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Oral single high-dose aspirin results in a long-lived inhibition of anodal current-induced vasodilatation

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1 Acetyl salicyclic acid (aspirin) irreversibly blocks cyclo-oxygenase (COX). This effect is short-lived in endothelial or smooth muscle cells due to resynthesis but long-lived in platelets devoid of synthesis ability. Aspirin blocks the anodal current-induced vasodilatation, suggesting participation by prostaglandin (PG). We analysed the time course of the effect of aspirin as an indirect indicator of the origin of the PG possibly involved in anodal current-induced vasodilatation.

2 In healthy volunteers, vasodilatation, estimated from the peak cutaneous vascular conductance (CVC_{peak}), was recorded in the forearm during and in the 20 min following 5 min, 0.10 mA transcutaneous anodal current application, using deionized water as a vehicle. CVC_{peak} was normalized to 44°C heat-induced maximal vasodilatation and expressed in per cent values. Experiments were performed before and at 2 and 10 h, 3, 7, 10 and 14 days after blinded 1-g aspirin or placebo treatment.

3 CVC_{peak} (mean ± s.d.mean) after aspirin *vs* placebo was 13.6 ± 14.5 *vs* 65.0 ± 32.1 ($P<0.05$) 14.7 ± 4.2 *vs* 87.5 ± 31.9 ($P<0.05$), 18.1 ± 10.2 *vs* 71.6 ± 26.8 ($P<0.05$), 42.5 ± 23.4 *vs* 73.3 ± 26.8 (non significant, NS), 60.2 ± 24.3 *vs* 75.2 ± 26.9 (NS), 52.1 ± 18.5 *vs* 67.9 ± 32.1 (NS) at 2 and 10 h and at days 3, 7, 10 and 14 respectively.

4 Aspirin inhibition of anodal current-induced vasodilatation persists long after endothelial and smooth muscle cyclo-oxygenases are assumed to be restored. This suggests that the PG involved in this response are not endothelial- or smooth muscle-derived. The underlying mechanism of this unexpected long-lived inhibition of vasodilatation by single high dose aspirin remains to be studied. *British Journal of Pharmacology* (2002) **137,** 384–390. doi:10.1038/sj.bjp.0704868

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Abbreviations: AU, arbitrary units; COX, cyclo-oxygenase; CVC, cutaneous vascular conductance; LDF, laser doppler flow; NS, non-significant; PG, prostaglandin; s.d., standard deviation

Introduction

Cyclo-oxygenase (COX) derivatives from arachidonic acid play a key role in the vasomotor control of the microcirculation, either as vasodilator or vasoconstrictor mediators depending on the subtype of eicosanoid agent synthesized and released. Prostaglandin (PG) can be released by a large variety of cells in humans, among which are endothelial cells, neural afferents, smooth muscle cells and platelets. Aspirin irreversibly blocks COX. Then, the effect of aspirin is reversed either when COX is re-synthesized in nucleated cells (such as endothelial or smooth muscle cells) or when non-nucleated cells, which are unable to re-synthesize COX, are replaced (e.g., platelets). As a result, the duration of COX blockade depends on the cell studied, from a few hours in nucleated cells to up to 10 days in platelets. (Heavey *et al*., 1985; Hla & Bailey, 1989; Zucker *et al*., 1961). In cardiovascular disease, this difference in the duration of the effect of aspirin is the rationale for the use of 50–1500 mg day^{-1} of this drug with long interdose-intervals (usually once a day) in an attempt to inhibit thromboxane production in platelets while sparing the vasodilator prostaglandins of nucleated cells (see for review: Patrono *et al*., 1998). But, although multiple studies have been performed on the short-lived vascular effects of high dose aspirin, assumed to result from the blockade of vasodilator prostanoid synthesis, little is known on the longer-term effects of high dose aspirin on vasodilator mechanisms.

