Prolonged aspirin inhibition of anodal vasodilation is not due to the trafficking delay of neural mediators

Laboratoire de Physiologie et Explorations Vasculaires, Centre Hospitalier Universitaire, 49033 Angers cedex, France

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Among our previous results, we showed that aspirin inhibited vasodilation induced by prolonged current application, suggesting the participation of PG (10). We also demonstrated that, when a 1-min current application was repeated, an amplified vascular response occurred compared with 2 min of all-at-once delivered currents of the same intensity. This amplification was assumed to rely on sensitization of primary afferent after the first current delivery. The amplification mechanism was also aspirin sensitive (11). Aspirin leads to a direct and irreversible inhibition of PG synthesis through the blockade of cyclooxygenase (COX) (40). PG are synthesized in a large variety of cells including nerves (16), and most PG exhibit vasodilator effects. In the vasodilation resulting from primary afferent excitation, PG involved could act through their direct vasomotor properties but could also act indirectly via sensitization of nociceptive C fibers to forthcoming stimulation (27, 28). We recently reported a long-lasting (up to 7 days) inhibitory effect of aspirin on (all-at-once delivered) 5-min, 0.1-mA current-induced vasodilation (13). It is not known whether vasodilation resulting from repeated 1-min anodal application would show the same long-lasting effects. If a prolonged effect of aspirin on the sensitizing mechanisms is observed (>3 days), it is unlikely that this could rely on the effect of aspirin on PG of endothelial or smooth muscle cell origin (21, 30). Indeed, in vitro COX is resynthesized in 24 to 36 h in endothelium and in 3 h in smooth muscle (21, 22). Then, past 24–36 h after treatment, aspirin should not affect PG synthesis in these nucleated cells. In nerves, COX, as other proteins, is resynthesized in the cytoplasm close to the nucleus and conducted to the nerve endings through active transport mechanisms. Transport of proteins along the axon (i.e., nerve trafficking) occurs at a maximal rate of 40 cm/day (17). It is possible that the time required to supply nerve endings with unblocked resynthesized COX would result in prolonged inhibition of current-induced cutaneous vasodilation. Then, once we confirmed the long-lasting effect of aspirin on this model, we compared the recovery of the response to repeated current application at proximal and distal sites on the arm (minimal distance between the 2 sites:...
50 cm) after a single high-dose aspirin intake. We hypothesized that, if trafficking of neural mediators was the cause of the long-lasting effect of aspirin due to the longer distance between nucleus and nerve ending at the distal location, an earlier recovery of the response after repeated current application should be observed at the proximal location.

**METHODS**

We performed two different experimental protocols on two groups of nonsmoking healthy volunteers with no clinical signs of, or risk factors for, vascular disease. In *protocol 1*, volunteers were 27.3 ± 6.5 (mean ± SD) yr; 2 females, 5 males; height: 173.7 ± 9.9 cm; weight: 63.9 ± 14.2 kg (n = 7). In *protocol 2*, they were 28.6 ± 5.9 yr; 2 females, 6 males; height: 172.6 ± 8.7 cm; weight: 63.4 ± 13.2 kg (n = 8). Volunteers had not taken any drugs in the 3 wk before the beginning of this study. Moreover, a minimal period of 3 wk passed between any two experiments on the same subject. Each subject gave written consent to participate in this institutionally approved study after being informed of the methods and procedures. 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 24 ± 1°C. Each trial began after 15 min of rest in this environmental condition for thermal and cardiovascular adaptation.

