Prevention of Inflammation-Induced Endothelial Dysfunction
A Novel Vasculo-Protective Action of Aspirin

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Background—Inflammation and infection may initiate and promote atherosclerosis or its complications by adverse effects on the vascular endothelium. The mechanisms by which aspirin reduces cardiovascular risk might involve anti-inflammatory actions or direct effects on the endothelium in addition to its antiplatelet action. We investigated the role of aspirin in modulating endothelial dysfunction induced by an experimental inflammatory stimulus.

Methods and Results—An inflammatory response was generated in healthy volunteers by Salmonella typhi vaccination. Venous occlusion plethysmography was used to assess resistance vessel responses (16 hours before and 8 hours after vaccination) to the endothelium-dependent dilator bradykinin (BK) and the endothelium-independent dilator glyceryltrinitrate (GTN). Twelve subjects were randomized to receive either aspirin 1.2 g orally or placebo 2 hours before vaccination. After vaccination alone there was suppression of the response to BK in the placebo group ($P<0.01$), with no change in response to GTN. In the aspirin group there was no change in the response to either BK or GTN after vaccination. Aspirin treatment prevented vaccine-induced elevation of interleukin-1 receptor antagonist but enhanced the generation of tumor necrosis factor-$\alpha$ compared with placebo. In an additional 5 individuals, local intrabrachial aspirin (10 mg/min for 15 minutes) failed to restore responses to BK after vaccination.

Conclusions—Experimental inflammation produces endothelial dysfunction, which can be prevented by pretreatment with aspirin. Locally administered aspirin does not reverse vaccine-induced endothelial dysfunction once established. The protective effects of aspirin on inflammation-induced endothelial dysfunction may be through modulation of the cytokine cascade. (Circulation. 2002;105:2600-2604.)

Key Words: arteries ■ aspirin ■ bradykinin ■ endothelium ■ inflammation

Aspirin is widely used in the secondary prevention of coronary heart disease and stroke. It is assumed that its beneficial effects are solely attributable to its antiplatelet actions. However, aspirin has potent anti-inflammatory properties and may directly affect prostanoid production in other circulating cells or vascular cells as well as having actions on platelets, and it is possible that these mechanisms are also important. Indeed, in a recent study, aspirin seemed to be most effective at reducing cardiovascular events in individuals with an elevated C-reactive protein (CRP).2

Epidemiological and observational studies have suggested an association between inflammation and risk of cardiovascular disease, and changes in endothelial function may underlie this association. We have shown previously that experimental inflammation profoundly impairs endothelium-dependent dilatation in the arterial and venous circulation.3,4 Loss of the normal vasodilator, antiplatelet, and antithrombotic properties of the vascular endothelium would be expected to tip the balance in favor of vasospasm, thrombosis, and inflammation.

In the present study, we tested the hypothesis that aspirin protects the arterial endothelium from inflammation and explored whether the observed effects resulted from inhibition of vascular prostanoid synthesis or through modulation of the inflammatory response.

Methods

Subjects
The protocol was approved by the University College London Hospitals Research Ethics Committee (institutional approval), and all participants gave written informed consent. All studies were performed in a temperature-controlled laboratory (24°C to 26°C). A total of 17 subjects (aged 21 to 24 years) who stated that they were healthy, taking no medication, and had not received typhoid vaccination in the previous 6 months were included.
Generation and Measurement of the Inflammatory Response
Salmonella typhi capsular polysaccharide vaccine 0.025 mg (Typhim Vi, Pasteur Mérieux MSD) was administered as previously described. Temperature (by mercury thermometer) and pulse were recorded before and hourly for 8 hours after administration of the vaccine. At each of these time points, blood samples were taken for the measurement of cytokines.