Iontophoresis is a fascinating tool for non-invasive pharmacological studies of the human microcirculation, but its use faces the problem of the 'non-specific' effect of the current used for drug diffusion (Grossmann *et al*., 1995). We previously reported that high dose aspirin can inhibit current-induced vasodilatation, suggesting a participation of PG in the vasodilator mechanism (Durand *et al*., 2002a, b), but other conclusions can be found from the literature (Morris & Shore, 1996; Berliner, 1997a; Abou-Elenin *et al*., 2002), possibly due to different experimental conditions. The origin of PG that participates in the current-induced vasodilatation in our experimental conditions remains unclear. We hypothesized that studying the time course of aspirin inhibition of current-induced vasodilatation could provide useful information on the possible origin of those PG that are involved in the current-induced vasodilatation. For this purpose, we analysed over 2 weeks the time course of the effect of a single 1-g oral dose of aspirin or placebo on the responses observed following a 5 min application of a 100 μA anodal current to forearm skin. An anodal current is a monopolar DC current delivered at the anode when the cathode is considered as the

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reference. The exact mechanism responsible for the anodal current-induced vasodilatation is still debated, but axon reflex due to nociceptor's excitation (Berliner, 1997a), local acidosis (Berliner, 1997a; Durand *et al*., 2002c), neurogenic inflammation (Durand *et al*., 2002b) or break excitation (Durand *et al*., 2002c), have been proposed. The present study provides evidence that aspirin inhibition of anodal current-induced vasodilatation is long-lived and persists far after endothelial and smooth muscle cyclo-oxygenases are assumed to be restored. This finding suggests that PG involved in anodal current-induced vasodilatation might not be of endothelial or smooth muscle cell origin.

Methods

Non-smoking healthy volunteers with no clinical signs of, or risk factors for vascular disease were involved in this study. They were 27.7 ± 6.2 (mean $\pm$ standard deviation (s.d.)) years old, two females, five males; height: 173.7 ± 9.9 cm, weight: 63.9 ± 14.2 kg. Volunteers had not taken any drug in the 3 weeks prior to the beginning of this study, and a minimum of 3 weeks elapsed between any two experiments on the same subject. After receiving information about the methods and procedures, all subjects gave their written consent to participate in this institutionally approved study. Experiments were carried out in accordance with the declaration of Helsinki. Subjects were placed supine in a quiet room with the ambient temperature set at $23 \pm 1^{\circ}$C. Each trial began after 15 min of rest for thermal and cardiovascular adaptation.

Forearm cutaneous blood flow was recorded using laser Doppler flowmetry (Periflux PF4001, Perimed, Sweden). Two laser Doppler probes were used. The first one was a multifibre laser Doppler probe, specially designed to allow for simultaneous cutaneous blood flow recording, current application and local heating (probe 481-1, Perimed, Sweden; a schematic representation of this probe is presented in Durand *et al*., 2002b). This 'active' probe is 22 mm in diameter and is constituted of a small optic fibre 90° to the skin surface, centred in a cylindrical thermostatic holder. The thermostatic holder has a circular chamber of ~ 1 cm^2 allowing for the positioning of a specially designed disposable sponge. A hole in the middle of the sponge allows for the recording of cutaneous blood flow through the optic fibre. The sponge was wet with 0.2 ml of deionized water before each experiment, and the probe was fixed to the skin with double-sided adhesive rings. The other probe (PF408, Perimed, Sweden) was used as a reference to confirm the absence of response to the current application at an unstimulated site 5 cm adjacent to the 'active' probe. A disposable Ag/AgCl adhesive electrode (Care 610, Kendall, Neustadt, Deutschland), was placed 5 cm from the laser probes to form an equilateral triangle. The 'active' probe was connected to a temperature-regulated heating system (Peritemp PF4005, Perimed, Sweden) and to the anode of an intensity-regulated current supplier (Periiont, Micropharmacology System, PF 382 Perimed, Sweden) allowing for the delivery of constant continuous currents for programmable durations. The disposable Ag/AgCl electrode served as the cathode. The current application consisted of the transcutaneous delivery of a 100 μA current for 5 min, which was never felt as painful by the subjects. Twenty minutes after the end of the current application, the site was locally warmed to 44°C to cause maximal cutaneous vasodilatation (Taylor *et al*., 1984; Johnson *et al*., 1986; Savage & Brengelmann, 1994; Saumet *et al*., 1998).

