Biochemical Pharmacology, Vol. 34, No. 12, pp. 2097–2101, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

EFFECTS OF COMMON CAROTID ARTERY DAMAGE ON ANTI-PLATELET-AGGREGABILITY AND PGI_2 PRODUCTION IN RATS

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(*Received* 1 *May* 1984; *accepted* 18 *October* 1984)

Abstract—The effects of vascular damage on platelet function were investigated in rats. Common carotid artery specimens 4 mm in length were dissected from male Wistar rats and exposed to ultrasonic treatment or rubbing of the intimal surface. In some cases, carotis was dissected after ligation for 4 hr. The specimens were fixed on a stirring bar and agitated in platelet-rich plasma added with ADP. After determining platelet aggregability, the plasma was subjected to radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$ (6-K-$PGF_{1\alpha}$). Anti-platelet-aggregability in the arterial specimens was significantly greater in the sonicated, rubbed or ligated specimens than in the intact group, probably due to the effect of prostacyclin (PGI_2). The measurement of 6-K-$PGF_{1\alpha}$ levels yielded simlar results. Endogenous arachidonic acid in the vessel wall seems to be utilized in the production of PGI_2, which probably acts only on local platelets. The findings indicate the presence of a mechanism that inhibits the formation of platelet thrombi by the accelerated production of PGI_2 under conditions favorable for the generation of thrombosis.

There have been many reports that platelet function is enhanced in cerebral infarction [1–4], yet it is still questionable whether hyperfunctioning platelets always play a primary role in the formation of thrombi [5]. Platelet aggregability is known to vary in the acute or chronic stage of cerebral infarction [6], and in chronic infarctions in cortical or penetrating branches [7], whether angiographically detectable or not [8]. These findings suggest that interaction between platelets and cerebral vessels may be also involved in the generation of cerebral infarction.

Moreover, although a number of authors have reported the results of clinical studies of anti-platelet agents including aspirin in transient ischemic attacks (TIA†), cerebral infarction and coronary arterial disease, agreement has not been reached as to their effects. Some investigators claim that further clinical trials without clarification of basic problems as to platelets may be dangerous [9].

From this point of view, we have directed our attention to the relationship between vascular injury and platelet function. Damage in the arterial wall is regarded as an important factor in triggering adhesion and aggregation of platelets and inducing arterial thrombosis and microembolus, which is considered to be a cause of TIA. Baumgartner [10] experimentally demonstrated that the detachment of vascular endothelium and the subsequent contact of platelets with the subendothelial structure cause the formation of platelet thrombi. If vascular injury, especially in the intima, is involved in the process, however, our knowledge of homeostasis suggests the existence of some kind of feed-back mechanism. The discovery and understanding of such a system are essential for effective drug therapy in the prevention of thrombosis. In the present study, the effect of damaged common carotid arteries on plasma platelets which were in the process of aggregation and prostacyclin (PGI_2) production was experimentally examined in rats.

MATERIALS AND METHODS

Sampling and preliminary treatment of common carotid arteries. The common carotid arteries of male Wistar rats weighing 200 g were dissected in sections 4 mm in length, placed in ice-cold physiological saline, and cut along the longitudinal axis to expose the endothelium. Contact with the specimen was kept to a minimum. The exposed vessel wall was pierced with a stirring bar through a hole (1 mm in diameter) in the center, so that the specimen could be rotated in the cuvette of the aggregometer. Some specimens were treated ultrasonically for 10 sec or were rubbed on the intimal surface three times with a toothpick, others were immersed in a 50 μg/ml 15-hydroperoxy arachidonic acid (15-HPAA; Ono-Yakuhin-Kogyo Co. Ltd., Osaka, Japan) solution for 15 min or in a 2 mM ice-cold aspirin (Midorijuji Co. Ltd., Osaka, Japan) solution for 30 min. These agents were dissolved in buffered saline (pH 7.4). In another group of rats, one carotid artery was ligated at two points, 6 mm apart, and 4 hr later the portion between the two ligations was processed as above.

