Review

The antithrombotic profile of aspirin. Aspirin resistance, or simply failure?

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Introduction

Cyclooxygenase-1 [COX-1, prostaglandin synthase] catalyses the transformation of arachidonic acid to the unstable intermediate prostaglandin PGH_2. Subsequently, thromboxane synthase acts on PGH_2 to form TXA_2, a transient biological product that induces platelet aggregation and is a powerful vasoconstrictor. Aspirin acts primarily by interfering with the biosynthesis of cyclic prostanoids: TXA_2, prostacyclin, and other prostaglandins. It irreversibly inhibits COX-1 by acetylation of serine-530 and induces a long-lasting functional defect in the platelets. The resultant decrease in production of prostaglandins and TXA_2 probably accounts for much of aspirin's antithrombotic effect [1,2]. The plasma half-life of aspirin is only 20 min in circulating blood. It is rapidly deacetylated and converted to salicylate in vivo. Salicylate does not affect COX-1 or COX-2 activity [3].

Because platelets cannot generate new COX, the effects of aspirin last for the duration of the life of the platelet [10 days]. After a single dose of aspirin, platelet COX activity recovers by 10% per day in parallel with platelet turnover. Although it may take 10 days for the total platelet population to be renewed, it has been shown that if as few as 20% of the platelets have normal COX activity, hemostasis may be normal.

TXA_2 and PGI_2 have opposing effects on hemostasis but the data suggest that the antithrombotic effects of TXA_2 inhibition predominate over the possible prothrombotic effects of PGI_2 inhibition [2].

The usual anti-thrombotic dose of ASA ranges from 80 to 500 mg/day. Platelet inhibition, as indicated by aggregometry, occurs very rapidly: within 5 min of ingestion of 320 mg lysine acetylsalicylate, or within 30 to 60 min of oral 320 mg acetylsalicylic acid administration [4].

As COX-1 inhibition by aspirin is irreversible, there is a cumulative inhibition of TXA_2 generation by platelets when low doses of aspirin are administered chronically [5]. There is a non-linear relationship between inhibition of platelet TXA_2 generation and inhibition of TXA_2-dependent platelet aggregation. More than 95% inhibition of TXA_2 generation is necessary to influence function [6]. With low aspirin doses, platelet thromboxane falls below 95% only after several days. Nevertheless, low-dose [40 to 60 mg] as well as high-dose [500 mg] aspirin suppresses thromboxane A_2 synthesis by >95% [7,8].

Although aspirin at high doses is anti-inflammatory due to inhibition of COX-2, it is 170-fold more potent in inhibiting COX-1 than COX-2 [9]. Low aspirin doses that have little or no measurable anti-inflammatory effect leave vascular prostacyclin formation almost intact [10]. Doses of aspirin in excess of 80 mg/d result in substantial inhibition of endogenous prostacyclin biosynthesis [11]. Two hours after 150 mg aspirin intake, 81 to 100 per cent inhibition of prostacyclin synthesis was demonstrated [12].

Epidemiological studies have indicated that very low and very high doses of aspirin [30–1500 mg] have equivalent anti-thrombotic effects, suggesting that inhibition of

platelet COX-1 is indeed the crucial target of aspirin [10]. This supports the view that inhibition of platelet COX-1 by low doses is sufficient to explain the cardio-protective effect of aspirin observed in clinical trials [1]. Aspirin reduces the risk of secondary events by about 25% in cardiovascular diseases [13] but some patients have recurrent vascular events in spite of aspirin therapy. It has been proposed that these patients are resistant to aspirin's antithrombotic effect.

Nevertheless, as published by Syrbe et al., [14] individual dose of aspirin seems to be necessary to full inhibition of ex-vivo platelet aggregation. Thus adjusted dose of aspirin for individuals with cardiovascular diseases could be necessary to prevent failure (aspirin resistance?) of therapy

Failures of aspirin to protect against arterial thrombotic events

Failure of aspirin to produce the expected inhibition of platelet function might be attributed to several mechanisms. Many individuals treated with aspirin do not achieve the inhibitory response anticipated on the basis of laboratory measurements of platelet activation and aggregation, a phenomenon termed "aspirin resistance" [15]. Antiplatelet drugs that are effective and safe in one individual may be ineffective in another. Aspirin is a weak platelet inhibitor, so on its own it does not provide sufficient antithrombotic therapy in some clinical or experimental circumstances. It seems that resistance to aspirin may be associated with an increase of arterial thrombotic events in spite of chronic intake. In ex vivo assays using aggregometry, with sodium arachidonate as agonist, aspirin inhibits platelet aggregation irreversibly in most people. However, in several patients, aspirin does not afford the degree of platelet inhibition needed to preclude events according to in vitro assessments [16-18].

