Inhibition of Prostacyclin by Treatment of Endothelium with Aspirin

CORRELATION WITH PLATELET ADHERENCE

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ABSTRACT Aspirin treatment of cultured endothelial cells from the umbilical vein increased the adherence of ^{51}Cr-platelets when thrombin was present. If the cyclooxygenase activity of endothelium was inhibited by aspirin, as it is in the platelet, reduction of endogenous prostacyclin (PGI_2) production could have been responsible. By correlating thrombin-induced adherence of platelets to endothelial monolayers with PGI_2 release (as measured by radioimmunoassay for 6-keto-prostaglandin $FI_{1\alpha}$ [6-keto-$PGF_{1\alpha}$]), we have demonstrated an inverse relationship between platelet adherence and PGI_2 levels. Untreated endothelial monolayers exposed to thrombin and platelets resulted in 4% platelet adherence and 107 nM 6-keto-$PGF_{1\alpha}$. With 0.1 mM aspirin treatment, which is known to block platelet cyclooxygenase, adherence was 5% and 6-keto-$PGF_{1\alpha}$ decreased to 45 nM. Increasing the aspirin concentration to 1 mM resulted in 44% adherence and <3 nM 6-keto-$PGF_{1\alpha}$. When 25 nM exogenous PGI_2 was added to 1 mM aspirin-treated endothelium, adherence returned to 5%. The increase in thrombin-induced platelet adherence to 1 mM aspirin-treated monolayers was reversed 2 h after removal of the aspirin solution. 6-Keto-$PGF_{1\alpha}$ returned to 37% of the untreated monolayer value. Recovery from the aspirin effect did not occur when cycloheximide, an inhibitor of protein synthesis, was present during the 2-h period.

INTRODUCTION

We have reported recently that minimal platelet adherence to endothelium occurred if thrombin, platelets, and endothelium were incubated simultaneously (1). This thrombin-induced platelet adherence could be enhanced by preincubation of the endothelium with a high dose of aspirin. The increased adherence was seen even when aspirin-treated platelets were used. The aspirin effect on the endothelium was temporary.

The present study was undertaken to determine whether prostacyclin (PGI_2)[1], the inhibitor of platelet aggregation found predominantly in the vascular intima (2), was involved in the aspirin modification of thrombin-induced platelet adherence to endothelium. This approach was suggested by our initial finding that cultured endothelial cells, but not our fibroblast or smooth muscle cells, yielded 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable end product of PGI_2, in response to thrombin (1). The thrombin-induced release of PGI_2 from confluent monolayers of cultured human umbilical vein endothelium has been detailed in subsequent studies (3, 4).

METHODS

The preparation of confluent monolayers in 35 × 10 mm culture dishes of primary human umbilical vein endothelium ($7–8 \times 10^5$ cells; 0.2–0.3 mg protein) and passed umbilical artery fibroblasts has been described elsewhere (1). Empty-dish controls were culture dishes that were handled exactly as dishes that contained cells. Incubation medium (IM): 8.6 g/liter bovine serum albumin, 140.3 mM NaCl, 5.8 mM KCl, 2.7 mM $CaCl_2$, and 16.3 mM Tris, pH 7.4. Monolayer washing buffer: Hank's balanced salt solution without $NaHCO_3$, and 15 mM Hepes, pH 7.4. The source of reagents and the preparation of solutions not specifically detailed below have been reported (1, 4).

Adherence or PGI_2 procedure. The adherence of ^{51}Cr-

This work was presented in part at the 51st Annual Meeting of the American Heart Association, Dallas, Texas, 15 November 1978.

[1] *Abbreviations used in this paper*: ASA, acetylsalicylic acid or aspirin; IM, incubation medium; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGI_2, prostaglandin I_2 or prostacyclin.

platelets to endothelium previously treated with acetylsalicylic acid or aspirin (ASA) has been described (1). Studies to determine PGI_2 release were identical to the adherence studies through the monolayer incubation procedure with thrombin and platelets, except that unlabeled washed platelets were employed. Briefly, 1 ml ASA in IM or 1 ml IM (control) was incubated with the monolayer for 30 min at 37°C with rocking. The preincubation solution was removed and the monolayer was washed twice. 1 ml 0.5 U/ml bovine thrombin in IM or 1 ml IM was added, followed immediately by 0.5 ml Tyrode's solution with human ^{51}Cr-platelets (for adherence) or unlabeled platelets (for PGI_2 studies). The dish was rocked 30 min at 37°C.