Measurement of Cytokines
Plasma, obtained by centrifugation, was placed in aliquots and stored at −70°C for the measurement of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1 receptor antagonist (IL-1ra) using commercially available enzyme-linked immunosorbent assay (ELISA) antibody pairs (Cytoset, Biosource International). Preliminary experiments optimized manufacturers guidelines to achieve a lower detection limit of 3 pg/mL for IL-6 and TNF-α and 10 pg/mL for IL-1ra. Levels of C-reactive protein (CRP) were measured using ELISA antibody pairs from Dako (Ely), with a lower detection limit of 10 ng/mL. All samples were analyzed in duplicate by an individual blinded to treatment protocol.

Assessment of Endothelial Function in Forearm Resistance Vessels
Mercury-in-silastic strain gauge plethysmography was used to measure forearm blood flow (mL/100 mL forearm/min) in both arms as described previously. For each study, the brachial artery of the nondominant arm was cannulated with a 27-G needle (Cooper’s Needle Works) inserted under local anesthesia (2 mL 1% lignocaine). Drugs or normal saline (sodium chloride 0.9% wt/vol) were infused continuously at 0.5 mL/min. During recording periods, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mm Hg. Forearm blood flow responses to intrabrachial infusions of 2 vasodilator drugs, bradykinin (BK) and glyceryl trinitrate (GTN), were measured in each subject on 2 occasions at 4 PM on 2 consecutive days, 16 hours before and 8 hours after vaccine administration. Measurement of basal blood flow was made over a 15-minute period before the infusion of drugs. The order of drug infusions was varied between studies. Saline was infused for 15 minutes between each drug infusion, and blood flow recordings were then made for an additional 2 minutes before drug infusion. The ratio of flow in the infused/noninfused (control) arm was calculated for each measurement period. Vasodilator responses were expressed as the percentage increase in the ratio of forearm blood flow (infused/noninfused arm) relative to the immediately preceding baseline flow, as described previously.

Study Protocols
Study 1: Effect of Oral Aspirin (1.2 g) Pretreatment on Vaccine-Induced Endothelial Dysfunction
Twelve individuals were studied in this protocol. The study design was double-blind, randomized, placebo-controlled. After the initial forearm blood flow study (BK and GTN) on day 1, a random number table was used to allocate subjects to either 1.2 g active aspirin (n=6) or matched placebo (n=6). Aspirin (or matched placebo) were self-administered at 6 AM on day 2 of the study, 2 hours before vaccination. Aspirin and placebo were prepacked by University College London Hospitals pharmacy, and the investigator conducting the vascular study was blind to the treatment allocation of the volunteer. The second forearm blood flow study (BK and GTN) was performed 8 hours after vaccination. In one individual who received placebo, the intra-arterial needle dislodged on the second day after the dose-response curve to BK. Therefore, for this group, the GTN data includes 5 individuals.

Study 2: Effect of Local Intrav-Arterial Aspirin on Established Vaccine-Induced Endothelial Dysfunction
In a second open-label study, forearm blood flow responses to BK were compared before and at 8 hours after vaccination in 5 individuals. After the second dose-response curve to BK, aspirin (Aspergic) was infused at 10 mg/min for 15 minutes (total dose, 150 mg) directly into the brachial artery, and 15 minutes later the forearm blood flow response to BK was measured for a third time. The dose-response curves were constructed 15 minutes after the aspirin was given, because this dose of aspirin has been reported by some investigators to cause a small fall in resting flow that returns to baseline within 15 minutes. This effect may be attributable to the pH of the solution.

Drug Infusions
BK was obtained from Clinalfa AG, and GTN was from David Bull Laboratories. Drugs were prepared as stock solutions, which were aliquoted and stored at −20°C until use. Cumulative dose-response curves were constructed to BK (20, 40, and 80 pmol/min, each dose for 3 minutes) and GTN (4, 8, and 16 nmol/min, each dose for 3 minutes). Aspirin (Aspergic, Labotoires Synthelabo) was freshly made up for each study to a solution of 20 mg/mL.

