Aspirin therapy: An attempt to explain the events of prothrombotic complications after treatment discontinuation

Christian Doutremepuich; Omar Aguejouf; Vanessa Desplat; Francisco X. Eizayaga
Laboratoire d’Hématologie, Université Victor Segalen Bordeaux 2, Bordeaux, France

Summary
Aspirin remains the most widely used drug for prevention of vascular events. Recent observational epidemiological evidence has raised the concern that aspirin withdrawal for treatment non-compliance, surgery or side effects can carry an increased thrombotic risk. The delay to the thrombotic event was between 7 to 30 days in most reports and most frequently 7 to 10 days. The mechanism underlying this effect remains poorly understood. Using an in vivo model of laser-induced thrombosis, aspirin injected in one single dose of 100 mg/kg bw has also shown a prothrombotic activity in the rat 8 to 10 days after injection in the normal rat. The hypothesis was made that minimal concentrations of aspirin or ultra-low dose aspirin (ULDA) could induce this effect. ULDA showed prothrombotic properties in the same model of induced thrombosis that were very similar to those described after aspirin withdrawal, but the effect was observed only one hour after aspirin administration. This prothrombotic effect of ULDA is very similar to the effect observed after COX 2 selective inhibition with NS 398. The administration of both the selective COX 2 inhibitor and ULDA did not produce further changes. High-dose ASA counterbalances the lack of COX 2 with an antithrombotic effect. No effect of residual ASA was observed in COX 2 -/- mice, thus confirming the existence of a COX 2 inhibition pathway. COX 2 inhibition produced by residual ASA is the probable cause of ischaemic accidents and drug-eluting stents thrombosis a few days after ASA withdrawal.

Keywords
Aspirin withdrawal, thrombosis, COX inhibition, “knock-out” mice

Introduction
Aspirin remains the most widely used drug for prevention of vascular events. Taken alone or in association with clopidogrel, it has shown a positive effect in acute myocardial infarction patients or for the prevention of atherothrombotic events (1, 2). Recent observational epidemiologic evidence has raised the concern that aspirin withdrawal for treatment non-compliance, surgery or side effects can carry an increased thrombotic risk. This risk appears to be increased for ischaemic stroke (3–5), cardiovascular problems (6–12), and acute lower limb ischaemia (13). The delay to the thrombotic event was between 7–30 days in most reports and most frequently 7–10 days. The possibility of a rebound effect after withdrawal of non-steroidal anti-inflammatory drugs (NSAIDs) was raised in previous papers, but the mechanism remains poorly understood (14). For Sibon (3) reported that 4.5% of their patients with ischaemic stroke reported discontinuation of anti-platelet drugs within the previous month, and 85% of them were taking aspirin. The stroke occurred 7.4 ± 1.26 days after discontinuation of the treatment. Fisher (6) reported a 1.52-fold risk of acute myocardial infarction (95% confidence interval [CI], 1.33 – 1.74) in patients who stopped NSAIDs between one and 29 days before testing compared to non-users. Ferrari (7) support the hypothesis that aspirin withdrawal may represent a real risk for the occurrence of a new coronary event. In a series of 1,236 patients hospitalised for acute coronary syndrome, 4.3% of the events but 13.3% of the recurrences were present 10 ± 1.9 days after aspirin withdrawal. Similar observations were reported by Collet (9) in a series of 1,358 patients. Burger (8) and Biondi-Zoccai (11) in two meta-analyses of 49,590 and 50,279 patients, respectively, suggested that aspirin withdrawal or non-adherence preceded 10.2% of acute coronary syndromes and was associated with a three-fold higher risk of major cardiac events (odds ratio [OR] = 3.14 [1.75–5.61], p = 0.0001). The time interval in days between discontinuation and the events was 14.3 ± 11.3 for stroke, 8.5 ± 11.3 for acute coronary syndromes and 25.8 ± 18.1 for acute peripheral syndromes (8).