Cutaneous blood flow was recorded using laser-Doppler flowmetry (LDF) with multifiber laser probes. On the area of current application, probes used were specially designed to fit on iontophoretic electrodes. The whole system allows for simultaneous concentric cutaneous blood flow recording, current application on a 0.8-cm² area, and local heating at the very site where current was applied on ~2 cm² (probe 481–1, Perimed, Sweden). Specific description of this probe, hereafter referred to as the “active probe,” can be found in previous reports (10). We also used a standard probe (PF408, Perimed, Sweden) as a reference to confirm the absence of response to the current application at an unstimulated site on the forearm. Probes were connected to laser-Doppler flowmeters (Periflux PF4001, Perimed, Sweden). The active probes were also connected to a temperature-regulated heating system (Peritemp PF4005, Perimed) and to the anode of regulated 9-V current suppliers (Perioint, Micropharmacology System, PF 382, Perimed). This current device allows for the delivery of constant continuous current for programmable durations. The cathodes were positioned on disposable Ag/AgCl adhesive electrodes (Care 610, Kendall, Neustadt, Deutschland), 5 cm away from the laser probes. Current application consisted of transcutaneous delivery of 100-μA current and was never felt as painful by the subjects. Temperature for local heating was set to 44°C for 24 min to cause maximal vasodilation, because multiple studies support the conclusion that cutaneous vasodilation is at maximal levels during prolonged local warming between 42 and 44°C (24, 32, 33, 37). Local cutaneous temperature was measured using a surface thermocouple probe at the forearm level. The thermocouple was connected to an electronic thermometer (BAT-12, Physitemp Instruments).

Systemic blood pressure was monitored using a Finapres 2350 (Ohmeda) positioned on the second or third finger of the hand contralateral to the sites of LDF measurement.

**Procedures**

*Protocol 1: evidence for a long-lasting effect of 1 g aspirin on vasodilation induced by repeated 1-min anodal current application.* Current applications were performed at the forearm level. The two LDF probes were positioned at a distance of 5 cm apart. The experiment consisted of a 2-min resting period followed by the first min 0.10-mA anodal current application (corresponding to a charge of 6 mC) by the active probe. A second 1-min, 0.1-mA current delivery was repeated after a 10-min interval, followed by a 20-min recovery period. The last step of the experiment was the 24-min local heating period at the site where the current was applied.

An experiment without treatment (reference experiment) was first performed, and then the subjects were assigned randomly to two protocols (aspirin or placebo treatment) separated by a minimum period of 3 wk. For each protocol, the experiment was repeated six times after 1 g aspirin or placebo treatment (hours 2 and 10, and at days 3, 7, 10, and 14 after the treatment). Position of the probes from one experiment to another was chosen randomly on the forearm and between both sides, but previous sites of current application were avoided to prevent any possible influence of previous current application.

Aspirin (Catalgine 1 g, Lipha Santé, Lyon, France) was dissolved in a 125-ml glass of orange juice to disguise the taste and appearance, whereas nothing was added to the orange juice in the placebo experiments. Two hours before the first experiment of each protocol, subjects drank the 125 ml orange juice, blinded from the presence or absence of aspirin in the glass.

*Protocol 2: response to repeated 1-min anodal current application performed simultaneously at a proximal and a distal site and recovery after a 1-g aspirin treatment.* To show a possible difference in the recovery of the response between a proximal and a distal site, after aspirin treatment, repeated current applications were performed as in *protocol 1* but with two active probes. One probe was attached to the shoulder (proximal site) and the other one to the ipsilateral forearm (distal site). A control laser-Doppler probe was positioned 5 cm from the active probe at the forearm level. Each subject underwent a series of five experiments performed before and at 2 h and 3, 5, and 10 days after aspirin treatment. Positions of the probes were randomized between the right and left side from one experiment to another. No current application was performed on a site previously stimulated.

**Measurements**

Skin blood flow responses were expressed in arbitrary units (AU) on a computer via an analog-to-digital converter (Biopac System) with a sample rate of 3 Hz on 16 bits. For data analysis, cutaneous blood flow was indexed as cutaneous vascular conductance (CVC) calculated as the ratio of LDF and mean arterial pressure and normalized to maximal levels as achieved with local heating to 44°C. Normalization of blood flow to maximum achievable at high temperature was used as in previous experiments to decrease the variability of single-point laser-Doppler measurements (12, 25, 31).

**Analysis of Results**

For data analysis, resting values for each subject (Rest) were defined as the average of the resting values recorded during the 2-min resting period. Characteristic points during and after current application are presented in Fig. 1 and
RESULTS

In all experiments, compared with starting values, no significant changes were observed in mean arterial pressure, local skin temperature, or skin blood flow (LDF) at the control probe.

