CHANGES OF PLASMA 6-KETO-PGF₁α AND THROMBOXANE B₂ LEVELS AND PLATELET AGGREGATION AFTER TOURNIQUET ISCHEMIA ON THE UPPER LIMB IN NORMAL SUBJECTS AND PATIENTS WITH ISCHEMIC HEART DISEASE

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To investigate the pathophysiology of ischemic heart disease (IHD), tourniquet ischemia on the upper limb was done and changes in platelet aggregation, plasma 6-keto-PGF₁α concentrations and plasma thromboxane B₂ (TXB₂) concentrations were studied. At rest, platelet aggregability and plasma TXB₂ concentrations were significantly increased in IHD patients compared with those in normal subjects (p < 0.001 and p < 0.001, respectively). In normal subjects, platelet aggregability, plasma 6-keto-PGF₁α concentrations and plasma TXB₂ concentrations rose significantly during ischemia (p < 0.05, p < 0.02 and p < 0.05, respectively). In addition, plasma 6-keto-PGF₁α concentrations were significantly lower in IHD patients than in normal subjects during ischemia (p < 0.005), though there was no significant change in the level of either group at rest.

These results suggest that increase in prostacyclin synthesis in normal subjects during tourniquet ischemia may be a defense mechanism to maintain the balance between prostacyclin and thromboxane A₂ (TXA₂) and to prevent platelet aggregation induced by the procedure. Increase in platelet aggregation and TXA₂ generation in IHD patients at rest indicates a close correlation between IHD and platelet reactivity. Tourniquet ischemia induced a significant increase in prostacyclin generation in normal subjects but not in IHD patients, which suggests that production of prostacyclin was impaired in IHD patients during ischemia. A marked difference was obvious in prostacyclin and TXA₂ generation between IHD patients and normal subjects, and this difference may play an important role in the pathogenesis of IHD.

CLINICAL, pathologic and experimental studies have suggested that platelet aggregation within the myocardial microcirculation may play an important role in the evolution of ischemic heart disease (IHD).¹ ² Spontaneous platelet aggregation has been demonstrated during acute myocardial infarction.³ Exercise induces in vivo formation of circulating platelet aggregates in patients with severe coronary artery disease.⁴ In addition, in animal experiments acute myocardial infarction has been induced by injection of ADP, catecholamines, thromboxane A₂,⁵ ⁷ or by stress reactions induced by cold or heat.⁸ ⁹ Such provocations are generally followed by

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formation of extensive platelet aggregation and acute myocardial infarction. Moreover, it is suggested that platelets can also be involved in coronary artery spasm, as in Prinzmetal’s angina, because coronary vascular tone may be modulated by the vasoactive prostaglandin precursors (endoperoxides and thromboxanes) released by platelets.

Platelet reactivity may be affected by prostacyclin (PGI$_2$) and thromboxane A$_2$ (TXA$_2$). Prostacyclin and TXA$_2$ are the main products of the cyclooxygenase pathways of arachidonic acids in endothelial cells and platelets, respectively. Prostacyclin is a powerful vasodilator and an inhibitor of platelet aggregation. On the other hand, TXA$_2$ is a strong vasoconstrictor and a stimulator of platelet aggregation. Thus, a balance between prostacyclin and TXA$_2$ may have a critical role in the homeostasis of the circulation not only under the physiological condition but also in the pathophysiology of IHD.

The present study was undertaken to investigate the changes of platelet aggregation, plasma prostacyclin concentrations and plasma TXA$_2$ concentrations in normal subjects and IHD patients, at rest and during tourniquet ischemia on the forearm, as a means to determine the physiological changes in healthy subjects and the pathophysiologic changes in IHD.

SUBJECTS AND METHODS

Subjects

Thirty-four normal subjects (27 males and 7 females) and 67 IHD patients (45 males and 22 females) were studied. The IHD patients included 57 cases of angina pectoris and 10 with old myocardial infarction. Patients with acute myocardial infarction were excluded. The ages were 29–69 with a mean of 42 years in normal subjects and 30–69 with a mean of 53 years in IHD patients. Diagnostic criteria of angina pectoris included clinical history (anginal pain, relieved by nitroglycerin), ST-T changes on electrocardiography (at rest and exercise test), and in some of these patients with coronary arteriographic findings. Diagnosis of old myocardial infarction was based on clinical history, abnormal Q waves on electrocardiogram, and in some with coronary arteriographic findings. Subjects with a history of diabetes mellitus, lipid abnormalities, manifest atherosclerotic vascular disease, kidney or heart disease, hypertension and thyroid dysfunction were excluded from the control group in this study.

