In PRP from eight of the subjects the ingested dazoxiben completely inhibited the release reaction induced by NaAA. Examples of the pattern obtained for two of these subjects are shown in Figures 2 and 3. In PRP from the other four subjects, the ingested dazoxiben did not completely inhibit the release reaction induced by NaAA. Examples of patterns found in two of these four subjects are shown in Figures 4 and 5. The four subjects in whom dazoxiben ingestion did not prevent NaAA-induced release reaction were the same four in whom 10⁻⁴M dazoxiben had been ineffective *in vitro*. Thus, individuals can be classified as "responders" or "non-responders" to dazoxiben on the basis of both *in vitro* and *ex vivo* experiments. For the eight "responders", the percentage release reaction induced by 1 mM NaAA was reduced from 60% to 0% after ingestion of dazoxiben. For the four "non-responders", it was reduced from 65% to 34% (range 25 to 51). The mean amount of NaAA that was

required to induce a release reaction in the "non-responders" changed from 0.37 mM before dazoxiben to 0.75 mM (range 0.6–1.0) after dazoxiben ingestion.

The mean amount of MDA that was produced by platelets from the "responders" before dazoxiben ingestion was 12.0 nmol/10⁹ platelets (range 5.4 to 15.7) and for the "non-responders" was 12.2 (range 9.9 to 13.2), so these were not different. After dazoxiben ingestion, the amount of MDA produced by platelets from the "responders" was 1.5 nmol/10⁹ platelets (range 0.5 to 2.4) and for the "non-responders" was 2.5 nmol/10⁹ platelets (range 0.9 to 5.9). These values are not significantly different from each other ($P>0.1$). So, unlike the release reaction, there were no "non-responders" in respect of MDA production after dazoxiben.

When PRP taken from each subject after ingestion of dazoxiben 200 mg was stimulated with 1 mM NaAA in the presence of a further 10⁻⁴M dazoxiben *in vitro*, the release reaction still occurred in three of the four "non-responders" (mean release=23%, range 10 to 42). In the fourth "non-responder", it was abolished.

After ingestion of aspirin 40 mg

Like dazoxiben, the ingestion of aspirin 40 mg also gave rise to two patterns of response to NaAA. In PRP from eight of the individuals, the effect of the aspirin was to completely prevent the release reaction induced by NaAA, whereas in PRP from the other four individuals the percentage release reaction induced by 1 mM NaAA was only reduced from 59% (55–67) to 51% (46–56). Two of the samples of PRP in which aspirin had not prevented the NaAA-induced release reaction came from the individuals who were "responders" to dazoxiben, whereas the other two came from "non-responders" to dazoxiben. Figures 2 and 4 show the results of two subjects in whom aspirin abolished the NaAA-induced release reaction. Figures 3 and 5 show the results from two subjects in whom aspirin did not abolish the NaAA-induced release reaction.

Unlike dazoxiben, the effectiveness with which aspirin inhibited the release reaction was related to the effectiveness with which it inhibited thromboxane synthesis. The mean amount of MDA produced by platelets from those for whom the NaAA-induced release reaction was prevented by aspirin was 1.1 nmol/10⁹ cells (range 0.2 to 2.1) and for these for whom the NaAA-induced release reaction was not prevented by aspirin was 6.4 nmol/10⁹ cells (range 5.2 to 8.4). This difference is highly significant ($P<0.001$).

After aspirin 40 mg and dazoxiben 200 mg together
After both aspirin and dazoxiben had been ingested,

the effect was to completely inhibit the NaAA-induced release reaction in PRP from all the subjects. The amount of MDA generated in response to 1 mM NaAA was 0.6 nmol/ 10^9 platelets (range 0.2 to 1.5). This was significantly less than the control value, and also significantly less than the values after ingestion of dazoxiben alone and aspirin alone ($P < 0.01$ in all three cases).

ADP-induced release reaction

The mean extent of the ADP-induced release reaction in PRP from the 12 subjects, before any drugs were taken, was 19% (range 8 to 32). One hour after dazoxiben 200 mg the mean extent of the ADP release reaction was 19% (range 7 to 38%). Clearly the ADP-induced release reaction was unaffected by dazoxiben. Twenty-four hours after aspirin 40 mg, the mean extent of the ADP-induced release reaction was 14% (range 5 to 32). This was not significantly different from the control value obtained before any drugs were taken. After both drugs had been ingested, the mean extent of the ADP-induced release reaction was 9% (range 4 to 16). This value was significantly different from the value obtained before drug ingestion ($P < 0.01$).

Adrenaline-induced release reaction

The mean extent of the adrenaline-induced release reaction in PRP from the 12 subjects before any drugs were taken was 49% (range 39 to 69). One hour after dazoxiben 200 mg the mean extent of the adrenaline-induced release reaction was 38% (range 0 to 57). This value was significantly lower than the value obtained when no drugs had been taken ($P < 0.01$). Twenty-four hours after aspirin 40 mg the mean extent of the adrenaline-induced release reaction was 30% (range 0 to 60). This value was significantly lower than the value obtained when no drugs had been taken ($P < 0.01$). After the combination of aspirin and dazoxiben, the mean extent of the release reaction induced by adrenaline was 19% (range 0 to 47). This was significantly lower than the control when no drugs had been taken ($P < 0.01$), and also significantly lower than the results obtained after each drug alone ($P < 0.01$).