Protocol 1: Long-Lasting Effect of 1 g Aspirin and Placebo on Vasodilation Induced by Repeated 1-min Anodal Current Application

Results for this protocol are presented in Table 1. Figure 2 presents the variation from reference experiment of Bpeak and B20 both for aspirin and placebo treatment. In brief, no difference was found between the placebo and aspirin treatment on rest values and Apeak. Compared with the reference experiment, aspirin treatment resulted in a complete abolition of current-induced vasodilation (Bpeak) at 2 h, 10 h, and 3 days, with a progressive normalization of the response over the following days, whereas placebo treatment did not affect the response. Therefore, a significant difference was found between placebo and aspirin treatment on both Bpeak and B20 until day 3, and a significant difference still existed on Bpeak but not on B20 at day 7.

Protocol 2: Response to Twice Repeated 1-min Anodal Current Application Performed Simultaneously at a Proximal and a Distal Site and Recovery After a 1-g Aspirin Treatment

A typical recording of the reference experiment of this protocol is presented in Fig. 3.

Whatever the localization of current application (forearm or shoulder), the effects of aspirin treatment were similar to those observed in the protocol 1. Compared with the reference experiment, a significant inhibition of current-induced vasodilation was observed 2 h after aspirin treatment and this inhibition was still significant on days 3 and 5. As can be noted from Fig. 4, no statistical difference between the distal site and the proximal site was observed in the recovery of the response to current application at each moment chosen after aspirin treatment.

Table 1. Values at Rest, Apeak, Bpeak, and B20, in percentage of the cutaneous vascular conductance maximal response to heating, and time for the occurrence of Bpeak from the end of the second period of current application observed for protocol 1

<table>
<thead>
<tr>
<th>Delay (from Aspirin Intake)</th>
<th>Time for Bpeak (min)</th>
<th>B20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.3 ± 0.2</td>
<td>17.4 ± 15.0</td>
</tr>
<tr>
<td>Aspirin 2 h</td>
<td>9.0 ± 0.2</td>
<td>10.7 ± 6.9</td>
</tr>
<tr>
<td>10 h</td>
<td>6.7 ± 0.4</td>
<td>10.8 ± 7.4</td>
</tr>
<tr>
<td>3 days</td>
<td>7.1 ± 0.4</td>
<td>10.1 ± 6.3</td>
</tr>
<tr>
<td>7 days</td>
<td>8.2 ± 0.4</td>
<td>12.4 ± 7.7</td>
</tr>
<tr>
<td>10 days</td>
<td>9.8 ± 0.3</td>
<td>14.2 ± 6.3</td>
</tr>
<tr>
<td>14 days</td>
<td>6.0 ± 0.3</td>
<td>16.0 ± 11.7</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>5.6 ± 0.2</td>
<td>9.7 ± 5.4</td>
</tr>
<tr>
<td>10 h</td>
<td>9.1 ± 0.7</td>
<td>18.9 ± 10.7</td>
</tr>
<tr>
<td>3 days</td>
<td>8.7 ± 0.3</td>
<td>13.3 ± 9.8</td>
</tr>
<tr>
<td>7 days</td>
<td>5.6 ± 0.2</td>
<td>8.8 ± 3.6</td>
</tr>
<tr>
<td>10 days</td>
<td>6.4 ± 0.2</td>
<td>14.1 ± 13.2</td>
</tr>
<tr>
<td>14 days</td>
<td>7.6 ± 0.3</td>
<td>17.9 ± 18.8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Anodal current was delivered in two 1-min periods with an interstimulation interval of 10 min. Placebo treatment does not affect the response as compared with reference experiment. Aspirin (1 g) inhibits this response shortly after intake but a progressive recovery occurs in the following days. *P < 0.05, aspirin vs. placebo, Apeak, maximal cutaneous vascular conductance after 1st period of current application; Bpeak and B20, peak value during and value 20 min after, respectively, the 2nd current application.
DISCUSSION

Experiments performed in protocol 1 of this study confirm a prolonged effect of aspirin (acetyl salicylic acid) on current-induced vasodilator mechanisms when repeated short-term current applications (1 min, 0.1 mA) are performed. We observed that a single high-dose aspirin treatment (1 g) can significantly inhibit the sensitization mechanism and microcirculatory vasodilation induced by repeated current applications, with a progressive recovery over 7 days. This delay is quite similar to the one we previously found with single 5-min current delivery (13) or with the duration of the impaired venodilation to arachidonic acid after aspirin treatment previously reported in human (5). This findings suggests that, as far as PG are involved in the current-induced vasodilation in our two experimental models, those of endothelial or smooth muscle cell’s origin are unlikely responsible for the long-lasting inhibition observed after aspirin. Furthermore, the comparable recovery of the microcirculatory response to the current application at a distal and a proximal site lead us to exclude that axonal transport of neural mediators is an explanation for this long-lasting effect of aspirin.