For the adherence determination, the suspension that contained unattached platelets was removed, and the monolayer was washed twice. The monolayer and attached ^{51}Cr-platelets were then solubilized and, after ^{51}Cr-radioactivity was determined in all solutions, percent adherence was calculated by dividing monolayer counts per minute, multiplied by 100, by the total counts per minute.

For the PGI_2 determination, the suspension that contained unattached platelets was removed and centrifuged at 1,100 *g* for 15 min. PGI_2 (or 6-keto-$PGF_{1\alpha}$) in the supernate was determined by means of a radioimmunoassay for 6-keto-$PGF_{1\alpha}$ (4).

Inhibition by exogenous PGI_2 of platelet adherence to endothelium. A stock solution of 1 mM synthetic PGI_2 in 50 mM Na_2CO_3 was stored under N_2 at −20°C. An aliquot of this PGI_2 was thawed immediately before use, diluted, and then incubated with platelets and the monolayer or empty dish within 10 min.

The monolayer or empty dish was pretreated with 1 mM ASA and washed, as described above. Added immediately in rapid succession were 0.5 ml IM with or without three times the final concentration of PGI_2, 0.5 ml IM with 1 U/ml bovine thrombin (with mixing), and 0.5 ml Tyrode's solution with ^{51}Cr-platelets (with mixing). The monolayer or empty dish was then rocked 30 min at 37°C. Adherence was determined as described above.

Determination of monolayer recovery from ASA. After the 1 mM ASA or control-buffer pretreatment and washing of the endothelium, the dish was immediately layered with 2 ml culture medium, with or without 2.5 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, Mo.). After a 2-h incubation without rocking as previously described (1), the medium was removed and the monolayer was washed twice. Thrombin and platelets were then rocked 30 min at 37°C with the endothelium. Platelet adherence or PGI_2 release into the supernate was determined as described above.

Statistical method. Student's *t* test was employed to determine statistical significance (5). Data represent a minimum of three experiments in duplicate. The handling of 6-keto-$PGF_{1\alpha}$ data below the 3 nM assay-detection limit has been described (4).

RESULTS

In Fig. 1, platelet adherence to untreated or ASA-pretreated venous endothelium was compared with the 6-keto-$PGF_{1\alpha}$ released into the incubation solution. In the absence of thrombin, ≅2% adherence was observed for untreated (1.7±0.4%) and ASA-treated monolayers (2.4±0.4%, 0.1 mM ASA; 1.4±0.1%, 1 mM ASA). 6-Keto-$PGF_{1\alpha}$ decreased from the 18.8±2.7 nM base-line level observed with untreated endothelium, to <3.0±0.5 nM with 0.1 mM ASA-treated

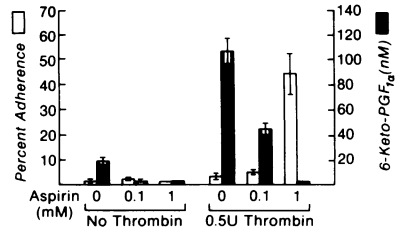

FIGURE 1 Platelet adherence to untreated and ASA-treated endothelium compared with 6-keto-$PGF_{1\alpha}$ release. ASA or buffer control was incubated with the monolayer for 30 min at 37°C with rocking. The preincubation solution was removed and the dish was washed twice. Thrombin or buffer control was added, followed immediately by ^{51}Cr-platelets (for adherence) or unlabeled platelets (for PGI_2 determinations). The monolayer was rocked 30 min at 37°C. Percent adherence was calculated by dividing counts per minute of cells attached to the monolayer, mutiplied by 100, by total counts per minute added to the dish. 6-Keto-$PGF_{1\alpha}$ released into the supernate was determined by radioimmunoassay.

endothelium, and then below the 3.0±0 nM assay-detection level with the 1 mM ASA-treated cells. It should be noted that the 18.8±2.7 nM 6-keto-$PGF_{1\alpha}$ value is obtained not from resting cells, but from untreated monolayers subjected to rocking. Previously, we have calculated (4) that this level is quantitatively similar to the PGI_2 reported in stirred suspensions of human umbilical vein endothelium (6).

In the presence of 0.5 U thrombin (Fig. 1), 3.8±0.9% platelet adherence and 106.7±10.2 nM 6-keto-$PGF_{1\alpha}$ were observed with the untreated monolayer. With 0.1 mM ASA-treated monolayers, adherence was 5.4 ±0.7%, whereas 6-keto-$PGF_{1\alpha}$ decreased to 44.9±4.5 nM. With 1 mM ASA-treated monolayers, adherence increased significantly to 44.2±8.2% and 6-keto-$PGF_{1\alpha}$ was <3.0±0 nM.