Statistical Analysis
Results are expressed as mean (SEM) unless otherwise stated. For resistance vessel studies, dose-forearm blood flow response curves were constructed, and comparisons were made by repeated-measures ANOVA or 2-way ANOVA as appropriate, with each subject as his own control. Cytokine levels after vaccination were expressed as a ratio relative to baseline for each subject. The time course over 8 hours was plotted, and comparisons between treatment groups were made by 2-way ANOVA. In all studies, P<0.05 was considered statistically significant.

Results
In the 8 hours after administration of capsular polysaccharide typhoid vaccine, pulse rate, temperature, or baseline blood flow did not change significantly in any group (data not shown).

Study 1: Effect of Oral Aspirin (1.2 g) Pretreatment on Vaccine-Induced Endothelial Dysfunction
Endothelial Function in Response to Vaccination
Placebo Group
Before vaccination, there was a dose-dependent increase in forearm blood flow to BK and to GTN. Eight hours after vaccination, the response to BK was reduced, whereas the responses to GTN were preserved (Figures 1a and 1b).

Oral Aspirin Group
Before vaccination, there was a dose-dependent increase in forearm blood flow to BK and GTN. In contrast to the placebo group, in this group, 8 hours after vaccination forearm blood flow responses to BK and GTN were maintained at prevaccination levels (Figures 1c and 1d).

Cytokine Response to Vaccination
In the placebo group, the level of IL-1ra rose to a peak at 3 hours and remained elevated until 8 hours after vaccination. At the 3-hour time point, this corresponded to a 30-fold increase from baseline values. In contrast, in the group treated with a single oral dose of aspirin, the concentration of IL-1ra after vaccination did not differ from baseline. The change in IL-1ra differed between the aspirin and placebo groups (P<0.05, 2-way ANOVA, Figure 2a).

In the placebo group, there was no rise in TNF-α at the time points studied. However, in the aspirin-treated group, TNF-α increased, peaking at 4 hours after vaccination. This
response was significantly different between the groups studied ($P<0.05$, 2-way ANOVA, Figure 2b).

There was a progressive increase in IL-6 in both the placebo and aspirin groups. There was a peak 5-fold increase at 4 hours after vaccination, with no difference between the groups ($P=\text{NS}$, 2-way ANOVA, Figure 2c). CRP levels and IL-8 and IL-10 did not change significantly in the 8 hours after vaccination (data not shown).

**Study 2: Effect of Local Intra-Arterial Aspirin on Established Vaccine-Induced Endothelial Dysfunction**

As expected, 8 hours after vaccination, there was a marked blunting of the response to BK ($P<0.01$ by repeated-measures ANOVA). Intra-arterial infusion of aspirin (10 mg/min for 15 minutes) had no effect on basal blood flow and did not restore the attenuated response to BK (Figure 3).

**Discussion**

This study confirms our previous observation that mild systemic inflammation impairs endothelium-dependent dilation in humans and provides direct evidence that pretreatment with an anti-inflammatory dose of aspirin protects from this endothelial dysfunction. These effects were associated with an alteration in the cytokine response to vaccination with a reduction in the induction of IL-1ra and an unexpected enhancement of the TNF-$\alpha$ responses after pretreatment with aspirin. This vasculoprotective effect of aspirin does not seem to be attributable to inhibition of vascular constrictor prostanoid synthesis, because locally infused aspirin did not restore endothelial function in vaccinated subjects. The abrogation of the cytokine response to inflammatory stimuli provides a novel mechanism by which aspirin can modulate endothelial function.