Gum, Topol and co-workers [19] found a significant correlation between aspirin resistance as measured by platelet aggregation and the composite primary outcome of death, myocardial infarction, or cerebrovascular accident in patients with stable cardiovascular disease. Of the 326 patients studied, 17 [5.2%] were resistant to 325 mg/day aspirin. Insufficient inhibition of platelet aggregation by aspirin ["aspirin resistance"] has been observed in 6–24% of patients with stable coronary artery disease: by optical aggregation, 5.5% of the patients were aspirin resistant and 23.8% were aspirin semi-responders.

The PFA-100 [Platelet Function Analyzer, Dade] is a device that simulates platelet function in vitro at high shear. The test is performed by combining 2 agonists in cartridge form: collagen/ADP and collagen/epinephrine. Closure time is the time required for platelets to effect full occlusion of an aperture cut into a membrane coated with

the pair of agonists. Under standardized flow conditions, platelet activation and aggregation slowly build a stable platelet plug at the aperture [20]. The PFA-100 system allows quantitative assessment of platelet function, reporting platelet aggregability as the time required to close the small aperture in the biologically active membrane. This analyzer detects no difference between the effects of low and high-dose aspirin on platelet function [21]. In the study by Gum and co-workers, 9.5% of patients were aspirin resistant [19] according to the PFA-100.

Aspirin resistance is significantly associated with an increased risk of death, myocardial infarction or cerebrovascular accident compared with aspirin-sensitive patients [24% vs. 10%, P = 0.03] [22]. This study suggests that aspirin non-responders might obtain less benefit with respect to cardiovascular events. Aspirin non-responder status may contribute to failure of aspirin therapy in the secondary prevention of cerebrovascular incidents in as many as 30–40 % of patients [23].

In the paper by Eikelboom et al. [24], 488 patients who suffered myocardial infarction or stroke or died from cardiovascular causes were compared with 488 patients who had no cardiovascular event; all 976 patients took aspirin. The patients in the highest quartile of urinary 11-dehydrothromboxane B_2 excretion levels [i.e. those who were least affected by aspirin intake] had a $3 \cdot 5$-fold higher risk of cardiovascular death compared with those in the lowest quartile [24].

With documented evidence of congestive heart failure [left ventricular ejection fraction <40% and New York Heart Association class II to IV symptoms], 88 outpatients who had been treated with aspirin 325 mg/day for ≥1 month were included in the study by Sane et al.[25]. Platelets were stimulated with 5 μM of adenosine diphosphate [ADP], 1 μg/ml of collagen or 5 μM of epinephrine, and aggregation was assessed using a Chronolog Lumi-Aggregometer. Whole blood aggregation was determined using the Chronolog device and the sample was stimulated with 4 μg/ml of collagen. Platelet receptor expression was assessed by flow cytometry. The effect of shear stress on platelet function was analyzed using the PFA-100. Closure times were determined using collagen/epinephrine test cartridges. Patients were considered aspirin non-responsive when 4 of the 5 parameters assayed were observed. Persistent platelet activation despite aspirin therapy was detected in 50 of the 88 patients [56.8%]. Using the criterion of closure time ≤193 s with the collagen/epinephrine cartridge [19], 49 of 88 patients [55.7%] could be considered aspirin resistant.

Patients undergoing coronary artery bypass grafting (CABG) have a high incidence of aspirin resistance. Before CABG, 10 µmol/L aspirin inhibited more than 90% of thromboxane formation within 15 min. In aspirin-resistant PRP, the inhibition of thromboxane formation was significantly delayed [18].

Aspirin may not be cardioprotective in patients with hyperlipidaemia. Platelet aggregation was measured using a final collagen concentration of 1.0 µg/ml in 56 patients receiving aspirin 325 mg/day who had a history of coronary heart disease. The 14 patients with poor responsiveness to aspirin had significantly higher mean concentrations of total cholesterol and LDL cholesterol than the 42 patients with good responsiveness. In total, 9/13 [69%] patients with hyperlipidemia had poor responsiveness to aspirin [26].

Recent data also indicate that other nonsteroidal antiinflammatory drugs [NSAIDs] might interfere with aspirin's effects [27,28]. Kurth et al [27] suggested that regular but not intermittent use of NSAIDs inhibits the clinical benefits of aspirin.