The main mechanism of the antithrombotic effect of aspirin is a definitive inhibition of cyclooxygenase (COX) 1 by acetylation that decreases the production of thromboxane (TXA2) in the platelets and the production of PGI2 (prostaglandin) in the endothelial cells. As platelets lack a cell nucleus and can not synthesise more COX 1, this inhibition remains for the lifespan of the platelets and decreases with the progressive generation of new platelets. In the endothelial cells, new COX 1 is synthesised and PGI2 production can be restarted. COX 2, an inducible form of COX, which is present in the endothelium but not mainly in the platelets, is also able...
to increase PGI₂ production. As a result of these effects, the normal balance between antithrombotic endothelial PGI₂ and prothrombotic platelet TXA₂ is switched to a new state in which the antithrombotic effect of endothelial PGI₂ prevails. Aspirin half-life is 13–19 minutes and is metabolised to salicylate, which has a half-life of 3.5 to 4.5 hours. The delay of several days observed between aspirin withdrawal and the thrombotic event raises the possibility that aspirin might have some long-lasting prothrombotic effect. The hypothesis of different aspects of the prothrombotic effects of aspirin have been studied in our laboratory.

Pharmacological evidence

The dose-dependent effects of aspirin

To observe in vivo the intimate nature of changes in thrombosis pathophysiology, endothelial cells, sub-endothelial collagen and platelets should be present in the most unmodified manner.

In 1992 we used a laser-induced thrombosis model in mesenteric vessels. To validate this model, higher doses of aspirin were initially tested (50, 100 and 200 mg/kg rat weight). The parameters used were the number of shots required to start embolisation, the number of emboli observed after the laser shot and the duration in minutes of the resulting embolisation. The three doses of aspirin increased the number of laser injuries required to start embolisation in a dose-dependent manner, and the number of emboli and the duration of embolisation were decreased (15).

The effects of different doses of aspirin, including very low decreasing doses of aspirin (dilution 5, dilution 9 and dilution 15) as described in the following first study, have been tested. The doses studied were saline control, salicylate 100 mg/kg, aspirin 100 mg/kg and decreasing doses of aspirin covering very extended values. Aspirin 100 mg/kg showed an antithrombotic effect with a decreased formation of emboli and duration of embolisation and a prolonged induced haemorrhage time (IHT). Importantly, the dilution 15 of aspirin showed a prothrombotic effect only in the laser study, whereas IHT was unchanged. Conversely, 100 mg/kg and 1 mg/kg of aspirin produced a clear prolongation of IHT, indicating not an opposite mechanism but likely a different one. The study demonstrated that the very low dose of aspirin-induced prothrombotic effect (16).

The time-dependent effects of aspirin

Frequently, the thrombotic events observed several days after aspirin withdrawal are considered as rebound effect or a return to the baseline of a patient with an already increased risk.

The half-life of a low dose of aspirin is approximately 13–19 minutes and that of salicylate 3.5 to 4.5 hours. Nevertheless, as the acetylation of COX 1 produced by aspirin is definitive, the effect remains for several days until new platelets are generated. The effect in time of one single dose of aspirin was studied with the injection of one antithrombotic dose of aspirin (100 mg/kg bw) and performing laser-induced thrombosis 2, 4, 6, 8, 10, 12, 14 and 16 days after the injection (17). There was a potent antithrombotic effect two days after injection, decreasing the number of emboli and the duration of embolisation. Nevertheless, thrombi formation was increased at days 8 and 10, expressing in the normal rat results similar to the observational epidemiological data described above. This experiment clearly demonstrates the antithrombotic effect of aspirin two days after injection and showed that a prothrombotic state can be observed several days after a single aspirin dose in a normal rat for a given period of time. A rebound effect could not be ruled out by this experiment but this effect is obviously also observed in the normal rat.

The aims of the two following studies, are then focused on the mechanisms implied in the effects described.

First study: Effects of COX selective inhibition in association with aspirin

This study was designed to elucidate the effects of aspirin when administered in different doses or and to explore the possible underlying mechanisms regarding it’s mechanism of action.