**Aspirin Pretreatment Preserves Endothelial Function**

Forearm arteriolar vasodilatation was assessed in both study groups before and after vaccination, with each study being performed at the same time of day. Dilator dose-response
lial dysfunction in various models, the observation that there is no increase in this cytokine in the placebo-treated group and that there is an increase seen after aspirin in the absence of endothelial dysfunction suggests that TNF-α is not the major mediator of vaccine-induced endothelial dysfunction. However, it is possible that the effects of aspirin to increase TNF-α production during inflammation may exert other detrimental effects, including procoagulant effects.8,9

Elevation of IL-6 is of particular interest in relation to cardiovascular risk, and IL-6 is presumed to be an important if not the principal stimulus to the synthesis of C-reactive protein, levels of which are predictive of the risk of cardiovascular events.11,12 However, our data show that aspirin protects endothelial function without modulating systemic IL-6. Furthermore, in our hand vein model, IL-6 did not induce endothelial dysfunction.4

It is not possible to define precisely which cytokine is responsible for endothelial dysfunction in the vaccine model, but our data suggest that interleukin-1 may be important. It induces endothelial dysfunction in humans, levels increase in response to vaccination, and its production/release is prevented by aspirin given in doses that prevent vaccine-induced endothelial dysfunction. Furthermore, IL-1ra levels are increased in patients with angina, and specific polymorphisms in the IL-1ra gene are associated with coronary artery disease.13,14 Studies with IL-1 antagonists would be required to test the causality of these associations. This study does not rule out contribution by other important proinflammatory and anti-inflammatory cytokines, and it is possible that the IL-1ra simply acts as a surrogate marker for some other cytokine or mediator that causes endothelial dysfunction.15,16 The vaccine model we have developed would allow exploration of such potential mechanisms and may also be of use to characterize the possible involvement of T-cell responses.

**Effects of Aspirin Are Not Attributable to Vasoconstrictor Prostanoids**

We investigated whether the effect of aspirin was attributable to a direct effect on vascular prostanoid production. In hypercholesterolemic subjects and patients with coronary artery disease, acute systemic administration of aspirin reverses endothelial dysfunction in the forearm bed.17,18 However, a dose of aspirin that has been shown previously almost completely to abolish prostanoid production in the forearm did not modify the impaired BK response produced by inflammation.19 This dose of aspirin achieves millimolar concentrations of aspirin in the forearm and causes irreversible acetylation of cyclooxygenase. The results strongly suggest that inflammation is not simply increasing the production of vasoconstrictor prostanoids to cause a reduction in the vasodilator response to BK. This part of the study was designed to investigate local mechanisms; however, the total dose of aspirin given directly into the bloodstream in this part of the study was 150 mg, which would be sufficient to significantly affect systemic prostanoid production. Although not designed specifically to test systemic effects, this part of the study also suggests that systemic inhibition of prostanoid generation does not reverse inflammation-induced endothelial dysfunction, at least in the short-term.
Clinical Implications
Systemic inflammation occurs in a wide variety of infective disorders and after iatrogenic procedures such as abdominal surgery. There is growing evidence that acute systemic inflammation is associated with a transient increase in the risk of cardiovascular events that may persist for days or weeks. There is also evidence that unstable angina is associated with inflammation, which might precede the onset of the syndrome.

Together our present findings and previous studies show that inflammation induces endothelial dysfunction in humans, that aspirin can prevent this effect, and that IL-1 may be important in the genesis of the effect. It would now be important to determine whether the findings we report here are important in acute inflammatory states in a clinical setting. Although the exact nature of inflammatory response to different stimuli may be variable, in 2 different experimental models we have shown reproducible inflammation-induced endothelial dysfunction in humans. Specifically the present study raises the possibility that protective effects of aspirin on endothelial function may contribute to the efficacy of this drug in reducing cardiovascular risk in certain situations. However, important clinical questions remain. First, do lower doses of aspirin given chronically have similar effects, or is this novel protective effect of aspirin seen only at high dose? Second, is this effect of aspirin important in its efficacy in reducing cardiovascular risk in certain situations? Third, is the finding that high-dose aspirin prevents inflammation-induced endothelial dysfunction only when given before the inflammatory insult of potential therapeutic importance with regard to the timing of aspirin administration in the clinical setting of cardiovascular events induced by inflammation?

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References
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