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Exercise-Induced Platelet Shape Change and Its Suppression by Trapidil in Normal Subjects

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Summary. **Most results on platelet activation during exercise in normal subjects and patients have recently been criticized as artificial activation *ex vivo*. The present study examined the shape changes of circulating platelets together with some other parameters in healthy subjects during a bicycle ergometer exercise. Striking shape changes revealed were spherification (decrease in the discoidness index) and pseudopod formation (increase in population of platelets with pseudopods). These changes in platelet shape persisted for 30 min after the exercise and after haemodynamic parameters returned to the pre-exercise level. The changes in the discoidness index and pseudopod formation during the exercise were found to be identical with our previous findings on platelets activated *in vitro* by ADP or thrombin. Trapidil (600 mg daily for 2 weeks per os), a modulator of platelets activity and vascular functions, significantly suppressed the shape change, suggesting a possible clinical benefit.**

INTRODUCTION

Effects of exercise on hemostatic parameters have been studied by many investigators because of frequent clinical events occurring during and after exercise in patients with vascular, especially thrombotic disorders. Increases in platelet count[1] with[2] or without[3] an increase in mean platelet volume, aggregability,[1] and plasma levels of alpha-granule contents,[4-9] and other findings suggesting platelet activation,[10-15] have been reported; some signs of activation[16,17] in both coagulation and fibrinolysis have also been recorded. However, several investigators have claimed that most of such findings of platelet activation could be artifacts during collection and sampling of the blood.[1,9,18] Some[7,19,20] have re-examined the suggestions by previous investigators[4-6,8] that enhancement of such activation might be related to cardiac ischemia. However, it has become likely that only an increase in platelet count is a consistent finding after exercise.[18] The platelet shape, known to change rapidly and easily, thus seems a sensitive parameter for possible activation during exercise. Douste-Blazy and colleagues[4] have reported an increase in activated forms of platelets after exercise in myocardial infarction survivors though they found no increase in those forms in healthy subjects.

In this study, with a special care to avoid *ex vivo* activation and using a quantification method of platelet shapes, occurrence of a significant platelet shape change could be proved during a bicycle ergometer exercise in normal healthy subjects. Furthermore, it was suggested that trapidil, a platelet function suppressant and test vasodilator,[21-28] was effective in suppressing such platelet activation.

MATERIALS AND METHODS

Subjects: Twenty-two healthy male volunteers (age 24-44) who have had no signs or symptoms of circulatory or thrombotic disorders, and had no drugs for two weeks before and during the experiment.

Experimental protocol: The studies progressed in three steps: In the first pilot study, 12 volunteers (age 24-40) were used to test the possibility of platelet shape change during exercise, using a bicycle ergometer method with increasing 3 min loads of 50, 75, 100 and 125 watts (W). Platelet shape and, as a parameter for

hemodynamics, the triple product to be described below were examined. In the second step of detecting possible platelet activation another five subjects (age 24-40) were examined while lying on a bed. Blood samples were taken with the procedures to be noted below, and the platelet shape, plasma β-thromboglobulin (β-TG) and thromboxane (Tx) B_2 levels were examined. In the third study on the effect of trapidil, the shape, β-TG, aggregability and retention rate (adhesion and aggregation onto glass beads) were examined in 12 subjects (five new ones and five from the first study; age 24-44) riding on a bicycle for exercise test before and during drug administration.

Blood sampling: an 18G siliconized butterfly needle was inserted into a thick cubital vein and maintained at the position during the tests. The time and procedure of blood sampling differed among the study group. In the first two groups, blood was sampled before and at the end of each loading, except 100 W, and 30 min after the exercise. In the third group, blood was sampled before the exercise, at the end of 125 W, and 30 min after the exercise. Normal saline, without heparin, was continuously infused through the needle at approximately 1 ml/min throughout the experiment. Before blood sampling, at least 5 ml of saline was rapidly infused and, under a light tourniquet, at least 3 ml of blood, from the orifice of a three-way stopcock placed between the needle and the infusion, was allowed to flow out and was discarded in order to avoid possible *in vivo* or *ex vivo* platelet activation caused by the retained needle or the infusion system. Subsequent 0.1-0.3 ml of blood was then directly poured and fixed in the fixative (see below) in the first pilot study. In the second and the third study group at least 3 ml of blood was drawn into a syringe and discarded and then 7 and 10 ml of blood, respectively, were drawn into another syringe and were used for various measurements.