Histological examination. Silver staining was employed to examine the extent of endothelial

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† Abbreviations: TIA, transient ischemic attack; PGI_2, prostacyclin; 15-HPAA, 15-hydroperoxy arachidonic acid; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PAR, platelet aggregation ratio; 6-K-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; and RIA, radioimmunoassay.

damage. The exposed specimens were immediately immersed in 0.5% silver nitrate for 5 min and then were transferred to a large volume of distilled water. The intercellular spaces of the endothelium were stained by exposing the specimens to sunlight. After dehydration the vessel specimens were placed on a glass slide, with the intimal side upward, and were observed under a light microscope. Cross-sections were made in a cryostat and stained with hematoxylin and eosine.

Determination of platelet aggregation. The blood collected from the abdominal aorta of rats was mixed with a 3.8% sodium citrate solution, at a volume ratio of 9:1, and centrifuged at 900 *g* for 7 min. The platelet count was adjusted to 300,000/mm^3 by adding platelet-poor plasma (PPP) to the platelet-rich plasma (PRP) separated by the centrifugation. One and one-half hours after the blood collection and at 40 min after the dissection the specimens were immersed in PRP. After adding ADP to a final concentration of 32 μM, platelet aggregation was measured with a platelet aggregometer (Autoram-31; Rikadenki Co. Ltd., Tokyo, Japan). This is a submaximal dose of ADP which induced 58.1 ± 6.6% (mean ± S.D.) maximum light transmittance in normal PRP. Platelet aggregability was expressed as the platelet aggregation ratio (PAR). PAR was calculated from the maximum light transmittance change induced by 32 μM ADP.

Determination of 6-keto-prostaglandin $F_{1\alpha}$ (6-K-$PGF_{1\alpha}$). Immediately after stirring the PRP in the aggregometer during the determination of aggregability for 5 min, indomethacin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to a final concentration of 0.1 mM. The mixture was centrifuged and stored at −20° until radioimmunoassay (RIA). RIA was performed within 2 months of the preparation of the plasma specimens with a 6-K-$PGF_{1\alpha}$ measurement kit ([^{3}H] RIA Kit; New England Nuclear, Boston, MA, U.S.A.; cross-reactivities of the antibody: 6-K-$PGF_{1\alpha}$, 100%; $PGF_{2\alpha}$, 2.7%; PGE_2, 2%: PGA_1 <0.3%; PGA_2, <0.1%; and thromboxane B_2, <0.1%). Samples were assayed without extraction and separation. Briefly, 100-μl duplicate aliquots of the plasma were incubated at 4° for 16 hr after the addition of the 6-K-$PGF_{1\alpha}$ antibody. We used standard concentrates of 6-K-$PGF_{1\alpha}$ added to 100-μl aliquots of the charcoal-treated plasma to draw up the standard assay curve. It was linear from 10 pg/tube to 500 pg/tube, and the assay limit was 10 pg/tube.

RESULTS

As shown in Fig. 1, nearly 80% of the normal intimal surface was covered by endothelial cells. In the arterial walls exposed to sonication or rubbing, the disapperance of the endothelium was evident by the total lack of the characteristic pattern. However, in the artery subjected to ligation for 4 hr, the endothelial structure was maintained. In cross-sections, the vessels treated by ultrasound showed a swelling throughout the entire wall, and those which were rubbed had irregularities in the intimal surface. No change was detected in the ligated vessels under light microscopy.