The fact that, in protocol 1, as previously observed (11), Apeak is not influenced by aspirin treatment suggests that the main effect of aspirin in this study is an inhibition of the sensitization mechanism to the renewed current application rather than an inhibition of the possible vasodilation that would result from a direct effect of released PGs. Indeed, PGs can act through direct vasomotor effect but can also act indirectly via sensitization of nociceptive C fibers (27, 28). Sensitization of primary afferents can also result from the effect of heat or protons on vanilloid receptors (VR1) or acid-sensing ion channels (ASIC). Sensitization by PGE2 is devoted to specific EP3C, EP4 receptors (35), which are not known to be directly affected by aspirin. Then, if sensitization is devoted to PGs, the effect of aspirin would be due to the impairment of PG synthesis through COX blockade rather than to the blockade of the effect of “normally” synthesized PGs on receptors. Recent reports suggest that aspirin may exert an inhibition of VR1 or ASIC (36, 41). We recently reported that protons could participate in the anodal current-induced vasodilation (12). Thus, assuming that the blockade of ASIC or VR1 would be irreversible, as is the blockade of COX, it could be suggested that the effect of aspirin could result from the time required to resynthesize receptors for proton sensitization.

Fig. 2. Variation from reference experiment of mean Bpeak (top) and B20 value (bottom) at 2 h (H2) and 10 h (H10) and 3 (D3), 7 (D7), 10 (D10), and 14 days (D14) after 1-g aspirin or placebo treatment. Bpeak and B20 are expressed as %maximal vasodilation obtained at the end of the heating period. Note that the placebo treatment does not affect Bpeak and B20 compared with the reference experiment, whereas the aspirin treatment leads to reversible variation corresponding to the inhibition of the current-induced vasodilation in the first days after 1-g aspirin intake. For Bpeak, significant variations from reference experiment can still be noticed 7 days after aspirin treatment. *P < 0.05 and **P < 0.01 aspirin vs. placebo.

Fig. 3. Typical recording of the reference experiment of protocol 2. After 2 min rest, current application consisted of a twice repeated 1-min anodal current application with a 10-min interstimulation interval at a distal or a proximal site. Twenty minutes after the end of the current application, the heating period was started for 24 min. Top to bottom, recordings are laser-Doppler flow (LDF) at the proximal site, LDF at the distal site, systemic arterial pressure, local skin temperature at a nonheated area 5-cm from heated probe, and reference LDF recorded at the distal level.
the authors' knowledge, nothing is known on the reversibility of this effect of aspirin on VR1 or ASIC. We estimate that the usual time necessary for the sole induction and expression of most of the proteins is too short to account for the long-lasting effect of aspirin. We assume that the long-lasting duration of the effect of aspirin does not rely on the delay for the sole resynthesis of unblocked COX enzymes or of unblocked receptors involved in sensitization.

In peripheral nerves, the synthesis of most molecules is located close to the cell nucleus. Thereafter molecules are transported to the periphery through active nerve trafficking. Both a slow and fast transport are described. Axonal slow- and fast-transport velocities are reported in the range 10 and 40 cm per day, respectively, in peripheral nerves (17). Most proteins are transported through fast axonal transport, but some (mainly those from the intermediate metabolism, surface associated, or cytoskeletal proteins) travel by the slower route (7, 9). Various methods have been developed to study the axonal transport. Mechanical stop-flow/crush of the axon (9) or “labeled” molecules in the intact axon (38) have largely been used. In the crush model, detection of the progressive concentration of the molecules on both sides of the crush allows for the study of anterograde and retrograde trafficking. By analogy with this technique, we assumed that the all-at-once irreversible blockade of COX (or sensitization receptors) in the whole nerve by a single dose of aspirin would be progressively compensated by unblocked proteins resynthesized and resupplied through nerve transport to the local skin nerve ending. The second assumption we made was that the progressive time course of the recovery of the vascular response from aspirin inhibition would reflect the progressive accumulation of resynthesized proteins involved in neural sensitization. Average distance of the distal probe to the ganglion was estimated as 60–70 cm and 15–20 cm at the proximal site. The distance between the two probes was ~50 cm in protocol 2. Considering the progressive recovery of the response to repeated current application after aspirin treatment, we hypothesized that time for the transport of proteins from the nucleus to nerve ending could be the cause for the long-lasting effects of aspirin. As a result of this hypothesis, a difference of amplitude should have been observed in the kinetics of the recovery of vascular responses between distal and proximal localization after aspirin treatment. Furthermore, the slower the true axonal transport might be, the larger the difference between proximal and distal recovery would be.