The ASA-induced increase in platelet adherence to endothelium was offset by the addition of exogenous PGI_2, as is shown in Fig. 2. For this study, the monolayer was treated with 1 mM ASA to completely inhibit PGI_2 release. Immediately before the addition of thrombin and platelets, synthetic PGI_2 was added to the dish. Platelet adherence to endothelium declined from the 49.2±2.5% observed in the absence of PGI_2 to 18.0±3.6, 12.3±1.8, 6.2±0.7, and 5.3±0.6% when a final concentration of 5, 10, 15, and 25 nM PGI_2 was used. This response to PGI_2 was significantly greater than that observed with the empty dish or fibroblast controls (Fig. 2). Adherence to the ASA-treated empty dish decreased minimally from 81.0±1.2% in the absence of PGI_2 to 74.4±1.7% with 25 nM PGI_2. Adherence to the ASA-treated fibroblast monolayer declined from 74.9±1.1% to 65.9±1.7% in the same PGI_2 range.

The duration of the ASA effect on endothelium, as reflected by thrombin-induced adherence and 6-keto-

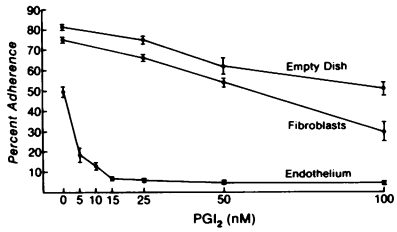

FIGURE 2 Inhibition by exogenous PGI_2 of 0.5 U thrombin-induced platelet adherence to ASA-treated endothelium, fibroblasts, and empty dishes. 1 mM ASA was incubated with the monolayer or empty dish for 30 min at 37°C with rocking. The ASA solution was removed and the cell layer or dish was washed twice. Added immediately were synthetic PGI_2, thrombin, and ^{51}Cr-platelets. The monolayer or empty dish was rocked 30 min at 37°C. Percent adherence was calculated by dividing counts per minute of cells attached to the monolayer, multiplied by 100, by total counts per minute added to the dish.

$PGF_{1\alpha}$ release, is shown in Fig. 3. In the absence of ASA, 0.5 U thrombin-induced platelet adherence was 10.9±5.0% and 6-keto-$PGF_{1\alpha}$ release was 124.0±11.7 nM for the monolayer incubated immediately with thrombin and platelets (0-h recovery). These values are not significantly different from those (not shown in Fig. 3) observed with endothelium not treated with ASA, which had been placed back into the growth medium with or without cycloheximide for 2 h.

With the 1 mM ASA-treated endothelium (Fig. 3), 46.2±5.8% platelet adherence and 6-keto-$PGF_{1\alpha}$ release below detectable levels (<3.0±0 nM) were observed when the monolayer was immediately incubated with 0.5 U thrombin and platelets. In contrast, 2 h after the ASA was removed from the endothelium, thrombin-induced adherence returned to 9.5±3.6%, the base-line level observed with endothelium not

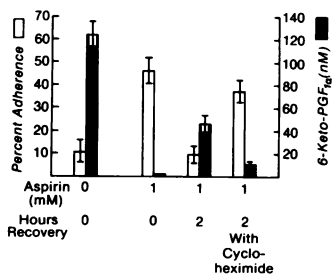

FIGURE 3 Duration of the ASA effect on endothelium as reflected by 0.5 U thrombin-induced platelet adherence and 6-keto-$PGF_{1\alpha}$ release. ASA or buffer control was incubated with the monolayer for 30 min at 37°C with rocking. The pre-incubation solution was removed and the monolayer was washed twice. The designated monolayers were layered with 2 ml culture medium with and without 2.5 μg/ml cycloheximide, incubated in a 5% CO_2 atmosphere at 37°C for 2 h (recovery period), and rewashed twice. All monolayers were then treated with thrombin and platelets as in Fig. 1.

treated with ASA. This was associated with an increase of 6-keto-$PGF_{1\alpha}$ to 46.3±7.0 nM, a concentration shown in the study with exogenous PGI_2 (Fig. 2) to inhibit platelet adherence to approximately the value observed here. When 2.5 μg/ml cycloheximide was present during the 2-h recovery period from ASA (Fig. 3), the high level of platelet adherence was maintained at 37.1±4.9%. This adherence was associated with <11.0±2.2 nM 6-keto-$PGF_{1\alpha}$.