Discussion

Dazoxiben is a selective inhibitor of thromboxane synthetase, with no activity against cyclo-oxygenase or prostacyclin synthetase (Tyler *et al.*, 1981). We have confirmed its effect on TXA₂ production (measured as MDA generation by platelets) after oral ingestion in healthy subjects. MDA production

in response to exogenous NaAA fell dramatically in PRP from all the subjects 1 h after ingestion of dazoxiben 200 mg. However, despite this consistent effect on MDA production, dazoxiben ingestion only abolished the release reaction in response to NaAA in PRP from some subjects, but not in others. NaAA-induced generation of MDA in PRP after dazoxiben was not significantly different in these two groups, so the difference in response to dazoxiben cannot be simply explained on the basis of differing degrees of TXA₂ inhibition.

The differential response to dazoxiben was consistently reproduced by *in vitro* testing of platelets from the same subjects in the presence of a standard concentration of 1×10^{-4} M dazoxiben. Thus, the subjects fell into two groups: those in whom dazoxiben inhibited MDA production and also abolished release reaction in response to NaAA, and those in whom release reaction was only slightly inhibited despite marked inhibition of MDA production. These categories correspond to the "responders" and "non-responders" described by Heptinstall *et al.* (1980) in *in vitro* studies using a related selective inhibitor of thromboxane synthetase, dazoxiben (UK 34787).

In three of the four "non-responders" in the present study, the release reaction was not abolished in PRP taken after ingestion of dazoxiben, even when it was incubated with further dazoxiben added *in vitro* at a concentration of 10^{-4} M. The "non-responders" in our group had platelets that tended to undergo a more extensive release reaction in response to NaAA, in the absence of any drug, than the "responders", although there was an overlap between individual results in the two groups. These results are similar to those described in the accompanying paper (Heptinstall & Fox, 1982) which reports on further studies of the effects of *in vitro* addition of UK 34787 to human PRP. It was found in that study that platelets that released most [¹⁴C]-5-HT in response to NaAA were also the ones that seemed to be most sensitive to TXA₂. In the present study there was a significant difference between "responders" and "non-responders" in the mean amount of NaAA that was required to induce a release reaction in the absence of dazoxiben. We are at present uncertain how to interpret this difference between platelets in the amount of NaAA required to induce release, or of its relevance to other aspects of platelet behaviour.

One possible explanation for the difference between "responders" and "non-responders" may lie in the fate of the cyclic endoperoxide intermediates PGG₂ and PGH₂ produced by the intact cyclo-oxygenase system. Both these substances are themselves potent inducers of platelet aggregation and release reaction, and it is possible that these en-

doperoxides can replace TXA₂ as mediators of platelet behaviour. However, platelets are capable of converting PGG₂ and PGH₂ into PGD₂, a potent inhibitor of platelet aggregation (Di Minno *et al.*, 1979), and it is possible that, in the presence of a thromboxane synthetase inhibitor, some platelets generate enough PGD₂ to inhibit platelet behaviour, while others do not. Further studies are required to test this hypothesis.

The inhibition of MDA generation induced by 1.0 mM NaAA in PRP from subjects 24 h after ingesting aspirin 40 mg was marked, but more variable than that seen 1 h after dazoxiben 200 mg. Inhibition of the NaAA-induced release reaction by aspirin was related to the extent of inhibition of MDA production. This is in contrast with the results seen with dazoxiben. The extent of inhibition of MDA production and release reaction by aspirin was not related to the "responder/non-responder" status of the subjects in relation to dazoxiben. The combination of aspirin 40 mg and dazoxiben 200 mg abolished the NaAA-induced release reaction in all subjects 25 h after aspirin ingestion, and 1 h after dazoxiben.

Neither aspirin 40 mg nor dazoxiben 200 mg alone had a significant effect on the ADP-induced release reaction, although adrenaline-induced release was significantly lower after each drug. A combination of aspirin 40 mg 25 h earlier, and dazoxiben 200 mg 1 h earlier significantly lowered both ADP- and adrenaline-induced release. Thus, if ADP- and adrenaline-mediated platelet behaviour are important in thrombogenesis, a combination of "low-dose" aspirin and dazoxiben might be of more therapeutic benefit than either drug alone, especially if it could be shown that vessel-wall prostacyclin production was not greatly altered by the combination. Alternatively, multiple doses of dazoxiben or of low-dose aspirin might have a cumulative effect on platelet behaviour while still preserving prostacyclin production. Before these theories can be put to clinical test, further work must be undertaken to provide more understanding of the effects of modulation of platelet and vessel wall prostaglandin pathways, and of the role of platelets in thrombosis.

E.W. Jones and S.R. Cockbill are both supported by a project grant from the Medical Research Council. Dazoxiben capsules were kindly supplied by Pfizer Ltd. We particularly thank Dr H.M. Tyler for help and advice. We are grateful to Professor J.R.A. Mitchell for reading the manuscript, and for constructive comment.

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