Medications (e.g., aspirin, sulfanaphylazon, diphenidamole and non-steroid antiinflammatory drugs) which may interfere with platelet reactivity and synthesis of prostaglandins were discontinued for at least 10 days prior to this study.

Collection of Blood Samples

Each subject was asked to lie comfortably on a bed for 10 min, and a 19 gauge needle was inserted into an antecubital vein, without the use of a tourniquet. To avoid the effects of pain and nervous tension induced by needle insertion, physiologic saline solution was infused slowly into the vein for 10 min. Blood was withdrawn after 10 min of infusion.

Subsequently, each subject was kept on a bed and a tourniquet with the pressure of 20 mmHg above systolic blood pressure was used on the other arm for 3 min. With this procedure, the skin became cyanotic and the pulse on the hand was impalpable. Following 3 min complete stasis of blood flow on the forearm, a 19 gauge needle was inserted into an antecubital vein and blood was withdrawn.

Platelet Aggregation

Platelet aggregation was studied according to optical density with Born’s method using an aggregometer and a flat-bed recorder (Rikadenki). Blood (4.5 ml) was added to a silicon-treated tube containing 0.5 ml of 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained by immediate centrifugation of the citrated blood at 110 x g for 5 min at room temperature. Platelet-poor plasma (PPP) was prepared by centrifuging PRP at 1000 x g for 10 min at room temperature. The aggregating agent was adenosin diphosphate (ADP, Sigma Chemical Co.) with a final concentration of 2 µM. The aggregation was measured for the maximum change in light transmission (measured in chart units) divided by the span of the original upper and lower limits set with PPP and PRP. All samples were tested within 3 hours of collection.

Measurement of Plasma 6-Keto-PGF$_1$α and TXB$_2$

Prostacyclin and TXA$_2$ are unstable in circulation: the half-life span is 2–3 min and 30 sec, respectively. Plasma levels of 6-keto-PGF$_1$α and TXB$_2$, the stable catabolites of prostacyclin and TXA$_2$, respectively, were determined. Blood was collected in cooled silicon-treated tubes. Aspirin with the final concentrations of 2 x 10$^{-5}$

PGI₂, TXA₂ and Platelet Aggregation in IHD

Fig. 1. Platelet aggregation, plasma 6-keto-PGF₁α concentrations and plasma TXB₂ concentrations in 67 IHD patients and 34 normal subjects at rest. *** = p < 0.001; NS = not significant.

Platelet aggregation of rabbits in vitro was assayed at 37°C for one hour. Following the addition of 0.1 ml dextran-coated charcoal (50 mg dextran, 500 mg NORIT A in 10 ml of isogel Tris buffer) to each tube, the tubes were centrifuged at 2500 rpm at 4°C for 5 min. Then the supernatant was decanted into a scintillation counting vial. Five ml of ACSII solution (Amersham Co.) was added to each vial and counted in a Beckman scintillation counter (Model LS 7500).

Antisera of 6-keto-PGF₁α used in this study had a cross-reaction of 0.55% with TXB₂, 11.2% with PGE₁, 12.2% with PGE₂, 5.2% with PGF₁α and 9.5% with PGF₂α. Cross-reactions of TXB₂ antisera were 0.037% with 6-keto-PGF₁α, 0.054% with PGE₁, 0.054% with PGE₂, 0.054% with PGF₁α and 0.087% with PGF₂α. The recoveries of various amounts of 6-keto-PGF₁α and TXB₂ added to plasma were 89% and 91%, respectively. The overall sensitivity of the procedure may be calculated to be around 10 pg/ml.

The values of platelet aggregation, plasma 6-keto-PGF₁α concentrations and plasma TXB₂ concentrations were compared between normal subjects and IHD patients using Student’s t-test. A paired comparison t-test was used to compare these values obtained from the rest and tourniquet ischemia in the same group. P-value of less than 0.05 was considered significant.

