

Recovery of Endothelial Cell Prostacyclin Production after Inhibition by Low Doses of Aspirin

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ABSTRACT Endothelial cells synthesize prostacyclin (PGI₂), an unstable prostaglandin that inhibits platelet aggregation and serotonin release. Because cyclooxygenase, which is necessary for synthesis of PGI₂, is inactivated by aspirin, we examined the effect of aspirin on PGI₂ production by cultured human endothelial cells. Endothelial cells synthesize PGI₂ (20.1 ± 7.2 ng/10⁶ cells, mean \pm SD) when stimulated with 20 μ M sodium arachidonate for 2 min. PGI₂ production is inhibited by low-dose aspirin (5 μ M); the $t_{1/2}$ of inactivation is 6.0 ± 1.3 min (mean \pm SEM, $n = 3$). Thus, endothelial cell cyclooxygenase is as sensitive to aspirin as the enzyme in platelets. After 1 h incubation with aspirin, endothelial cell PGI₂ production was inhibited 50% by 2.1 ± 0.4 μ M aspirin and was inhibited 90% by 6.2 ± 0.9 μ M aspirin (mean \pm SEM, $n = 4$). When endothelial cells were incubated with 100 μ M aspirin, washed, and recultured, their ability to synthesize PGI₂ returned to control levels in 35.6 ± 1.0 h (mean \pm SEM, $n = 4$). Recovery of endothelial PGI₂ production after aspirin depended on de novo protein synthesis because treatment with cycloheximide (3 μ g/ml) inhibited recovery by 92%.

These results indicate that although endothelial

cell cyclooxygenase in vitro is inhibited by low concentrations of aspirin, endothelial cells rapidly resynthesize their cyclooxygenase after the aspirin is removed. This rapid resynthesis of cyclooxygenase lessens the likelihood that aspirin used in clinical doses promotes thrombosis.

INTRODUCTION

Prostacyclin (PGI₂)¹ is a labile prostaglandin that inhibits platelet aggregation and serotonin release and causes vasodilation (1). We have recently shown that cultured human endothelial cells synthesize PGI₂ when stimulated with thrombin, the calcium ionophore A23187, trypsin, or sodium arachidonate (2, 3). The initial step in the synthesis of PGI₂ in endothelium or thromboxane A₂ in platelets from arachidonic acid is catalyzed by the enzyme cyclooxygenase. In platelets, cyclooxygenase is irreversibly inactivated by low doses of aspirin (4), and this is the basis for the use of aspirin as an antithrombotic agent. However, cyclooxygenase is also required for PGI₂ synthesis from arachidonate so that inhibition of endothelial cell cyclooxygenase would decrease endothelial cell PGI₂ production and might promote thrombosis. Because aspirin is now commonly used in the treatment of thrombo-embolic disorders, we have examined the effect of aspirin on endothelial cell PGI₂ production.

In this paper we show that although endothelial

Dr. Jaffe is the recipient of a National Institutes of Health Research Career Development Award (5 KO4 HL 00237) and a Career Scientist Award from the Irma T. Hirsch Trust.

Received for publication 21 November 1978 and in revised form 15 December 1978.

¹Abbreviation used in this paper: PGI₂, prostacyclin.

cell PGI₂ synthesis is indeed inhibited by low doses of aspirin, recovery of PGI₂ production occurs rapidly.

METHODS

Endothelial cell monolayer cultures. Human endothelial cells derived from umbilical cord veins were cultured in replicate 35-mm Petri dishes in medium 199 containing 20% pooled human serum, as previously described (5), and used when confluent. Cell counts in dishes used on a given day varied by <15%.

Treatment of endothelial cells with aspirin. Aspirin (Sigma Chemical Co., St. Louis, Mo.) stock solutions (0.1 M in absolute ethanol) were always freshly prepared and sterilized by filtration. Before addition to endothelial cells, the aspirin was diluted with sterile water and 0.01 vol of diluted aspirin solution added to the culture medium to achieve the desired final concentration. The endothelial cells were incubated in 5% CO₂ at 37°C for varying periods of time after which the culture medium was removed and the cells washed twice with Buffer A (10 mM Hepes pH 7.3 at 37°C, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose). The cells were then either assayed immediately for PGI₂ production or washed and recultured for additional time periods and then assayed. Control endothelial cell monolayers treated with 0.1% ethanol alone were included in each series of experiments. Ethanol at this concentration had no effect on PGI₂ synthesis.