Drug administration: Trapidil (Rocornal^R) (Mochida, Japan) 600 mg—200 mg three times—daily was given orally to the subjects for 2 weeks. The exercise experiment was performed before and on the final day of the administration (2-3 h after ingestion).

Shape analysis: Blood (0.1-0.3 ml) was dropped (directly fixed) in 10 ml of 1% glutaraldehyde containing one tenth volume of 3.8% sodium citrate in a polycarbonate container as reported previously.[29,30] Platelet shape analysis was performed according to our method[29,30] using a light microscope (Olympus, Japan, $\times$1000, with oil immersion) by an experienced investigator who did not know the protocol of the samples. The results were expressed by frequency (%) of each type of platelets, i.e., disc, spheroid or hemisphere, sphere, bipolar, other irregular shapes as well as forms with pseudopods, (%Ps). A discoidness index is represented by: (disc−sphere)/(disc+spheroid+sphere) for expressing how many platelets are discoid. The special types, bipolar and other, usually make up less than 10% of the total.[31] The reproducibility of this evaluation is satisfactory; i.e., in

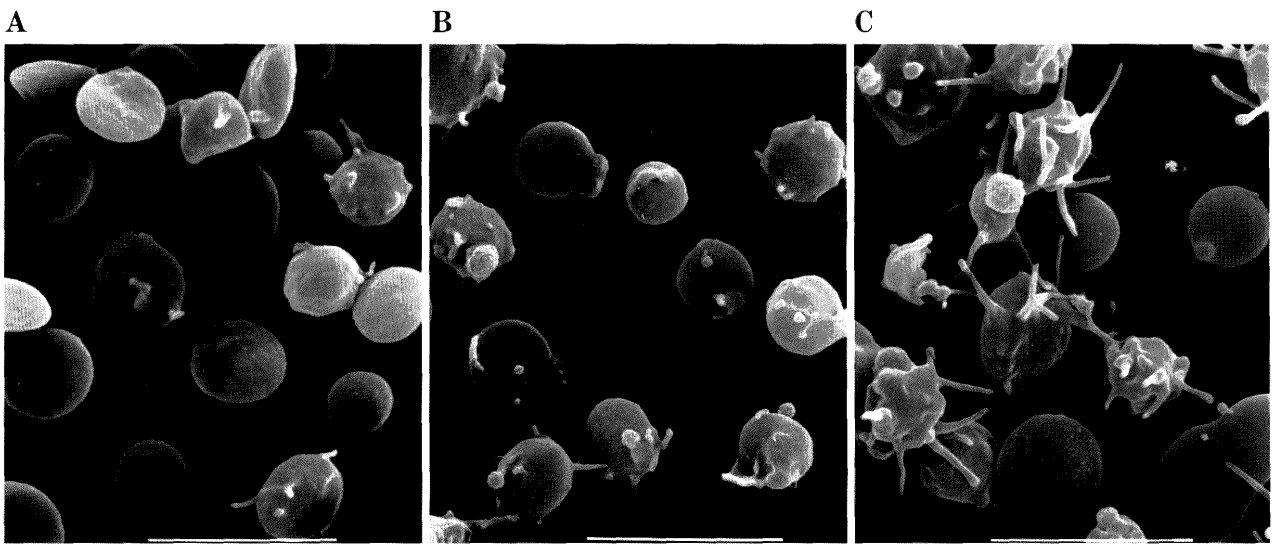

Fig. 1. SEM images of resting and activated platelets in relation to shape parameters. A: Resting platelets with few pseudopods. In this sample the discoidness index is calculated 0.87 and % pseudopod is 21.3%. B-C: Slight or moderately activated platelets. The indices and %pseudopod are 0.19 and 52.5%(B), and -0.16 and 64.7%(C) respectively. White bars represent 1 μm.

ten analyses of one sample SD does not exceed 0.1 for the discoidness index, and 5% for each shape type. Fig. 1 illustrates a correlation of these parameters to scanning electron microscopic images.