RESULTS

Platelet Aggregation, Plasma 6-keto-PGF₁α Concentrations and Plasma TXB₂ Concentrations in IHD Patients and Normal Subjects at Rest

As shown in Fig. 1, platelet aggregations induced by ADP 2 μM were 58 ± 3% in IHD patients and 33 ± 3% in normal subjects (mean ± SEM). IHD patients had a significantly higher level of platelet aggregation than normal subjects (p < 0.001). Plasma TXB₂ concentrations were 238 ± 12 pg/ml in IHD patients and 188 ± 13 pg/ml in normal subjects (mean ± SEM). IHD patients had a significantly higher level of plasma TXB₂ concentrations than normal subjects (p < 0.001). Plasma 6-keto-PGF₁α concentrations were 122 ± 9 pg/ml in IHD patients and 146 ± 11 pg/ml in normal subjects (mean ± SEM). There was no significant change in plasma 6-keto-PGF₁α
concentrations between the two groups.

**Changes in Normal Subjects during Tourniquet Ischemia**

As shown in Fig. 2, tourniquet ischemia on the forearm for 3 min in 34 normal subjects induced increases of platelet aggregation from $33 \pm 3$ to $38 \pm 4\%$, of plasma 6-keto-PGF$_1\alpha$ concentrations from $146 \pm 11$ to $176 \pm 13$ pg/ml and of plasma TXB$_2$ concentrations from $188 \pm 13$ to $260 \pm 30$ pg/ml (mean $\pm$ SEM). As compared to the values obtained at rest, tourniquet ischemia on the forearm for 3 min induced significant increases in platelet aggregation, plasma 6-keto-PGF$_1\alpha$ concentrations and plasma TXB$_2$ concentrations in normal subjects ($p < 0.05$, $p < 0.02$ and $p < 0.05$, respectively).

**Changes in IHD Patients during Tourniquet Ischemia**

In IHD patients, the values obtained during tourniquet ischemia were $59 \pm 3\%$ in platelet aggregation, $116 \pm 12$ pg/ml in plasma 6-keto-PGF$_1\alpha$ concentrations and $307 \pm 24$ pg/ml in plasma TXB$_2$ concentrations (mean $\pm$ SEM) (Fig. 2). No significant change in platelet aggregation, plasma 6-keto-PGF$_1\alpha$ concentrations or plasma TXB$_2$ concentrations were observed in IHD patients during tourniquet ischemia when compared with the values obtained at rest. These results differed from those in normal subjects, in whom tourniquet ischemia on the forearm induced a significant increase in platelet aggregation and plasma concentrations of 6-keto-PGF$_1\alpha$ and TXB$_2$. Therefore, during tourniquet ischemia, plasma 6-keto-PGF$_1\alpha$ concentrations were significantly lower in IHD patients than in normal subjects ($p < 0.005$).

**DISCUSSION**

Many techniques are currently available to evaluate platelet reactivity in vitro, including measurement of platelet aggregation, platelet adhesion, platelet survival and platelet-specific proteins. Among these methods, the measurement of platelet aggregation is one of the simplest and least time-consuming tests to indicate the increased tendency of platelets to form aggregates. However, platelet aggregation may be influenced by a number of factors including the age and sex of patients, physical activity, mental stress, cigarette smoking and a wide range of drugs. Hence, the present procedure, as stated above, was to take blood samples in order to keep variables to a minimum.

In this study, ADP $2 \mu$M was used as the platelet aggregating agent, which was suggested as a suitable concentration by Mishina.$^{19}$ In our previous experiments, when the concentrations of ADP were lower than $2 \mu$M, both the platelet aggregation and the ADP concentrations showed a significantly correlative increment; while for ADP concentrations upper than $2 \mu$M, no significant change in platelet aggregation was observed. With a $2 \mu$M ADP, a significant change in platelet aggregation was induced in normal subjects against the values obtained at rest and during tourniquet ischemia. It also induced a significant increase in platelet aggregation in IHD patients compared with normal subjects. These results indicate that as low as $2 \mu$M of ADP may be suitable for the platelet aggregation test in IHD.

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patients.