We found no difference. In addition, the time required to restore the vascular response at the proximal site was at least twice as high as the one resulting from our hypothesis. It could be suggested that insufficient local concentration of proteins at the proximal site could also explain the proximal delay (longer than expected from the sole time required for nerve trafficking), but it cannot account for the absence of difference between the proximal and distal restoration of the response. As a conclusion, assuming that the technical approach we used is valid for the study of axonal transport velocity, our findings are not consistent with nerve trafficking as the cause of the long-lasting effect of aspirin in our experiments.

If the long-lasting effect of aspirin on current-induced vasodilation cannot be explained by nerve trafficking of blocked VR1, ASIC, or COX in nerves or other nucleated cells, what other mechanisms could be proposed? Platelets are unable to synthesize proteins. As a result, platelet COX blockade by aspirin persists during the whole platelet life. Then the effect of a single dose of aspirin on platelet function is expected to decrease progressively over 10 days (45). This delay is very similar to the one reported in this study and consistent with the presence of vasodilator PGs in platelets. In vitro evidence exists of a direct platelet-mediated vasorelaxation in rat preconstricted arteries (44) or of the participation of platelet PG in neurogenic inflammation (15). However, to the best of our knowledge, there is no in vivo proof of a direct platelet-mediated vasodilation specific to humans. Salicylates may also interfere with gene expression of various molecules involved in inflammatory processes, such as nuclear factor-κB, cytokines, or COX-2 (8, 26, 43). Recent evidence has been provided that cytokines from glial cells participate in hyperalgesia observed in peripheral inflammation (42). Nevertheless, nothing is known about 1) the duration of actions of aspirin on gene expression or 2) the potential involvement of these inflammatory molecules or of glial cells in our experimental conditions.

Fig. 4. Recovery at 2 hours and 3, 5, and 10 days after 1-g aspirin treatment of the mean Bpeak and B20 values recorded on a distal (open column) or a proximal (solid column) site compared with the reference experiment of protocol 2. Bpeak and B20 are expressed as % maximal vasodilation obtained at the end of the heating period. No significant difference was noted in the recovery of the response between the distal and the proximal site.
In summary, the present study confirms that aspirin has long-lasting effects on vasodilation resulting from repeated anodal current applications, but failed to show a difference between simultaneous proximal and distal recordings in the recovery after aspirin intake. Although this does not exclude the involvement of neural COX in the response to anodal current applications, it suggests that the long-lived aspirin inhibition of anodal vasodilation is not due to the trafficking delay of neural mediators. Whether this long-lasting effect of aspirin could account for the increased risk of cardiovascular failure observed in the week after non-steroidal inflammatory drug absorption (19, 23, 29) is pure speculation, but is an interesting hypothesis for future experiments. Repeated current applications are frequently used in iontophoresis experiments to obtain dose-response curves (14). In these experimental designs, the fact that sensitization of primary afferents by the current is still almost abolished at 3 days of aspirin treatment (whereas smooth muscle and endothelial COX are assumed to be restored) could be useful as an indication that the long-lived aspirin inhibition of platelet COX in the response to anodal current application in the skin relies on aspirin-sensitive mechanisms. J Physiol 540: 261–269, 2002.

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REFERENCES

10. Durand S, Fromy B, Bouyé P, Saumet JL, and Abraham P. Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin-sensitive mechanisms. J Physiol 540: 261–269, 2002.