Local cutaneous temperature was measured using a surface thermocouple probe positioned 5 cm from the 'active' laser probe. The thermocouple was connected to an electronic thermometer (BAT-12, Physitemp Instr. Inc., Clifton, NJ, U.S.A.). Mean systemic blood pressure was monitored using a Finapres 2350 (Ohmeda, Englewood, CO, U.S.A.) positioned on the 2nd or 3rd finger of the hand contra-lateral to the sites of Laser Doppler Flow (LDF) measurements.

Each subject underwent a series of 13 measurements of current-induced vasodilatation. In order to avoid side effects or prolonged local effects of a previous experiment, position of the probes on the forearm and arm studied (right or left) were chosen randomly for each experiment. A reference experiment without treatment was performed and then the subjects participated in each of two protocols (aspirin or placebo treatment), in random order, separated by a minimum period of 3 weeks. For each protocol, experiments were repeated six times after aspirin or placebo treatment. Aspirin (Catalgine 1-g Lipha Santé, Lyon, France) was dissolved in 125 ml orange juice in order to disguise the taste and appearance of aspirin, whereas nothing was added to the orange juice in the placebo experiments. Before each series of experiments, subjects were given the 125 ml orange juice, blinded relative to the presence of aspirin. Measurements of current-induced vasodilatation were repeated at: 2 and 10 h and 3, 7, 10 and 14 days following treatment with aspirin or placebo.

Measurements

The data were expressed in arbitrary units (AU) and recorded on a computer *via* an analogue to digital converter (Biopac System, Inc., CA, U.S.A.) with a sample rate of 3 Hz, on 16 bits. Due to instantaneous variability of microcirculatory blood flow related to vasomotion, all results were averaged over 5-s periods (15 raw samples) for future analysis. To index active changes in the cutaneous vasculature at the active probe, vasodilatation was assessed as cutaneous vascular conductance (CVC) and calculated as the ratio of LDF to mean arterial pressure for each 5-s interval. Maximal vasodilatation to local heating (CVC_{max}) was calculated from each experiment from the average of CVC values observed over the last 2 min of the heating period. Last, CVC values were normalized for each subject to CVC_{max}, to better compare responses among sites and subjects (Kellogg *et al*., 1998; Peters *et al*., 2000). It appears to us more satisfactory to normalize blood flow to the maximum achievable at high temperature rather than baseline. Baseline blood flow is extremely variable, with minor differences in ambient temperature. Furthermore, normalization to maximal flow achieved through temperature or chemically-induced vasodilatation seems a largely validated technique and maximal flow is assumed to be comparable through thermal or chemical approach (Saumet *et al*., 1998). Results are expressed as percentages of CVC_{max}.

Analysis of results

We compared the last resting value of LDF on the control probe and local cutaneous temperature to the last values recorded during the experiment to confirm the absence of significant haemodynamic changes throughout the experiments.

Since previous reports suggest that galvanic current application to the skin could induce tissue damage (Burnette & Ongpipattanakul, 1988), we aimed to determine whether the vasodilatation observed during the experiments could be the result of a post-ischaemic response. We postulated that a reactive hyperaemia could only occur as a result of a prolonged ischaemia and arbitrarily defined the minimal duration of this ischaemia to 20 s. Therefore, we searched for an eventual decrease of CVC from resting values of at least 20 s, during and following current application. For this purpose, we calculated the mean and standard deviation of the 24 CVC values recorded in the resting period of each protocol, and search for the eventual occurrence of four (minimum) consecutive 5-s intervals resulting in CVC values below two standard deviations from the mean of the resting values. Finally, to synthesize the results, CVC_{rest} for each subject was the last value recorded during the 2-min resting period. CVC_{peak} represents the mean of individual values observed at the time where the peak value was observed on averaged data during, or in the recovery period following, current application. CVC_{20} represents the values observed at twenty min following current application.