The present findings showed that plasma TXB₂ concentrations were significantly increased in IHD patients compared with normal subjects (Fig. 1). In addition, the platelets from patients with IHD were significantly more sensitive to the aggregating effect of ADP than those from normal subjects. TXA₂ is produced by platelets and TXA₂ can induce platelet aggregation. Therefore, the increase in platelet aggregation and the plasma TXB₂ concentrations in IHD patients indicated a close correlation between platelet reactivity and IHD. It is accepted that the atheroma formed in the damaged vessels causes a turbulent flow in the irregular narrow vessels and traumatizes the platelets sufficient to render them unduly sensitive to in vitro aggregating agents. Moreover, Gazes²⁰ suggested that the abnormal platelet activity may be due to plasma factors released from the damaged myocardium or vessel walls such as catecholamines. Nagazawa²¹ indicated that the acquired platelet abnormalities resulted from the effect of antiplatelet antibodies causing a release phenomenon. These hypotheses help account for the fact that platelet aggregability and plasma TXB₂ concentrations were increased in IHD patients in the present study.

In normal subjects, tourniquet ischemia on the forearm for 3 min induced an increase not only in platelet aggregation and plasma TXB₂ concentrations but also in plasma 6-keto-PGF₁α concentrations. Although the antisera of 6-keto-PGF₁α had approximately a 12% cross-reaction with other prostaglandins, the 6-keto-PGF₁α values were considered indicators of prostacyclin. Increased platelet aggregation during tourniquet ischemia may have resulted from stasis of blood flow, change in blood pH, catecholamine activity, TXA₂ activity and other biological changes. Increased TXA₂ generation in plasma may have correlation with the increased platelet aggregation. In normal subjects, increased prostacyclin production during tourniquet ischemia is most likely a defense mechanism, as reported by Neri Serneri²². This mechanism may maintain the balance between prostacyclin and TXA₂ activities in normal subjects during ischemic attack and may prevent formation of platelet emboli in healthy subjects during transient ischemia. However, in this study, platelet aggregation was tested at 1-3 hours after blood sampling. By that time, the platelets in the samples had undergone their release reaction in circulation and the prostacyclin and TXA₂ had lost their biological activities in the blood samples. Hence with tourniquet ischemia, the normal subjects showed an increase in platelet aggregation in vitro in spite of a balance between prostacyclin and TXA₂.

On the other hand, tourniquet ischemia induced no significant change in platelet aggregation, plasma 6-keto-PGF₁α concentrations and plasma TXB₂ concentrations in IHD patients. These results directly contradict those observed in normal subjects. O'Brien²³ reported that platelets in vivo may become activated and undergo a release process in disease, and then the partially exhausted platelets continue to circulate. Thus, the platelets in the circulation of IHD patients may will have been partially emptied, so the tourniquet ischemia induced only a slight but not significant increase in the TXA₂ generation. For the same reason, tourniquet ischemia induced no significant change in platelet aggregation in IHD patients.

It is generally recognized that prostacyclin generation by arteries is suppressed in atherosclerosis²⁴,²⁵. Using tourniquet ischemia for 60 min on the hind legs of a pig, Zahavi²⁶ found that the blood vessels distal to the tourniquet were deendothelialised and prostacyclin release from the damaged veins was decreased. It is unlikely that reduced release of prostacyclin in IHD patients during tourniquet ischemia might have resulted in vascular injury from this procedure, since the duration of ischemia in the IHD patients and normal subjects was similar, while prostacyclin generation increased in normal subjects. This indicates that the vessels distal to the tourniquet were not injured by 3 min tourniquet ischemia. Thus, the decreased prostacyclin production in IHD patients suggests that vessels in the hands of IHD patients may be abnormal. On the other hand, IHD patients may have a subclinical atherosclerotic change in vessels other than the coronary arteries. The impaired prostacyclin generation in the atherosclerotic vessels suggests exhaustion of arachidonic acids and/or decrease in prostacyclin synthetic enzymes (cyclooxygenase and/or prostacyclin synthetase) in the endothelial cells.

Atherosclerosis is considered as the main cause of IHD. It may involve not only the coronary arteries but also the arteries in many other organs. Experimental coronary ligation in man is impossible, although it can be done in animals. Therefore, tourniquet ischemia is done on the forearm instead of ligation of the coronary arteries in order to investigate the changes in
platelet aggregability and plasma levels of prostacyclin and TXA$_2$ during ischemic attack. The results in this study accord with those of Sakai in our laboratory. He found that ligation of coronary arteries in dogs induced a significant increase in plasma 6-keto-PGF$_1\alpha$ and TXB$_2$ concentrations. It suggests that a tourniquet test may be a useful method to study the pathophysiology of IHD in man. The present results suggest that an increase in platelet aggregability and TXA$_2$ generation as well as a decrease in prostacyclin production have a close correlation with IHD.

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