Methods

Laser-induced thrombosis

Animals were anaesthetised with 200 mg/kg of thiopental sodium (Pentothal®, Laboratoires Abbott, Rungis, France) a median laparotomy was performed. The intestinal loop was placed on the microscope table and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France). The wavelength used was 514 nm and the energy was adjusted to 120 mW. The laser beam was applied during 1/15 seconds. The dynamic-course of thrombus formation was continuously monitored with an inverted microscope (Axiolabor, Zeiss, France). Microscopic images were recorded through a digital camera (DX L107, color camera CCD, Basler, Vision Technologies, Ahrensburg, Germany) to visualise and digitalise data coupled to a Dell monitor. A schematic view of the apparatus used has been previously described (16). Arterioles between 15 and 25 μm diameter were used. Two parameters were assessed during each procedure: the number of platelet emboli removed from the thrombus by blood flow after an injure produced by the laser shot and the duration of embolisation, defined as the time between the first and the last emboli occurring after thrombus formation, expressed in minutes.

Drugs tested

The amounts of 1 mg/ml and 100 mg/ml were obtained by diluting a solution of acetylsalicylate (Aspegic, Sanofi-synthelabo, France) 500 mg/5 ml. Aspirin dilutions were prepared as follows: 1 g of pure, finely powdered aspirin was suspended in 99 ml of alcohol (70%). After being vigorously shaken, 1 ml of this dilution was...
then mixed with 99 ml of distilled water and vigorously shaken (dilution 1). The latter process was repeated until obtaining desired dilutions: four times (dilution 5), eight times (dilution 9) and 14 times (dilution 15). The dilutions of aspirin were done with separated volumetric flasks and a piston driven air displacement pipette for accuracy. Alcohol and sterilised water following the above-mentioned procedures without adding the aspirin was used as placebo of dilutions 5, 9 and 15. Sterilised water for injectable preparations was used as placebo for groups receiving 100 mg/kg or 1 mg/kg of aspirin. Aspirin in high dose, in dilutions or in the corresponding placebo was subcutaneously administered at a final volume of 1 ml/kg rat weight. Subcutaneous administration was chosen because previous unpublished studies of our laboratory determined more accurate and reproducible results with aspirin dilutions by this method. The different placebos were used to avoid interferences due to the different kinds of preparations of aspirin used.

Selective inhibitors of COX 1, SC-560 and of COX 2, NS-398, were purchased from Cayman Chemical (Ann Arbor, MI, USA), and suspended in Carboxy-Methyl-Cellulose (CMC) 0.5 g/l at a final volume of 1 ml/kg rat weight. The CMC solution without adding the inhibitors was used as placebo. COX selective inhibitors were used at 2.5, 5, 7.5 or 10 mg/kg when used alone and at a dose of 10 mg/kg when both were simultaneously administered. COX selective inhibitors were administered per os.

SC-560 is a member of the diaryl heterocycle class of COX inhibitors which includes celecoxib (Celebrex) and rofecoxib (Vioxx). However, unlike these selective COX 2 inhibitors, SC-560 is a selective inhibitor of COX-1. Using human recombinant enzymes, the IC_{50} value for SC-560 with respect to COX-1 is 9 nM, while the corresponding IC_{50} value for COX-2 is 6.3 μM. Thus, SC-560 shows 700-fold selectivity for the COX-1 enzyme. SC-560 is orally active in the rat, where 10 mg/kg completely abolishes the ionophore-induced production of thromboxane B_{2} in whole blood (18–20).

NS-398 is a selective inhibitor of COX 2. The IC_{50} values for human recombinant COX 1 and ~2 are 75 and 1.77 μM, respectively. The IC_{50} values for ovine COX 1 and 2 are 220 and 0.15 μM, respectively (21, 22).

Animals
Male Wistar rats (200–250g) purchased from Delpre Breeding Center (St. Doulchard, France) were housed separately and acclimatised before use under conditions of controlled temperature (25 ± 2°C) and illumination (12 hour light/dark cycle). They were fed with standard rat chow and water ad libitum. Animals received care in compliance with the European Convention of Animal Care.