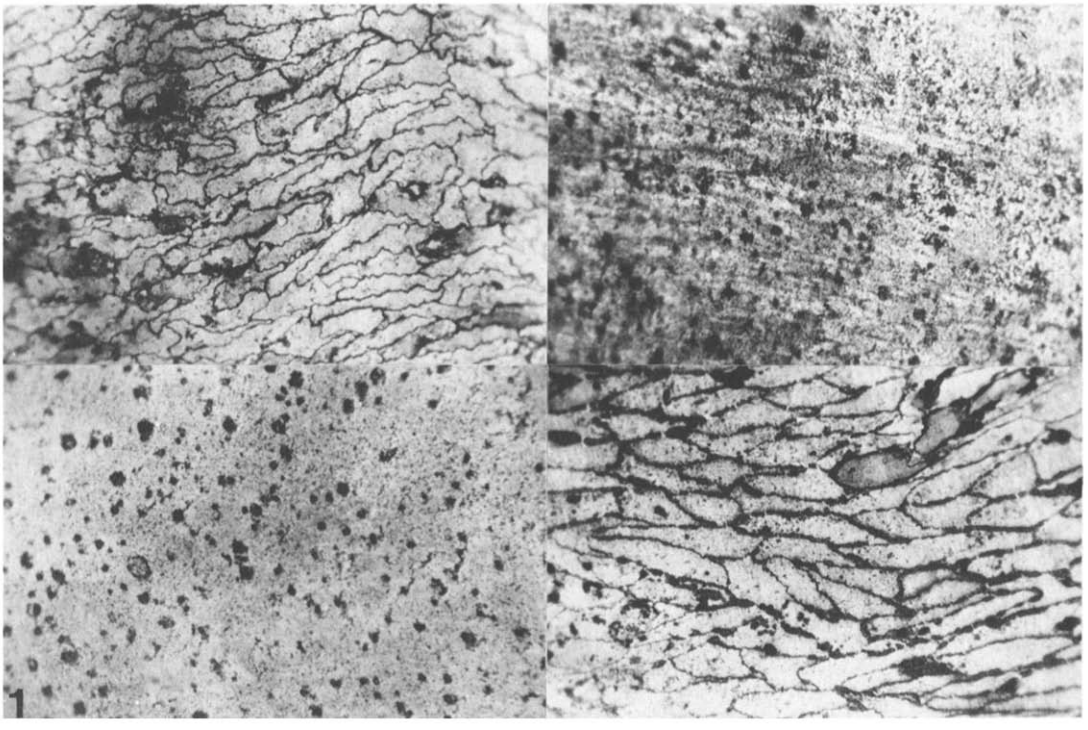

Fig. 1. Endothelial cells stained by silver impregnation (×400). Nearly 80% of the intimal surface of the intact (upper left) and ligated (lower right) common carotid arteries was covered by the endothelium, while the sonicated (upper right) and rubbed (lower left) vessels were completely devoid of endothelial cells.

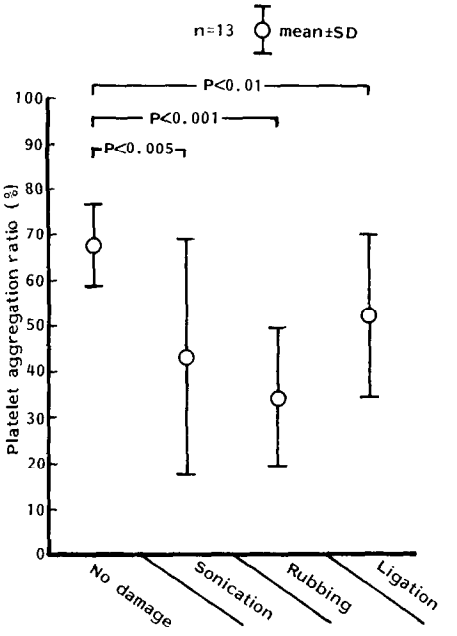

Fig. 2. Platelet aggregation ratios expressed as percentage of the maximal aggregation ratio in the plasma without contact to the vessel specimen (100%). Aggregation was induced by ADP (32 μM).