Platelet preparations. Washed platelet suspensions for the thrombin release assay (see below) were prepared as previously described (3).

Assay for PGI₂ production. Monolayers of endothelial cells were washed twice with Buffer A and then covered with 1 ml of Buffer B (15 mM Tris HCl pH 8.6, 140 mM NaCl, 5.5 mM glucose), which preserves PGI₂ activity (6). Sodium arachidonate (final concentration, 20 μM) was then added and the monolayer incubated for 2 min at 37°C. Buffer B was then removed and either assayed immediately for PGI₂ or frozen at -70°C until assayed. PGI₂ activity was measured by quantitating the release of [³H]serotonin from pre-labeled, washed, aspirin-treated platelet suspensions stimulated by thrombin in the presence or absence of authentic PGI₂ or supernatant fluids containing PGI₂. This assay system is a modification of the method of Baenziger et al. (6) and has been described previously (3). PGI₂ (a gift of Dr. John Pike, Upjohn Co., Kalamazoo, Mich.) standards ranging from 0.1 to 100 nM were included in all experiments. Because the percent change in thrombin concentration producing 50% of maximal serotonin release (ΔT₅₀) varied in a dose-response fashion with PGI₂ concentration, a curve of ΔT₅₀ vs. log PGI₂ was plotted and used to convert our ΔT₅₀ data to PGI₂ concentration.

Cycloheximide inhibition of the recovery of PGI₂ production. Endothelial cells were incubated with 100 μM aspirin for 1 h, washed, and cultured for 18 h with cycloheximide (0.1, 1, or 3 μg/ml) (Sigma Chemical Co.). The cells were then assayed for PGI₂ production and compared to cells processed in parallel in the absence of cycloheximide.

RESULTS

Time-course of inhibition of endothelial cell PGI₂ production by aspirin. Endothelial cells stimulated for 2 min with 20 μM sodium arachidonate produced 20.1±7.2 ng PGI₂/10⁶ cells (mean±SD, n = 4). This represents a concentration of PGI₂ in the supernate

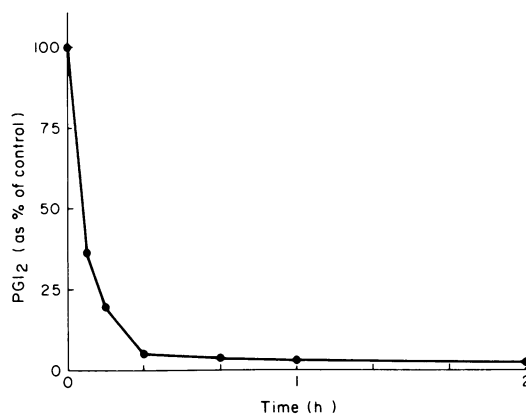


FIGURE 1 Time-course of inhibition by aspirin of endothelial PGI₂ production. Replicate cultures of human endothelial cells were treated with 5 μM aspirin for increasing times, washed (see Methods), and then stimulated with 20 μM sodium arachidonate for 2 min. The supernatant fluids were then removed from the monolayers and assayed for PGI₂ using the [³H]serotonin release assay described in Methods. The quantities of PGI₂ produced are expressed as percent of aspirin-free controls.

(1 ml) of 28.2±14.7 nM (mean±SD). Inactivation of endothelial cell cyclooxygenase by aspirin was measured as the reduction in PGI₂ synthesis observed after stimulation with arachidonate (20 μM) for 2 min. When endothelial cells were exposed to 5 μM aspirin for varying periods of time, PGI₂ production rapidly declined (Fig. 1). After 5 min of aspirin exposure PGI₂ production fell to 36% of control values, and after 1 h endothelial cell PGI₂ production was <3% of control.

Because a plot of log (PGI₂ _{t=∞}/PGI₂ _{t=0}) vs. time was linear for the first 20 min, the experiment initially followed pseudo first order kinetics for the acetylation reaction. With calculations similar to those of Burch et al. (7), the t_{1/2} of decrease of endothelial cell PGI₂ production in the presence of 5 μM aspirin was 6.0±1.3 min (mean±SEM, n = 3), and the rate constant for the inhibition of PGI₂ production by 5 μM aspirin (K_{apparent} = [ln2]/t_{1/2}) was 0.116 min⁻¹. Defining a potency term as K_{apparent}/aspirin concentration (7), the inhibitory capacity of aspirin on endothelial cell PGI₂ production was 23,100/min⁻¹ M⁻¹. Because Burch et al. calculated a potency of aspirin toward platelet cyclooxygenase of 21,700/min⁻¹ M⁻¹ (7), it appears that endothelial cells and platelets are similarly sensitive to aspirin.