All values are expressed as mean$\pm$s.d.mean in the text. Differences between experiments and between resting and other values within an experiment, as well as differences between aspirin and placebo experiment for a defined delay from treatment, were studied with two-tailed paired *t*-tests. For all statistical analyses, a P value $\leqslant 0.05$ was considered significant. Non-significant results are reported NS.

Results

Figure 1 shows the responses to anodal current and local heating recorded in the same patient at 2 h and 3, 7 and 14 days following placebo or aspirin treatment. Note that at day 3 after aspirin intake, the response to current application is almost abolished in this patient.

Figure 2 shows the mean response to anodal current observed at 2 and 10 h, and 3, 7, 10 and 14 days following placebo or aspirin treatment.

No significant difference was noted within the experiments on cutaneous temperatures and on the control LDF probe, as reported in Table 1. No significant decrease was found in the averaged values from the resting periods of the 13 protocols performed, as would be observed during transient ischaemia.

In the pretreatment experiment, CVC_{rest} in our subjects was $7.4\pm3.1\%$. A slow vasodilatation occurred during current application and CVC was $19.7\pm7.5\%$ at the end of current application. CVC_{peak} after current application was $69.1\pm24.8\%$ and CVC_{20} was still 68.0 ± 21.4. Table 2 shows the results of CVC_{rest}, CVC_{peak} and CVC_{20} observed in the days following aspirin or placebo treatment. No difference was noted in CVC_{rest} among the different experiments or in CVC_{rest} between placebo and aspirin at the same period after treatment. Consistently, CVC_{peak} and CVC_{20} were not significantly different from one experiment to another after placebo treatment and were in the same range as that

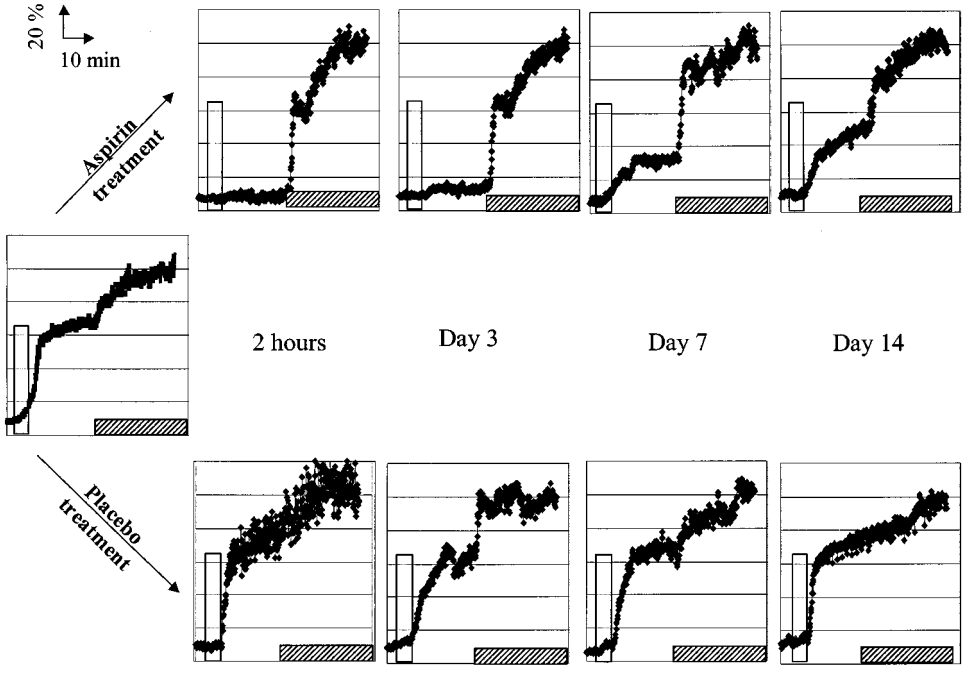

Figure 1 Example of the time course of the cutaneous vascular conductance, expressed as per cent of maximal heat-induced vasodilatation in one typical subject in the pretreatment experiment, at 2 h and at days 3, 7 and 14 following aspirin or placebo treatment. Grey bar schematizes the 5 min application of anodal 100 μA continuous application. Hashed bar is the period of local 44°C heating.