PAR induced by ADP was 67.7 ± 9.4% (mean ± S.D.) in the presence of undamaged specimens, while it was significantly lower in vessels subjected to sonication, rubbing or ligation, indicating a significant increase in anti-platelet-aggregability (Fig. 2). Although the plasma 6-K-$PGF_{1\alpha}$ levels varied widely in the plasma specimens after the determination of platelet aggregability, they were markedly higher in the plasma incubated with sonicated,

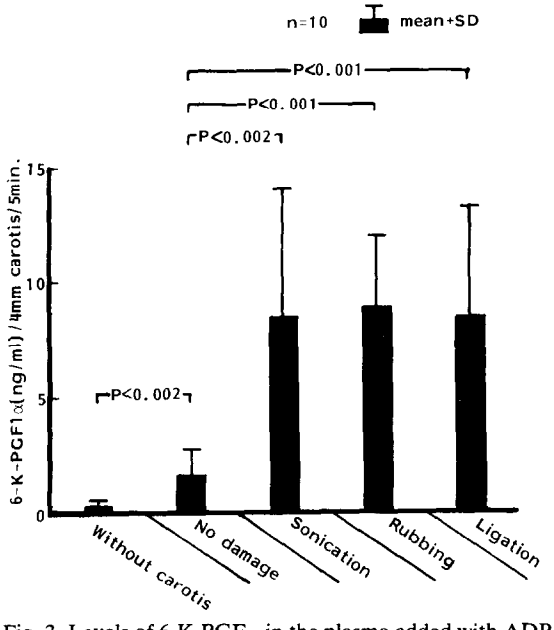

Fig. 3. Levels of 6-K-$PGF_{1\alpha}$ in the plasma added with ADP (32 μM) and incubated with vessel specimens for 5 min.

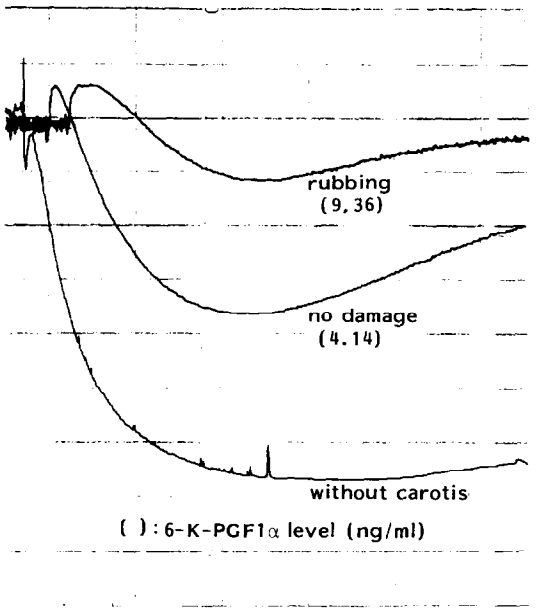

Fig. 4. Typical aggregation curves. Platelet aggregation was induced by ADP (32 μM).

rubbed or ligated vessels than in the plasma incubated with intact vessels (Fig. 3). In individual arteries, anti-platelet-aggregability and 6-K-$PGF_{1\alpha}$ levels were nearly parallel (Fig. 4).

The rubbed carotid arteries pretreated either with 15-HPAA for 15 min or with aspirin for 30 min completely lost anti-platelet-aggregability (four experiments; a representative result is shown in Fig. 5). The levels of 6-K-$PGF_{1\alpha}$ in the plasmas incubated with aspirin-treated vessel were notably lower than with the untreated vessel. By contrast, no such changes were observed in the 6-K-$PGF_{1\alpha}$ levels, when the rubbed arteries were incubated in PPP for 5 min under stirring (Fig. 6).

Finally, PRP prepared from the blood of the rats with ligated common carotid artery was analyzed in order to observe the systemic effect of the cessation of carotid artery blood flow on platelet function. No difference was observed in the maximum platelet aggregation ratio induced by 32 μM ADP between this PRP (58.9 ± 5.3%, mean ± S.D., N = 10) and PRP obtained from normal rats (58.1 ± 6.6%, N = 10).