Dose-response characteristics of inhibition of endothelial cell PGI₂ production by aspirin. Endothelial cells were incubated with increasing doses of aspirin for 1 h and then assayed for PGI₂ production after stimulation with 20 μM sodium arachidonate. As shown in Fig. 2, PGI₂ synthesis was inhibited by aspirin in a dose-dependent manner. Data obtained

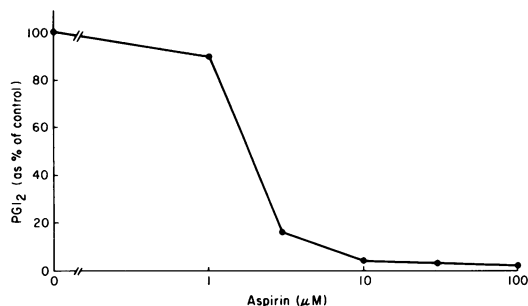


FIGURE 2 Inhibition of endothelial cell PGI₂ production as a function of aspirin dosage. Replicate cultures of endothelial cells were treated with increasing doses of aspirin for 1 h. The aspirin was removed, the cells washed, and then stimulated with 20 μM sodium arachidonate for 2 min. The supernatant fluids were removed and assayed for PGI₂ activity. The amounts of PGI₂ produced are expressed as percent of aspirin-free controls.

from curves of experiments such as those shown in Fig. 2 indicated that after 1 h endothelial cell PGI₂ production was inhibited 50% by 2.1 ± 0.4 μM aspirin and inhibited 90% by 6.2 ± 0.9 μM aspirin (mean \pm SEM, $n = 4$).

Time-course of recovery of endothelial cell PGI₂ production after inhibition by aspirin. Replicate cultures of endothelial cells were treated with 100 μM aspirin for 1 h at 37°C, washed, and then recultured for varying periods. The endothelial cells were then stimulated with 20 μM sodium arachidonate and assayed for PGI₂ production. The results of a typical experiment are shown in Fig. 3. The ability of endothelial cells to produce PGI₂ returned to control

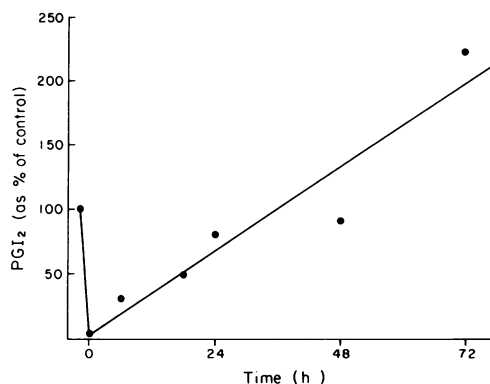


FIGURE 3 Time-course of recovery of endothelial cell PGI₂ production after inhibition by aspirin. Replicate cultures of endothelial cells were treated with 100 μM aspirin for 1 h at 37°C, washed, and then incubated with medium 199 containing 20% human serum for increasing time intervals. The endothelial cells were then washed and stimulated with 20 μM sodium arachidonate for 2 min, and the supernatant fluids were assayed for PGI₂ activity. The amounts of PGI₂ produced are expressed as percent of aspirin-free controls.

levels by 35 h. Data obtained from curves of experiments such as those seen in Fig. 3 showed that after a 1 h incubation with 100 μM aspirin, endothelial cell PGI₂ production returned to control levels in 35.6 ± 1.0 h (mean \pm SEM, $n = 4$). In three additional experiments in which recovery was monitored from 0 to 24 h, recovery curves were similar to those seen in Fig. 3. By extrapolation these curves yielded an estimated recovery time of 37.6 ± 4.9 h (mean \pm SEM, $n = 3$) which is consistent with the 36-h recovery time obtained in our other experiments.

Resumption of endothelial cell PGI₂ production after inhibition by aspirin was dependent on new protein synthesis. Cycloheximide inhibited the recovery of PGI₂ production in a dose-related fashion; thus cycloheximide (3 μg/ml) inhibited recovery of PGI₂ production by 92%.