Plasma β-TG, PF-4 and Tx B_2: blood in the second syringe was immediately poured into cooled tubes which contained EDTA, theophylline and/or indomethacin for preparation for radioimmunoassay (RIA Kits for β-TG from Amersham, England, those for PF-4 from Amersham, England, and those for Tx B_2 from New England Nuclear, USA), and the plasma levels were measured.

The triple product: Changes in hemodynamics were expressed by a conventional parameter in our laboratory, the triple product which is pulse rate (/min)$\times$ pulse pressure (mmHg)$\times$ejection fraction (sec) on carotid pulse tracing.[32]

Platelet function: aggregability (the maximum aggregation rate; MAR) was examined using citrated platelet rich plasma (platelet count 30×10^9/L) in a Niko Hematracer 1 aggregometer. Inducers used were ADP (at final concentration 1 and 10 μM) and arachidonate (AA) (2 mM). Retention rate (adhesiveness and aggregability of the platelets in native venous blood onto a glass bead surface in a column) was measured by Hellem II method.[33]

Statistical analysis was carried out by using paired t-test or Wilcoxon's test.

RESULTS

Effect of exercise on the hemodynamics and the platelet shape

As shown in Fig. 2, the triple product increased as the exercise load increased up to 125 W, in spite of a considerable variation among the subjects, and it returned to the pre-exercise level within 30 min after the exercise. The platelet shape changed significantly (Fig. 2), namely the discoidness index decreased from 0.7 ± 0.11 (mean$\pm$SD) to 0.19 ± 0.40, and, in a mirror image, %Ps increased from 29.2 ± 10.1% to $48.5\pm$ 16.6%. This means that many discoid platelets transformed into spheres or spheroids and issued pseudopods. A few platelets even assumed a typical activated form, spiny sphere. There was also significant variation in both parameters among the subjects, but no apparent correlation could be found between the shape parameters and the triple products in each subject. The bipolar or other irregular forms were