DISCUSSION

An inverse relationship appears to exist between thrombin-stimulated PGI_2 release from human umbilical vein endothelium, and thrombin-induced platelet adherence to the endothelial monolayer. In Fig. 1, low platelet adherence was associated with high 6-keto-$PGF_{1\alpha}$ release from untreated endothelium, whereas high adherence and 6-keto-$PGF_{1\alpha}$ values below the assay-detection level were observed with 1 mM ASA-treated endothelium. This inhibition by ASA of PGI_2 activity in components of the blood vessel wall has also been observed by others (7–13).

Evidence that release of endogenous PGI_2 is causative in inhibiting the thrombin-induced platelet adherence to endothelium was suggested by the data in Fig. 2. Adherence to endothelial monolayers that did not release endogenous PGI_2 was reduced by adding synthetic PGI_2 to the dishes before the incubation with thrombin and platelets. The study also indicates that the ≅100-nM endogenous PGI_2 capable of release by thrombin is more than sufficient to prevent adherence to the endothelium. However, factors other than PGI_2 may also be involved in preventing thrombin-induced platelet adherence to endothelium. Similar amounts of exogenous PGI_2 only partially decreased adherence to empty dishes or to fibroblasts (Fig. 2).

With 0.1 mM ASA-treated endothelium (Fig. 1), thrombin-induced platelet adherence after a 30-min incubation was not significantly increased over the level observed with the monolayer not treated with ASA. On the other hand, 6-keto-$PGF_{1\alpha}$ release was reduced to 44.9±4.5 nM, which is 42% of the non-ASA-treated monolayer level. A 40–50-nM concentration of synthetic PGI_2 has been shown to be more than sufficient to block thrombin-induced adherence to 1 mM ASA-treated endothelium (Fig. 2). It should be noted that a plasma level of ≅0.1 mM ASA is achieved 15 min after the ingestion of two ASA tablets (14). After a 20-min incubation, this same concentration of ASA effects a 95% decrease in platelet cyclooxygenase activity as determined by platelet malonaldehyde production (1, 15). Therefore, it appears that the cyclooxygenase that uses endogenous arachidonate in umbilical vein endothelium is more resistant to pharma-

cological doses of ASA than is its platelet counterpart. Burch et al. (7) have reported that the fatty acid cyclooxygenase in microsomal fractions from human thoracic aorta or coronary artery is also less sensitive to ASA than is the cyclooxygenase in intact or disrupted platelets.

If placed in fresh culture medium after treatment with 1 mM ASA, endothelium partially regains its capacity to inhibit adherence in 0.5 h (1), and completely recovers from the ASA effect in 2 h (Fig. 3). Thrombin-induced adherence to ASA-treated monolayers decreased from a high of 46.2±5.8% at 0 h to 9.5±3.6% at 2 h. The latter value is not statistically different ($P < 0.50$) from the 10.9±5.0% adherence observed with non-ASA-treated monolayers at zero recovery time (Fig. 3). However, complete recovery of 6-keto-$PGF_{1\alpha}$ release was not observed during the 2-h recovery period. 6-Keto-$PGF_{1\alpha}$ increased from below detectable levels, but only to 46.3±7.0 nM which is approximately 37% of the 124.0±11.7-nM level observed with endothelium without ASA at zero recovery time. This quick recovery from ASA is in agreement with Kelton et al. (12) who reported that the "thrombogenic" effect of high-dose ASA lasted about 2.5 h after its removal from rabbit tissue. In contrast, Gordon and Pearson (13) have reported that pig endothelium treated 24 h with 1 mM ASA only slightly recovered its ability to inhibit platelet aggregation 24 h after removal of the ASA (13). However, pig endothelium treated with 0.1 mM ASA substantially recovered during the same period.

Cycloheximide is a glutaramide antibiotic that inhibits protein synthesis in mammalian cells (16), and recently has been employed in studies with cultured umbilical vein endothelium (17). The ASA effect was maintained if this reagent was included in the culture medium during the 2-h recovery period (Fig. 3), suggesting that protein synthesis may be required for the regeneration of enzymatic activity necessary for PGI_2 production.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Ms. Judith Komlash and Ms. Connie Schroeder.

This work was supported in part by the National Heart, Lung, and Blood Institute through a Specialized Center of Research in Atherosclerosis (grant 14230), and by National Institutes of Health (grants HL-21239 and HL-14890).

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