Distribution of groups
The study comprised five experiments of 20 groups. Each group comprised 10 animals (n = 10):
- **Experiment 1**: Action of aspirin 100 mg/kg alone or associated with specific inhibitors of COX 1 (SC-560) or COX 2 (NS-398) tested at different doses (2.5, 5, 7.5 and 10 mg/kg) or their association at 10 mg/kg.
- **Experiment 2**: Action of aspirin 1 mg/kg alone or associated with specific inhibitors of COX 1 (SC-560) or COX 2 (NS-398) tested at different doses (2.5, 5, 7.5 and 10 mg/kg) or their association at 10 mg/kg.
- **Experiment 3**: Action of aspirin dilution N° 5 alone or associated with specific inhibitors of COX 1 (SC-560) or COX 2 (NS-398) tested at different doses (2.5, 5, 7.5 and 10 mg/kg) or their association at 10 mg/kg.
- **Experiment 4**: Action of aspirin dilution 9 alone or associated with specific inhibitors of COX 1 (SC-560) or COX 2 (NS-398) tested at different doses (2.5, 5, 7.5 and 10 mg/kg) or their association at 10 mg/kg.
- **Experiment 5**: Action of aspirin dilution 15 alone or associated with specific inhibitors of COX 1 (SC-560) or COX 2 (NS-398) tested at different doses (2.5, 5, 7.5 or 10 mg/kg) or their association at 10 mg/kg.

Statistical analysis
Data are expressed as mean ± standard error of the mean (SEM) and compared using Student t-test. A value of p<0.05 was considered as significant. Statistical calculations were performed using Graph Pad Prism version 4.00 for Windows (Graph Pad Software, San Diego, CA USA, www.graphpad.com).

Results
Effect of aspirin (Figs. 1 and 3)
Thrombus production was inversely proportional to the dose injected. Number of emboli (p<0.0002) and duration of embolisation (p<0.005) decreased significantly at 100 mg/kg and increased significantly (p<0.0006 and <0.005, respectively) at dilution 15. Aspirin at 1 mg/kg induced a mild decrease in both parameters (number of emboli: p<0.02 and duration of embolisation: p<0.012). There was no significant difference in both parameters at dilution 5. While there was a significant increment at dilution 9 (number of emboli p<0.02, duration of embolisation, p<0.01), it was smaller than that observed at dilution 15.

Effects of selective inhibition of COX 1 with SC-560 (Figs. 1 and 3)
COX 1 selective inhibition with SC-560 produced a decrease in number of emboli and duration of embolisation that was proportional to the dose administered. Similar results were obtained with SC-560 and aspirin at 1 mg/kg and at 100 mg/kg. The decreases in number of emboli and duration of embolisation observed with aspirin at 100 mg/kg were not accentuated by adding increasing doses of SC-560. The pro-thrombotic effect of increasing number of emboli and duration of embolisation with aspirin at dilution 15 was somewhat antagonised by COX 1 inhibition, but a clear pro-
A prothrombotic effect was observed compared to the matching placebo group with the same dose of COX 1 inhibitor.

**Effects of selective inhibition of COX 2 with NS-398 (Figs. 2 and 4)**

The selective inhibition of COX 2 with NS-398 increased number of emboli and duration of embolisation in a dose-dependent manner. This pro-thrombotic effect was strongly antagonised by aspirin at 100 mg/kg (number of emboli $p<0.0005$ and duration of embolisation $p<0.01$ for 10 mg/kg of NS-398) and to a lesser degree by 1 mg/kg (number of emboli $p<0.03$ and duration of embolisation $p<0.01$ for 10 mg/kg of NS-398). In both number of emboli and duration of embolisation, the pro-thrombotic action of COX 2 inhibition and aspirin at dilution 15 produced comparable alterations.