Experimental models have also shown that aspirin fails to prevent thrombosis-related activities. Thrombin generated at an endothelial lesion induces platelet activation, an activity not affected by aspirin [29]. Maalej and Folts [30] showed, in a canine model with coronary artery stenosis, that aspirin did not prevent the reduction in cyclic flow. As several potential agonists are released when the vascular endothelium is damaged, not only from platelets but also from the endothelial cells, erythrocytes and leukocytes, it may be supposed that the inhibitory effect of aspirin may also be overwhelmed in vivo. This study might explain why both platelet aggregation and platelet count are significantly lower in the coronary venous blood than in the aortic blood of patients with coronary artery disease, though not in normals; this was found many years ago by Metha et al. [31].

Santos and colleagues [32] found that the synergism between red cells and platelets in promoting thrombosis was not prevented by low-dose aspirin but only by 500 mg/day. The implication of these studies is that patients taking aspirin may not be fully protected against arterial thrombosis [33]. In 1986 and 1988 [34,35], through ex vivo aggregation experiments with platelet-rich plasma from volunteers taking aspirin, we showed that the inhibitory effect of aspirin on platelet aggregation induced by sodium arachidonate was overcome by the synergism between sodium arachidonate and platelet activating factor, or ADP or collagen. We employed a mixed agonist system in an attempt to better reflect the multiple stimuli that platelets encounter during in vivo activation. Under these conditions a normal platelet aggregation pattern was obtained, although the thromboxane level measured in the stimulated platelet-rich plasma was less than 5% in all aspirinated samples. Since this constitutes a "physiological effect at the site of eventual endothelial damage" it can not be called aspirin resistance.

As was also suggested by Bertele et al. [36], our study indicated no correlation between platelet function and thromboxane level, implying that aspirin does not prevent an agonist potentiation effect when low doses or a daily high dose are administered. These results are in line with those obtained by Cerletti et al. [37]

On the other hand, it is possible that in the PFA-100 device where a collagen/epinephrine or ADP/epinephrine coated cartridge is used, the synergistic effect of these two agonists has a similar outcome to that observed in our PRP aggregation studies. We suggest that this can not be called aspirin resistance and that the PFA-100 system used to define this concept, was overestimated

About the Definition of Aspirin Resistance
As pointed out by Patrono [38], recurrent vascular events despite the chronic use of aspirin should be defined as treatment failure instead aspirin resistance [unless we attribute treatment failure to aspirin resistance]. Aspirin resistance is a poorly defined term. It can imply a clinical inability of aspirin to protect individuals from arterial thrombotic events; or laboratory indications of the failure of aspirin to inhibit platelet activity, mainly platelet aggregation; or a close-to-normal urinary concentration of thromboxane metabolites. Possible mechanisms of aspirin resistance were detailed by Gaetano and Cerletti [39] and were summarized by Cambria-Kiely and Gandhi [40]. They include: **1.** Bioavailability of aspirin; **2.** Platelet function; **3.** Polymorphisms; **4.** Platelet interactions with other blood cells and cell-derived products; **5.** Several other factors i.e. stimulation of platelet aggregation by cigarette smoking; ASA resistant platelet aggregability by increased levels of norepinephrine, as seen during excessive exercise or periods of mental stress; biosynthesis of F[2]-isoprostane 8-iso-prostaglandin [PGF2 alpha], a bioactive product of arachidonic acid peroxidation; and increased platelet sensitivity to collagen.

The urinary concentrations of the metabolite 11-dehydrothromboxane B_2 indicate the level of TXA_2 generation. Eikelboom et al [24] indicated that in aspirin-treated patients, urinary concentrations of 11-dehydrothromboxane B_2 predict the future risk of myocardial infarction or cardiovascular death. These authors also support the view that failure to suppress thromboxane generation defines aspirin resistance [24]. This hypothesis assumes a direct association between the rise of urinary 11-dehydrothrom-

boxane B_2 levels and increment of vascular events [myocardial infarction, stroke and cardiovascular death].

Poor platelet responsiveness to aspirin was defined by Friend et al. [26] as aggregation of $\geq$ 50% of platelets using the PFA-100 device. Gum et al [19] defined aspirin resistance on the basis of the platelet aggregation assay: aggregation of $\geq$ 70% with 10 μM ADP, and of $\geq$ 20% with 0.5 mg/ml arachidonic acid, constituted aspirin resistance. They also defined aspirin semiresponders as meeting one but not both of these criteria. There seems to be no correlation between the results obtained by aggregometry and by the PFA-100 device, as showed by Gum et al [19]: of the 18 patients who were aspirin resistant by aggregation, only 4 were aspirin resistant by PFA-100.