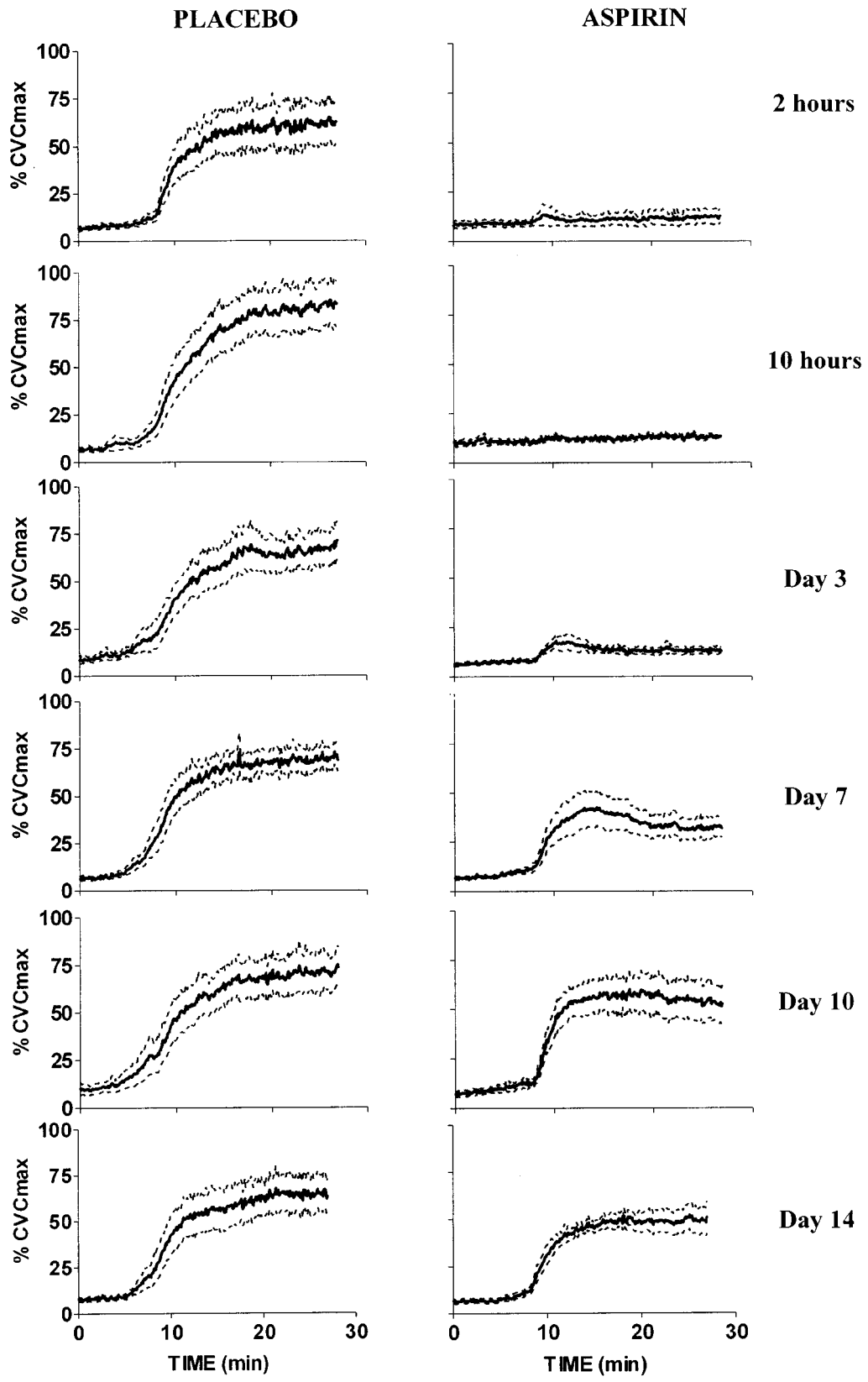

Figure 2 Mean cutaneous blood flow response ± s.e.mean (estimated from cutaneous vascular conductance (CVC)) observed in the 2 min preceding, during and in the 20 min following a 5-min anodal current application, at different intervals from aspirin (right graphs) or placebo (left graphs) treatment: 2 and 10 h and at day 3, 7, 10 and 14. Results are expressed as per cent of maximal heat-induced CVC (% CVC_{max}). The heating period is not presented to simplify the graph.