**Simultaneous inhibition of COX 1 and COX 2 (Figs. 5 and 6)**

When COX 1 and 2 inhibitors were administered simultaneously, we observed a decrease in number of emboli and duration of embolisation similar to that observed with COX 1 inhibition. This effect was normalised by the lowest concentrations of aspirin ($p<0.0001$ at 10 mg/kg of inhibitors and aspirin in dilution 15).
Discussion of the first study

Aspirin in 100 mg/kg has shown a decreased thrombi formation. This effect of the higher dose of aspirin was similar to the effect of selectively inhibiting COX 1. It is interesting to see that the addition of COX 1 selective inhibition with SC 560 in 0, 2.5, 5, 7.5 or 10 mg/kg. Addition of COX 1 selective inhibition with SC 560 or 100 mg/kg of aspirin had the same effect, and that these effects act not in a synergistic way, as both are supposed to work through the same mechanism over thrombi production (23). COX 2 selective inhibition, having a clear pro-thrombotic effect, was not capable of modifying the antithrombotic effect of aspirin at the highest dose.

Aspirin in 1 mg/kg has shown a less marked effect decreasing mildly the number of emboli and duration of embolisation but not producing a haemorrhagic tendency. The lower end of aspirin dosing used in this study has shown an almost opposite effect. Significant increases in the number of emboli and duration of embolisation after one dose of aspirin at dilution 15 were also described in previous studies (16). After COX selective inhibition in different doses, aspirin at dilution 15 shows a reaction that is radically different from that of aspirin in 100 mg/kg. Whether this last high dose has an antithrombotic effect that is not modified by COX 1 inhibition and is similar in nature to it, with aspirin dilution 15 we have a pro-thrombotic reaction similar to the effect of COX 2 inhibition. The administration of dilution 15 aspirin to rats previously treated with the COX 2 selective inhibitor produced no further effect. These observations led us in a previous study to conclude that high dilutions...
of aspirin have a COX 2-inhibiting effect (23, 24). This prothrombotic reaction is not modified by COX 2 inhibition although is decreased somewhat with the antithrombotic effect of COX 1 selective inhibition. This decrease observed in the prothrombotic effect of aspirin at dilution 15 could be explained by a partially protective effect in the platelet against COX 1 inhibition. Another possible explanation may be the prothrombotic effect of COX 2 inhibition counterbalanced to some extent by the decrease in TXA\(_2\) platelet production observed after COX 1 selective inhibition.

Aspirin modifies thrombi production decreasing the response with higher doses and increasing it with the lowest dilutions. The lowest dilution of aspirin studied had an effect of protecting COX 1 against inhibition or directly inhibiting COX 2 leading to a strong pro-thrombotic state.

**Second study: Effects of aspirin in “Knock-out” mice (COX 1-/- or COX 2-/-)**

To confirm these results, we hypothesised the inhibition of COX2 by low dilutions of aspirin and designed an experiment using 72
genetically modified male homozygous mice without COX 1 (COX 1 -/-) and 72 lacking COX 2 (COX 2 -/-) where we studied induced haemorrhage time (IHT) and laser induced thrombosis to evaluate primary haemostasis.

Aspirin doses used were 100 mg/kg/bw, 1 mg/kg/bw and aspirin 1/100 dilutions number 5 (dilution 5), 9 (dilution 9) and 15 (dilution 15), which were obtained by successive 1/100 as discussed above. Sterilised water was used as placebo. All drugs were injected subcutaneously at a final volume of 1 ml/kg/bw.

Methods

Animals

Male homozygous COX 1 -/-: The COX-1 mouse was developed in the laboratories of Robert Langenbach at NIEHS and Oliver Smithies at University of North Carolina in 1995. The model was created by targeting the PtgS1 gene in E14TG2a embryonic stem cells derived from 129P2/OlaHsd mice and injecting the targeted cells into C57BL/6J blastocysts. Resultant chimeras were backcrossed to C57BL/6J mice. Taconic received stock in September 1998 for embryo transfer derivation into the NIEHS repository. The model was transferred from NIEHS to Taconic in 2002 and derived by embryo transfer. The line is maintained on a mixed B6;129P2 background by breeding homozygous males to heterozygous females. A separate wild-type colony is maintained (25).