DISCUSSION

Endothelial cells are considered to play an important role in maintaining the fluidity of blood. Their injury, therefore, seems to be an essential factor in the formation of thrombi. Close histological observations, both *in vitro* and *in vivo*, have been made on the process of platelet adhesion to the subendothelial structure and subsequent platelet aggregation [11, 12]. Moreover, the adhesion and aggregation of platelets were demonstrated to increase with the rate of blood flow, indicating a greater role of endothelial detachment in arterial than venous thrombosis. This endothelial detachment and the subsequent for-

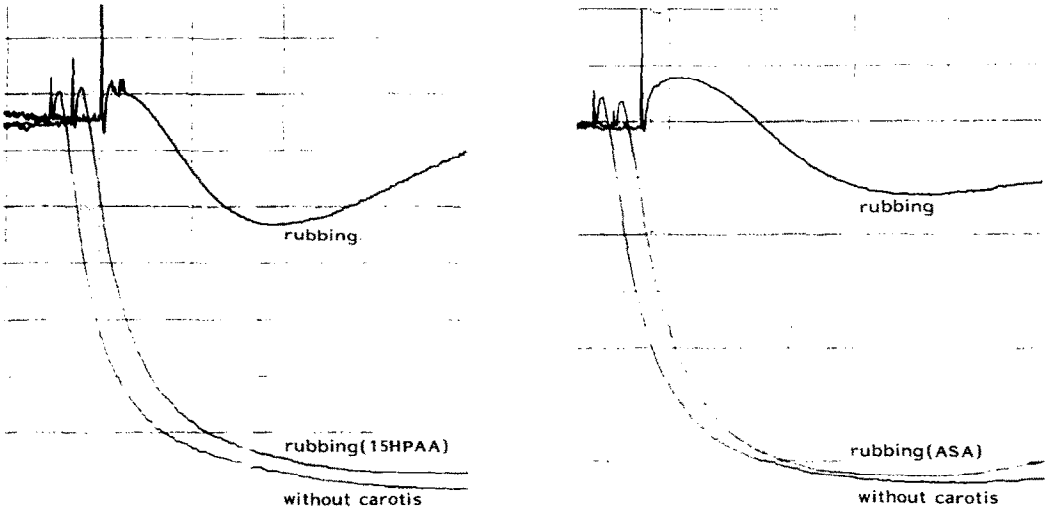

Fig. 5. Anti-platelet-aggregability of the rubbed samples pretreated with 15-HPAA or aspirin (ASA). Platelet aggregation was induced by ADP (32 μM).

mation of platelet thrombi may lead to the growth of the smooth muscle cells of the media, induced by platelet derived growth factor, and, eventually, to atherosclerosis [13]. Thus, negative aspects of the effect of endothelial injury on organisms have been emphasized.

Circulating endothelial cells, first detected in patients with thrombotic diseases by Bourvier *et al.* [14], were also found in healthy subjects by Iwata *et al.* [15]. This suggests the frequent occurrence of endothelial detachment even in individuals free of thrombosis. *In vitro* experiments also revealed that the growth of platelet thrombi at the site of intimal detachment is limited and that they tend to diminish with time [9]. These findings indicate the presence of a protective mechanism against the development of platelet thrombi and the occurrence of arterial thrombosis.

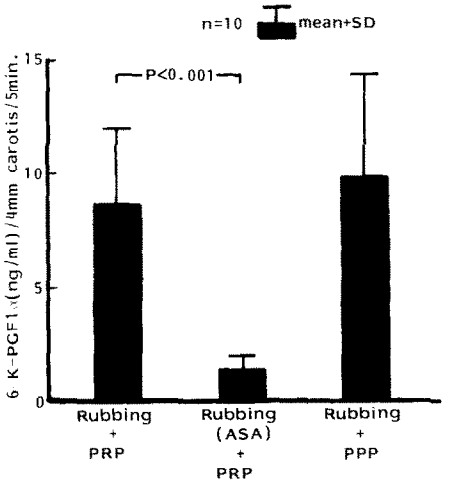

Fig. 6. Production of 6-K-$PGF_{1\alpha}$ and aspirin (ASA) pretreatment. Platelet aggregation was induced by ADP (32 μM).