Table 1 Laser Doppler Flow (LDF) in arbitrary units (AU) at the control probe and local cutaneous temperature (temp in degree celsius (°C)) in the different protocols both before and after and at various intervals following, either aspirin or placebo treatment

	LDF Rest (AU)	*LDF End* (AU)	*Temp Rest* (°C)	*Temp End* (°C)	*LDF Rest* (AU)	*LDF End* (AU)	*Temp Rest* (°C)	*Temp End* (°C)
Before	5.5±1.6	7.0±3.5	33.3±0.7	34.0±1.2	x	x	x	x
After			Aspirin				Placebo	
2 h	10.3±5.7	11.7±6.3	31.9±1.1	32.8±1.5	7.7±3.1	9.2±4.7	32.8±0.9	32.7±1.9
10 h	5.0±2.5	6.1±4.0	33.7±1.0	34.6±0.7	6.2±1.4	7.5±4.3	33.5±0.8	34.5±1.2
3 days	9.4±2.4	8.5±3.6	32.5±0.7	32.8±0.7	8.1±4.8	8.5±3.0	31.8±3.6	33.5±0.8
7 days	8.6±3.0	8.5±6.3	32.4±1.2	32.9±1.3	9.3±8.7	7.9±6.1	32.9±0.7	34.0±2.2
10 days	8.5±3.9	8.7±3.4	32.4±1.5	32.1±1.4	8.2±4.3	8.4±2.3	32.7±0.6	33.6±1.2
14 days	8.9±3.9	10.1±4.5	32.7±0.9	33.2±1.2	5.3±1.8	5.8±2.0	33.0±0.8	33.5±0.7

Values reported are: the value at the end of the initial resting period of 2 min, just before the beginning of the electrical stimulation (Rest); the last recorded value at the end of the experiment (End).

Table 2 Summary of cutaneous vascular conductance (CVC) in percentage of maximal CVC: at rest (CVC_{rest}), at peak (CVC_{peak}) and at 20 min following current application (CVC_{20}), under aspirin or placebo treatment at 2 h (H2) and 10 h (H10) and at days 3, 7, 10 and 14 (D3, D7, D10 and D14)

	Aspirin treatment			*Placebo treatment*		
	CVC_{rest} (%)	CVC_{peak} (%)	CVC_{20} (%)	CVC_{rest} (%)	CVC_{peak} (%)	CVC_{20} (%)
H2	8.6±4.8	13.6±14.5**	12.8±8.8**	7.4±3.7	65.0±32.1	62.7±30.9
H10	10.0±3.3	14.7±4.2**	13.5±2.2**	6.7±3.7	87.5±31.9	81.5±31.4
D3	6.5±2.1	18.1±10.2*	12.9±4.7**	9.4±4.3	71.6±26.8	71.6±26.8
D7	6.8±2.6	42.5±23.4	32.1±12.9*	6.7±3.2	73.3±26.8	68.9±16.3
D10	8.4±3.9	60.2±24.3	52.9±23.4	10.5±7.3	75.2±26.9	73.7±27.7
D14	6.4±3.3	52.1±18.5	50.9±22.5	8.1±3.4	67.9±32.1	63.4±25.4

**$P<0.01$; *$P<0.05$ aspirin vs placebo.

observed in the initial pretreatment experiment. Aspirin treatment almost abolished current-induced vasodilatation at 2 and 10 h, and markedly inhibited vasodilatation for both CVC_{peak} and CVC_{20} on day 3 as compared to placebo experiments. No difference in CVC_{peak} was found at day 7, 10 and 14 after aspirin treatment, but instead of the plateau observed in placebo experiments, CVC decreased slowly during the recovery period at day 7 (as can be noted from Table 2). A significant difference from placebo experiment in CVC_{20} was noted at day 7 under aspirin treatment. Figure 3 shows the ratio of CVC_{peak} and CVC_{20} to CVC_{rest} illustrating the progressive restoration of the vascular response to current application in the days following a single oral dose aspirin.