COX 2 -/-: The COX-2 mouse was developed in the laboratories of Robert Langenbach at NIEHS and Oliver Smithies at University of North Carolina in 1995. The model was created by targeting the Ptgs2 gene in E14TG2a embryonic stem cells derived from 129P2/OlaHsd mice and injecting the targeted cells into C57BL/6J blastocysts. Resultant chimeras were backcrossed to C57BL/6J mice. Taconic received stock in September 1998 for embryo transfer derivation into the NIEHS repository. The model was transferred from NIEHS to Taconic in 2002 and derived by embryo transfer. The line is maintained on a mixed B6;129P2 background by breeding homozygous males to heterozygous females. A separate wild-type colony is maintained (26).

Mice were purchased from Taconic Farms Inc. (Hudson City Centre, NY, USA), were housed separately under conditions of controlled temperature and illumination. They were fed with standard mouse chow and water ad libitum. Animals received care in compliance with the European Convention of Animal Care.

Induced haemorrhagic time (IHT)

IHT was performed 10 minutes before thrombosis induction by laser. The tail of the mouse was immersed in water for 5 minutes at 37ºC and sectioned 6 mm from the extremity and is expressed as the time between the tail section and the end of bleeding, expressed in seconds.

Thrombus induction

See the above described procedure.

Drugs tested

The preparation of aspirin is made in the same way that in the study of the effects of aspirin in the presence of the specific inhibitors of the COX.

Sterilised water (placebo) or aspirin were subcutaneously administered at a final volume of 1 ml/kg mouse weight. The groups were treated with placebo or aspirin in 100 mg/kg, 1 mg/kg or dilutions 5, 9 or 15 (n= 9–11 mice/group).

Distribution of groups

“Knock-out” mice were distributed in six groups for COX 1 -/- and six groups for COX 2 -/- (n= 12/ group), respectively:

Group 1: Placebo (sterilised water).
Group 2: aspirin 100 mg/kg.
Group 3: aspirin 1 mg/kg.
Group 4: aspirin dilution 5.
Group 5: aspirin dilution 9.
Group 6: aspirin dilution 15.

Statistical analysis
Data are expressed as mean ± SEM and compared using one way analysis of variance (ANOVA) followed by Dunnet’s multiple comparison test. A value of p<0.05 was considered as significant. Statistical calculations were performed using Graph Pad Prism version 4.00 for Windows (www.graphpad.com).

Results and discussion of the second study
The IHT model (Fig. 7) is especially sensitive to the effect of high doses of aspirin. However, mice without COX 1 did not react to the higher doses of aspirin. The highest dilution (dilution 15) of aspirin significantly shortened IHT in COX 1-deficient mice, confirming that its strong prothrombotic effect is not mediated by COX 1. No significant changes in IHT were observed after aspirin in COX 2-deficient mice.

The highest dose of aspirin produced a decreased number of emboli and duration of embolisation (Fig. 8) with placebo in COX 1-deficient mice were clearly decreased when compared to COX 2-deficient mice, highlighting the importance of COX 1 generated TXA2 in the platelets. The highest dose of aspirin produced a decreased number of emboli in COX 1-deficient mice.

The highest dose of aspirin produced a decreased number of emboli in COX 1-deficient mice, indicating the presence of an anti-
thrombotic mechanism different to COX 1 inhibition at this dose. This model of COX 1 -/- mouse is known to produce alterations in platelet aggregation (25). Explanations for this effect may include compensation in COX 1 and 2 activity or interaction between COX and nitric oxide (NO) synthase, as suggested by Skill (27), or an effect of aspirin outside the mechanism of COX inhibition like increased NO synthesis by the endothelial cell, as suggested by Taubert (28). Although intermediate doses did not show significant results, there was a clear antithrombotic effect of high-dose aspirin in COX 2-deficient mice and a prothrombotic effect with the lowest dilution in COX 1-deficient mice. These results clearly confirm that although high dose aspirin exerts its main effect through COX 1 inhibition, dilution 15 of aspirin acts through COX 2 inhibition, thus inducing thrombosis.