In our sonicated or rubbed specimens of carotid arteries examined by silver staining, the endothelium was completely destroyed. Despite this damage, anti-platelet-aggregability of the carotid arterial tissue increased along with the production of 6-K-$PGF_{1\alpha}$, a stable metabolite of PGI_2. In addition, anti-aggregability of the vessels completely disappeared in the presence of 15-HPAA, an inhibitor of PGI_2 synthetase. These observations suggest that the inhibition of platelet aggregation may be ascribed to PGI_2. PGI_2 is known to be produced mainly in the intima and the media of blood vessels. Although its productivity per unit tissue weight is notably higher in the endothelium, about 60% of the total production occurs in organs other than the endothelium [16]. It is well known that smooth muscle cells of the media also produce PGI_2 [17, 18]. The distribution of PGI_2 production sites, as well as the results of our experiments, allows us to speculate that PGI_2 production increases in the subendothelium, probably in the smooth muscle cells of the media, upon endothelial destruction or removal.

It is known that PGI_2 production is stimulated by mild vessel injury [19]. Our observation that PGI_2, an inhibitor of platelet function, was increased by the extensive destruction of vascular endothelium is of particular interest, because the destruction of endothelium is an important factor in the formation of platelet thrombi. This observation, and the finding that PGI_2 production in the subendothelial structure after endothelial damage is inversely related to the extent of platelet adhesion and aggregation [20], indicate the physiologic importance of PGI_2 production in the subendothelial tissues.

Diabetes [21], arteriosclerosis [22] and obesity [23] are among the conditions associated with a decreased PGI_2 production in vessel walls, whereas hypoxia has been suggested as a state related to its increase [24]. Carlson and Wennmaln [25] also showed that cessation of blood flow in the upper arm increases a vasodilative metabolite of arachidonic acid, probably PGI_2. In our experiments with ligated vessels, acute

cessation of blood flow decreased platelet aggregability due to increased 6-K-$PGF_{1\alpha}$ (PGI_2) production in the ligated portion. This enhancement of PGI_2 production may have resulted from hypoxia of vessels. While in the sonicated or rubbed vessel walls the elevation of PGI_2 production seemed to take place in vascular components other than the endothelium, it probably occurred in the endothelial cells themselves in the ligated vessels. Our experiments with carotid ligation suggest the possibility that clinical findings such as a temporary depression of platelet function in cerebral infarctions in acute phase [6] or myocardial infarction [26] and arterial recanalisation frequently in cerebral embolism are due to an increase in PGI_2 resulting from acute cessation of blood flow. However, the present findings must be supplemented by further clinical observations.

Under certain conditions, prostaglandin endoperoxides from platelets seem to be utilized as a substrate for PGI_2 produced in vascular tissues [27, 28]. The marked decrease in 6-K-$PGF_{1\alpha}$ (PGI_2) generation in the rubbed vessels treated with aspirin, an inhibitor of cyclo-oxygenase, indicates that the PGI_2 in the tissue of injured carotid arteries was chiefly derived from endogenous arachidonic acid in our experiments. PGI_2 production, therefore, is not affected in vascular disorders by the use of drugs that inhibit only platelet function. This concept may have clinical significance.

Controversy still remains as to whether PGI_2, an extremely unstable and active substance, has only local platelet inhibiting or vasodilating effects or, also, has a role as a circulating systemic hormone [29–31]. Despite the marked increases in PGI_2 production and anti-platelet-aggregability in our vessel specimens 4 hr after ligation, no changes were observed in platelet aggregability in systemic blood. This suggests that PGI_2 only locally inhibited platelet function under the present experimental conditions.

In conclusion, our results indicate that arteries may acquire a potent anti-thrombotic capability by enhanced PGI_2 production when they are exposed to conditions that stimulate the formation of thrombi, such as the removal of the endothelium and acute cessation of blood flow.

Acknowledgement—This work was supported in part by a Research Grant for Cardiovascular Diseases (59 A-4) from the Ministry of Health and Welfare.

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