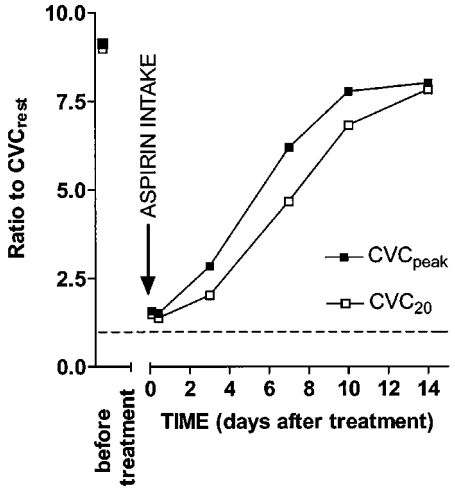

Figure 3 Time course evolution of the ratios of mean CVC_{peak} and mean CVC_{20} to mean CVC_{rest} in response to 5 min monopolar anodal current application before, at 2 and 10 h and at days 3, 7, 10 and 14 following 1-g single oral dose aspirin treatment. The dashed line represents the ratio of 1 for which no change is found from CVC_{rest}.

Discussion

The major result of the present study is that a single 1-g oral dose of aspirin nearly abolishes anodal current-induced vasodilatation for up to 3 days and still impairs the responses at day 7. The fact that aspirin may exert prolonged inhibition of a vasodilator mechanism has been previously observed in humans during intra-venous injection of arachidonic acid (Bhagat *et al*., 1995), but the underlying mechanisms for this response, and the exact duration of the inhibition past day 5, were not studied. Earlier results on the sensitivity of anodal current-induced vasodilatation to aspirin are controversial (Morris & Shore, 1996; Berliner, 1997a; Abou-Elenin *et al*., 2002; Durand *et al*., 2002a, b). Specifically, the prolonged effect of aspirin inhibition is a possible explanation of the absence of significant vasodilatation to anodal current application observed in some of these previous experiments (Abou-Elenin *et al*., 2002). Indeed, in that experiment the evaluation of current application through deionized water

was studied following an initial procedure using aspirin treatment in the same subjects. The absence of response may have resulted from the prolonged effect of aspirin treatment on the second experiments.

PG are synthesized through a well-known biochemical pathway in which COX play a key role (Vane, 1971). Acetylsalicylic acid is a powerful and irreversible inhibitor of COX. In nucleated cells (endothelium or smooth muscle) the blockade of COX by high dose aspirin is assumed to be restored within a few hours due to re-synthesis of COX (Heavey *et al*., 1985), whereas in platelets the blockade is irreversible through the remaining life of the platelet. *In vitro*, COX in smooth muscle cells is restored in 3 h whereas, in endothelial cells, synthesis is altered for up to 24 h (Hla & Bailey, 1989). It could be possible for aspirin to have longer effects *in vivo* than *in vitro* on endothelial or smooth muscle COX, as the study of Baghat *et al*. (1995) suggests, but we assumed from the *in vitro* results that, if experiments are performed 10–12 h following treatment as in the present study, a differentiation should be possible between endothelial and smooth muscle COX. The commonly accepted rate of peripheral platelet utilization and destruction is approximately 10% per day (Zucker *et al*., 1961). At that rate, half of the platelets are replaced within 5 days, and the effect of a single dose of aspirin on platelet function should still be noted beyond 24–36 h, when both endothelial and smooth muscle cell COX are assumed to be completely restored.

The fact that vasodilatation is largely impaired at day 3 in our experiments argues against the sole involvement of endothelial or smooth muscle COX in the underlying mechanisms of current-induced vasodilatation. Could PG of platelet origin contribute to vasodilatation? If not, what could explain the long lasting effect noted?