High-dose aspirin in COX 1-deficient mice has a mild effect by decreasing thrombosis and a strong prothrombotic effect at dilution 15. Both effects are independent of COX 1 activity. Dilution 15 had no effect in COX 2-deficient mice. The effect observed with the lowest dose suggests that it is directly due to residual amounts of aspirin rather than a rebound effect or protective effect over COX 1 inhibition, and that the complications observed after aspirin discontinuation may arise from this unrecognised effect.

General discussion

Compliance is often difficult to evaluate in everyday practice because of clinical and laboratory aspirin resistance and variability in the methods used for assessment of the effect (29, 30). Epidemiological evidences suggest that the withdrawal of aspirin because of non-compliance or for surgical interventions can carry an increased risk of thrombotic events. This increased risk is observed within the first month, and mostly within the first two weeks. The main result of this increased risk could be ischaemic stroke, myocardial infarction or lower limb ischaemia. The mechanism of this effect is not well understood and is often interpreted as a rebound effect in patients with an increased risk of ischaemic events. The delay between these events and aspirin withdrawal was almost reproduced by us in the normal rat, with a single injection of aspirin at the dose of 100 mg/kg on days 8 and 10 after administration. It is not clear whether this effect is caused by aspirin itself or by a rebound effect. Nevertheless, these data emphasise the presence of the same effect in the normal rat and not in a subject clearly predisposed to an ischaemic event.

The effects observed after very low doses of aspirin administration rule out the speculation about a rebound effect. A minimal dose of injected aspirin produces a clear prothrombotic effect, increasing the number of emboli and duration of embolisation after a laser-induced endothelial lesion, an effect achieved only one hour after the administration. This effect seemed to be directly induced by the this injection and not by a rebound effect.

The inhibition of COX 2 mRNA and protein level by low concentrations of aspirin or salicylate in isolated endothelial cells has been described in previous publications and reviewed by Wu (31).

Higher concentrations of aspirin were here described as enhancers of COX 2 protein level. This observation led us to postulate that very low doses of aspirin could induce a prothrombotic action, besides its inhibiting activity on platelet COX 1. Selective inhibition of COX 1 induced an antithrombotic effect, decreasing the number of emboli and the duration of embolisation. The administration of very low doses of aspirin counterbalances these effects. The evaluation of COX 2 inhibition showed similar effects on thrombus production in microcirculation to that of very low doses of aspirin, as both increased the duration of embolisation. Moreover, the successive administration of COX 2 selective inhibitor and very low doses of aspirin did not induce a further effect on thrombus induction, thus suggesting a possible common pathway for the effect.

The highest dose of aspirin produced a decreased number of emboli in COX 1-deficient mice, indicating the presence of an antithrombotic mechanism different to COX 1 inhibition at this dose. This model of COX 1 -/- mouse is known to produce alterations in platelet aggregation (26). Explanations for this effect may include compensation in COX 1 and 2 activity or interaction between COX and NO synthase, as suggested by Skill (27), or an effect of aspirin outside the mechanism of COX inhibition like increased NO synthesis by the endothelial cell, as suggested by Taubert (28). Although intermediate doses did not show significant results, there was a clear antithrombotic effect of high dose aspirin in COX 2-deficient mice and a prothrombotic effect with the lowest dilution in COX 1-deficient mice. These results clearly confirm that although high dose aspirin exerts its main effect through COX 1 inhibition, dilution 15 of aspirin acts through COX 2 inhibition, thus inducing thrombosis.

In conclusion, high-dose aspirin in COX 1-deficient mice has a mild effect by decreasing thrombosis and a strong pro-thrombotic effect at dilution 15. Both effects are independent of COX 1 activity. Dilution 15 had no effect in COX 2-deficient mice. The effect observed with the lowest dose suggests that it is directly due to residual amounts of aspirin rather than a rebound effect, and that the complications observed after aspirin discontinuation may arise from this unrecognised effect.

These results and finding could have important implications for public health. And they highlight the importance of extended
References