Anti-aggregatory treatment with ASA was considered by Tarjan et al. [17] to be ineffective if typical aggregation curves were obtained above the following final inducer concentrations: ADP: > 5 μM, epinephrine: > 5 μM, arachidonic acid: > 250 μM, collagen: > 2 μg/ml. Compliance by subjects was proven by HPLC determination of urinary metabolites of ASA, performed immediately after admission [17].

Sane et al. [25] considered patients to be aspirin non-responsive when 4 of the following 5 parameters were observed: collagen-induced aggregation >70%; adenosine diphosphate-induced aggregation >60%, whole blood aggregation >18 ohms; expression of active GP IIb/IIIa >220 log mean fluorescence intensity units; and P-selectin positivity >8%. When the PFA-100 device was used, aspirin resistance was defined in terms of a normal collagen and/or epinephrine closure time [< or = 193 seconds] [19].

Weber et al. [41] proposed to classify aspirin resistance into three categories. Type 1 [pharmacokinetic type] entails the inhibition of platelet thromboxane formation in vitro but not in vivo. Type 2 [pharmacodynamic type] is characterized by the inability of aspirin to inhibit platelet thromboxane formation both in vivo and in vitro. Type 3 [pseudoresistance] involves thromboxane-independent platelet activation.

According to Koksch et al. [42] aspirin resistance involves, besides thromboxane formation, an impaired inhibition of platelet aggregation and an increased expression of P-selectin, a marker of α-granule secretion associated with the progression of atherosclerosis.

Also Weber et al [43] suggested that the inducible isoform of cyclooxygenase in platelets, COX-2, confers aspirin resistance, although this opinion was challenged by Patrignani et al. [44].

Aspirin resistance may be caused by an increased sensitivity of platelets to collagen. A platelet aggregation study specific for collagen dose response may be useful for strict selection of ASA responders for low-dose ASA therapy, and for identifying ASA non-responders for high-dose ASA therapy [45].

Using a collagen/epinephrine coated cartridge in the PFA-100 [R], a prevalence of aspirin resistance of 29.2% was determined by Macchi et al. [46]. These authors support the view that hypersensitivity to adenosine diphosphate could provide a possible explanation for aspirin resistance.

Buchanan and Brister [47] used bleeding time to define responders and non-responders. Aspirin effected a dose-dependent prolongation of bleeding time in 60% of volunteers [ASA responders], which was associated with decreases in platelet TXA_2 and 12-hydroxyeicosatetraenoic acid [12-HETE] synthesis and in platelet aggregation and adhesion. However, in volunteers whose bleeding time was not prolonged [ASA non-responders], platelet 12-HETE synthesis and platelet adhesion were unchanged or increased [P < 0.001] despite platelet TXA_2 and aggregation being inhibited. Beside the problems of methodology, the dissociation between TXA_2 and bleeding time makes this test inadequate for defining non-responsive patients.

Andersen et al. [48] showed that the levels of TXA_2 were extremely low in both aspirin responders and non-responders. However, the levels of soluble P-selectin were significantly higher in non-responders than responders.

Resistance to other antiplatelet drugs has also been described. "Clopidogrel resistance" has been documented [49]. Clopidogrel non-responders were defined by an inhibition of ADP [5 and 20 mol/L] induced platelet aggregation that was less than 10% of the baseline value 4 h after clopidogrel 600 mg intake. Semi-responders corresponded to patients with an inhibition of 10 to 29%; responders are patients with an inhibition over 30%. Up to 4.7% of the patients undergoing coronary stenting developed thrombotic stent occlusion, despite intensive clopidogrel treatment; the parallel with aspirin resistance seems striking. However, as there is no standard definition of aspirin resistance, comparison between the results of different studies is difficult.

We support the view that aspirin resistance cannot be defined by the level of serum thromboxane or its urinary metabolites, because these measurements do not correlate with the reduction of inhibition of platelet aggregation in response to multiple stimuli, and also because:

1. Although most of the thromboxane is believed to come from the platelets, there are additional cellular origins: monocytes/macrophages are also a rich source of thromboxane A_2 [50].

2. Unlike the platelet, the macrophage is capable of synthesizing new COX-2 after aspirin has inhibited it. COX-2 is the enzyme responsible for most of the metabolism of arachidonic acid in the macrophage, and low dose aspirin is not sufficient to inhibit COX-2 [50] maximally.

3. Macrophages in atheromata may contribute significantly to the pool of thromboxane A_2 [51].

4. Aspirin only inhibits monocyte PGHS-2, which is inducible by inflammatory stimuli, transiently at very high concentrations [52].