Human platelets contain thromboxane A2, a powerful vasoconstrictor, as well as other PG that are known as vasodilator agents, such as PGE2. A previous study using current of 0.16 mA/cm^2 lasting 1 h showed that tissue alterations could occur as a result of current application to the skin (Burnette & Ongpipattanakul, 1988). It might be suggested that the current-induced vasodilatation could result from a post ischaemic reactive hyperaemia due to a local thrombotic event following platelet activation and thromboxane release. However (a) the current used by Burnette & Ongpipattanakul (1988) is of higher intensity and longer duration than that used in this experiment. (b) No significant decrease of CVC was noted following the initiation of current application that might reflect a transient thrombotic event. (c) The duration of the current-induced vasodilatation far exceeds the usual duration of post-ischaemic hyperaemia. Thus, we assume that a post-ischaemic reaction is not likely to be the primary cause of the vasodilatation that might be expected from a platelet-mediated thrombosis or transient thromboxane release. On the contrary, could our results be due to a platelet-mediated vasodilatation? *In vitro*, evidence exists that normal platelets are able to produce relaxation in rat or rabbit pre-constricted arteries (Yang *et al*., 1994; Oskarsson *et al*., 1997), but to the best of our knowledge, there is no *in vivo* proof of a direct platelet-mediated vasodilatation, specifically in humans. Reviews (Holzer, 1997) and recent experiments (Gecse *et al*., 1999) advocate for a participation of platelets in neurogenic inflammation. Further, experiments with local anaesthezia (Morris & Shore, 1996) or capsaicin desensitization of primary afferents (Durand *et al*., 2002b) indicate a participation of afferent fibres in anodal current-induced vasodilatation. Whether a neurogenic inflammation involving platelets could explain the long-lasting effect of aspirin observed in the present experiments is an interesting but unproven possibility.

Mechanisms other than platelet participation in vasodilatation are possible. First, non-steroidal anti-inflammatory drugs may selectively inhibit gene expression at various levels (Yuan *et al*., 2000; Paccani *et al*., 2002). Specifically, salicylates may interfere with the expression of nuclear factor-kappa B (Kopp & Ghosh, 1994) or interleukin-4 (Cianferoni *et al*., 2001). The duration of these interactions and the potential involvement of these inflammatory molecules in our experiments are unknown. Second, we recently demonstrated the involvement of primary afferents in current-induced cutaneous vasodilatation (Durand *et al*., 2002a). In primary afferent nerves, the nucleus is far away from nerve ending and axonal trafficking is needed to supply nerve endings with products synthesized close to the nucleus, such as PG. Axonal trafficking velocity is estimated to be a few centimetres a day (Ochs, 1972). In our studies, recordings were performed at the forearm level, $\sim$40–50 cm away from the dorsal root ganglion, where neural nucleus is located. Thus, duration of axonal trafficking may be an explanation for the duration of the aspirin effects we observed.

From a physiological point of view, further studies are needed using anti-aggregant agents and/or low dose aspirin to test the eventual involvement of platelet PG in the anodal current-induced vasodilatation found in our experiments and to search for an *in vivo* proof of a platelet-mediated vasodilatation in humans. Our results are also of practical interest, in clinical experiments studying vasodilator mechanisms. Consistent with previous reports (Bhagat *et al*., 1995), our results emphasize that, following high dose aspirin treatment, a period of at least 2 weeks should be allowed from the last aspirin intake. Lastly, anodal galvanic stimulation is widely used in iontophoretic experiments despite the well-known 'non-specific' effects of the current (Grossmann *et al*., 1995; Berliner, 1997b; Hamdy *et al*., 2001; Durand *et al*., 2002b). The fact that aspirin can essentially abolish the vasodilator effect of anodal current application for 3 days might be of advantage through pharmacological inhibition of this undesirable interfering effect in iontophoresis experiments, without significant impairment of endothelial or smooth muscle cell PG pathways.

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