DISCUSSION

Pharmacologic studies in man have shown that 650 mg of aspirin given orally will yield plasma aspirin levels of 128 μM at 10 min, 56 μM at 25 min, and 18 μM at 1 h (8). Our data (i.e., 6.2 μM aspirin for 1 h inhibits endothelial cell PGI₂ production by 90%) indicate that endothelial cell PGI₂ production is promptly inhibited by doses of aspirin comparable to those employed clinically. This is also true of platelet thromboxane A₂ production (7). It thus appears that endothelial cell and platelet cyclooxygenases are equally sensitive to aspirin inhibition. Endothelial cells begin to regain their ability to produce PGI₂ as soon as aspirin is removed (Fig. 3) and recover fully by ≈ 36 h. In marked contrast, however, platelets never regain cyclooxygenase activity after aspirin because acetylation of the enzyme is not reversible (7). The platelet cyclooxygenase, which becomes measurable 48 h after aspirin, represents that present in newly formed platelets (7). Thus, during the first 48 h after aspirin treatment, endothelial cells can produce PGI₂, whereas platelets cannot produce thromboxane A₂. Recovery of endothelial PGI₂ production after aspirin reflects new protein synthesis inasmuch as it is inhibited by cycloheximide.

In the early stages of recovery from aspirin inhibition, endothelial cell PGI₂ production may be $<10\%$ of normal (i.e., 10% of 28.2 nM or 2.8 nM in our culture system). The surface:volume ratio of the human vascular system ($1,000 \text{ m}^2/3,500 \text{ ml} = 2.86 \times 10^3 \text{ cm}^2/\text{ml}$)² is much greater than this ratio in a 35-mm Petri dish ($9.6 \text{ cm}^2/1 \text{ ml} \approx 9.6 \text{ cm}^2/\text{ml}$). Thus in vivo production of PGI₂ at 10% of maximum could theoretically yield a peak blood PGI₂ concentration of $\approx 830 \text{ nM}$

² Assuming an average vascular surface area of 1,000 m² (9) and an average plasma volume of 3,500 ml.

(2.8 nM \times 2.86 \times 10³/9.6). In vitro, such levels of PGI₂ have a profound inhibitory effect on platelet behavior. For example, 1.4 nM PGI₂ fully inhibited thrombus formation, and 57 nM PGI₂ inhibited platelet adhesion by 56% when human blood was exposed to rabbit aorta subendothelium (10). Furthermore, 47 nM PGI₂ caused a half-maximal increase in platelet cyclic AMP to a level of \approx 12 times basal level (11) when only a doubling of cyclic AMP was needed to inhibit platelet aggregation (12). Similarly, we have reported that 0.1 nM PGI₂ completely inhibited platelet aggregation in platelet-rich plasma induced by threshold levels of arachidonic acid (3). Thus, in man and experimental animals even a small fraction of the productive capacity for PGI₂ in normal blood vessels should result in more than enough PGI₂ to inhibit platelet function.

Recent animal studies support our contention that the effect of aspirin on endothelial cyclooxygenase is of brief duration. In rabbits, PGI₂ mediated decrease in thrombus size after standardized vascular injury was observed as soon as 2.5 h after infusion of aspirin (200 mg/kg) (13). Also, in rats given lysine acetylsalicylate (200 mg/kg), PGI₂ production in veins returned to 51% of control in 24 h and to normal levels in 48 h (14).

In summary, endothelial cell PGI₂ synthesis is inhibited by low doses of aspirin, but recovery to control levels occurs by 36 h. The ability of endothelial cells in normal blood vessels to produce PGI₂ seems to be far in excess of that needed to inhibit platelet function. Our data thus suggest that aspirin as used in clinical practice will not paradoxically promote thrombosis. We do, however, suggest that in patients with normal platelet turnover, aspirin administration be limited to once a day.

ACKNOWLEDGMENTS

We thank Christopher Ley, Tina Pavane, Emily Altman, Charles Dorso, Karen Tack-Goldman, and Andrea Frank for excellent technical assistance and Naomi Nemtzow for typing the manuscript.

This work is supported by the National Institutes of Health through a specialized Center for Thrombosis Re-

search grant (HL 18828), the New York Heart Association, and the Arnold R. Krakower Hematology Foundation.

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