Pleiotropic Effects of Aspirin

In patients undergoing aspirin therapy, despite synergistic platelet aggregation at endothelial lesions in vivo and close-to-normal concentrations of thromboxane in plasma and thromboxane metabolites in the urine, aspirin still prevents arterial thrombosis to some extent. It therefore appears that aspirin exerts antithrombotic effects through mechanisms other than its weak inhibition of platelet aggregation. These other mechanisms could be at least as important [53].

Aspirin significantly decreases the levels of inflammatory factors in animal models [54]. It markedly inhibits plaque growth. It inhibits vascular smooth muscle cell proliferation, and transforming growth factor β plays a significant intermediary role in this [55,56]. It diminishes in vitro thrombin generation in platelet rich plasma activated by sodium arachidonate [57]. Pretreatment with 1.2 g of aspirin preserves endothelial function and diminishes the increase of inflammatory markers after administration of salmonella vaccine [58]. In a subset of healthy men in the Physicians Health Study, patients within the highest quartile of C-reactive protein elevation showed a significant benefit from aspirin treatment [325 mg/day every other day] compared with those in the lowest quartile [59]. In patients with coronary artery disease, aspirin also seems to reduce C-reactive protein levels [60]. Low-dose aspirin [as well as sinvastatin] decreased IL-1β levels and platelet activation after a 2-month treatment [61]. Other effects of aspirin are: modulation of thrombolysis [62,63]; increase in fibrin gel porosity [64]; effects on membrane fluidity [65]; modulation of the formation of lipid bodies in leukocytes from which eicosanoids may be released [66]; facilitation of the inhibition of platelet activation by neutrophils, an effect that appears to be mediated by a nitric oxide [NO]/cGMP-dependent process [67]; protection of low density lipoprotein from oxidative modification [68];

improvement of endothelial dysfunction in atherosclerosis [69]; and acetylation of platelet membrane glycoproteins IIb-IIIa, which augments the inhibitory effects of abciximab by increasing its binding to platelets [70]. This range of effects of aspirin could be relevant to its pleiotropy; some of the effects could be more important than its anti-platelet activity. It is simplistic to suppose that suppression of aspirin's weak anti-aggregating action is the only cause for its failure to prevent arterial thrombosis.

Failure of Plaque "Passivation". Role of Inflammation

It is not surprising that a hypercoagulable state occurs in the plasma in acute coronary syndromes, as indicated by blood prothrombotic markers or by the presence of new cardiovascular events in the face of powerful antithrombotic therapy [71-78]. Inflammation can have a prothrombotic effect through the increase of tissue factor, platelet reactivity or acute phase reactant proteins such as fibrinogen, or through a decrease in fibrinolysis by increasing the level of plasminogen activator inhibitor-1 [PAI-1] [79].

Locally, thrombin is not only involved in coagulation; it has pro-inflammatory activity [80]. Thrombin can activate receptors on platelets and the vascular endothelium, leading to inflammation and tissue injury [81]. Activated platelets express CD40L and induce endothelial cells to secrete chemokines and to express adhesion molecules, indicating that platelets could initiate an inflammatory response of the vessel wall [82]. Interesting, it has recently been shown that besides their specific activity, lipid-lowering drugs [83,84], the novel group of antidiabetic drugs thiazolidinediones [85,86], and angiotensin-converting enzyme inhibitor, all exhibit anti-inflammatory properties. Their clinical benefits may to some extent derive from lowering inflammation [87].

The underlying inflammation of atheromata in acute coronary syndromes could be the basis of failure of intensive antithrombotic therapy [88]. COX-2 inhibition may decrease athero-inflammation, reducing monocyte infiltration and improving vascular cell function and plaque stability, resulting in a decrease of coronary athero-thrombotic events [53]. In our hands, the combination of a preferential COX-2 inhibitor, meloxicam, plus heparin and aspirin, proved superior to heparin and aspirin alone for reducing coronary thrombotic events in patients with acute coronary syndromes without ST-segment elevation [89].

In conclusion, aspirin resistance depends on circumstances independent of aspirin and could more aptly be termed aspirin failure. This is supported by the fact that increasing doses of aspirin can completely inhibit platelet

aggregation in patients who are unresponsive or only partially responsive to aspirin [cited by [90]]. Otherwise, thrombin generated at the endothelial lesion can induce platelet activation, which is not affected by aspirin.

All these findings indicate that, in acute coronary syndromes, there is a strong pro-clotting activity in the complicated atheroma. This activity is augmented by the underlying inflammation, which in several circumstances can overwhelm the inhibitory effects of single or combined anti-thrombotic drugs, including aspirin. Thus, the underlying endothelial and/or atheroma inflammation in coronary syndromes could explain why the antiplatelet effect of aspirin fails, irrespective of its anti-aggregating capacity or the urinary levels of thromboxane metabolites.

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