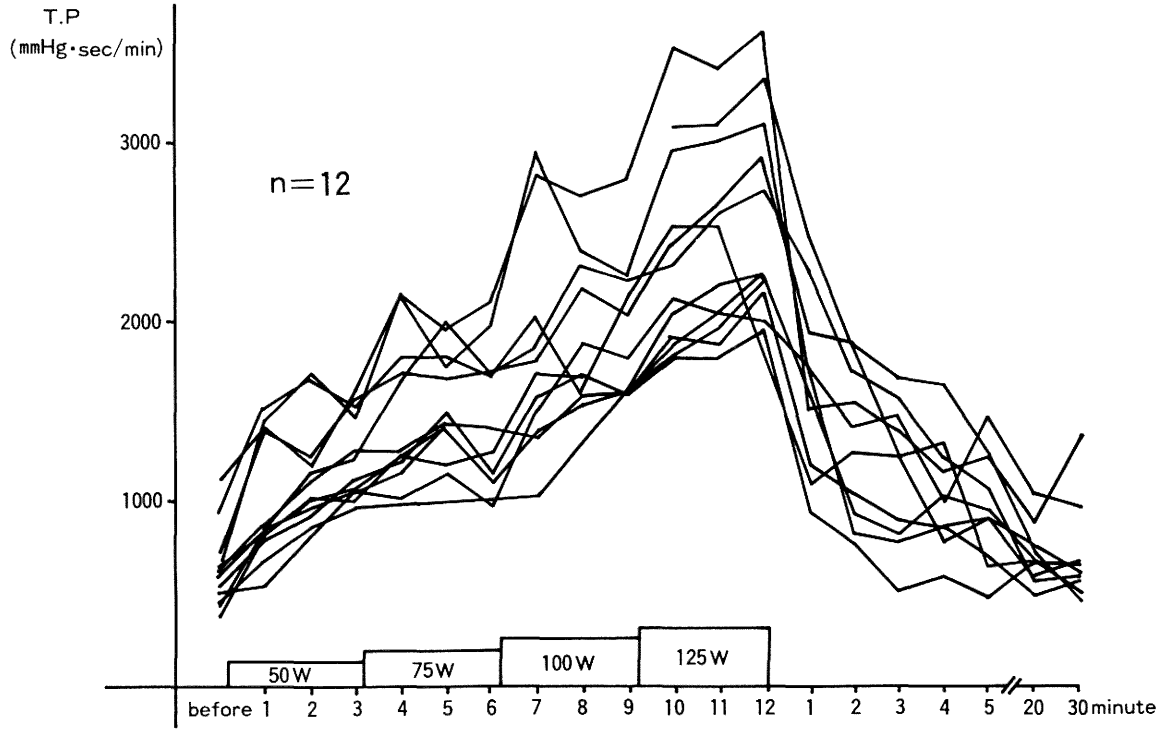

Fig. 2. Triple product (TP) during and after a bicycle ergometer exercise in normal subjects in the pilot (first) study. TP increased with the exercise loading, but returned to the rest levels within 30 min after the exercise.

not increased. In striking contrast to the triple product, the platelet shape change persisted until even 30 min after the exercise (Fig. 3).

When the correlation between the discoidness index and %Ps was examined (Fig. 4A) the plots for the former on the Y axis and those for the latter on the X axis lay in a narrow oblique zone between the starting point of ideally resting platelets i.e., the discoidness index 1 and %Ps 0%, and the point of

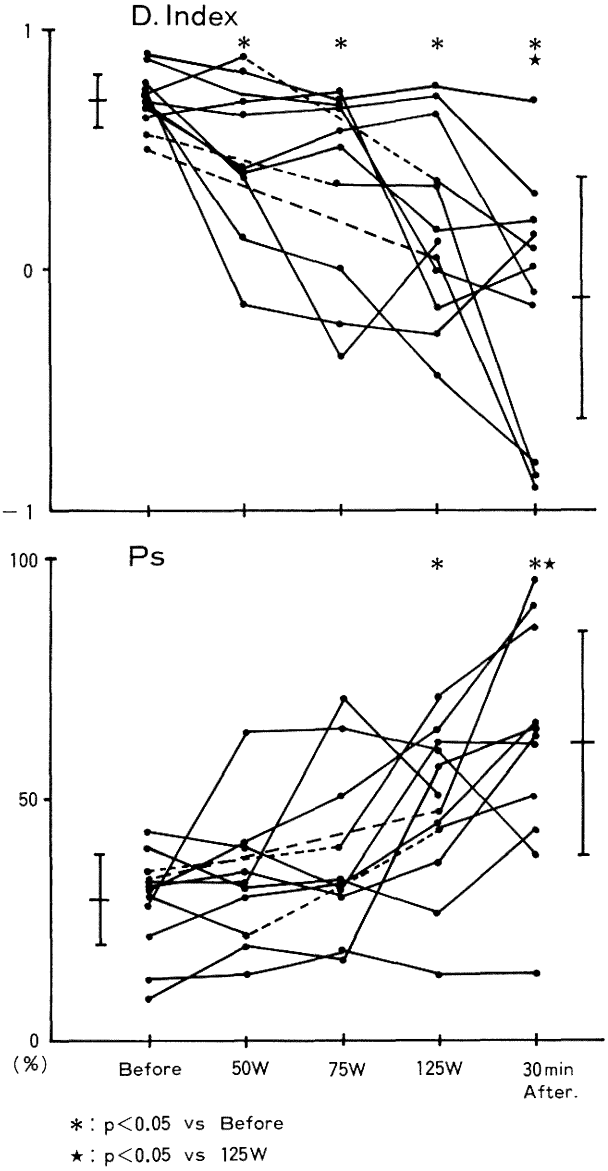

Fig. 3. Changes in the platelet shape as indicated by Discoidness (D) index and Ps (% platelets with pseudopods) during and after exercise. Both parameters changed with increasing loads, and the changes persisted 30 min after the exercise in contrast with TP.

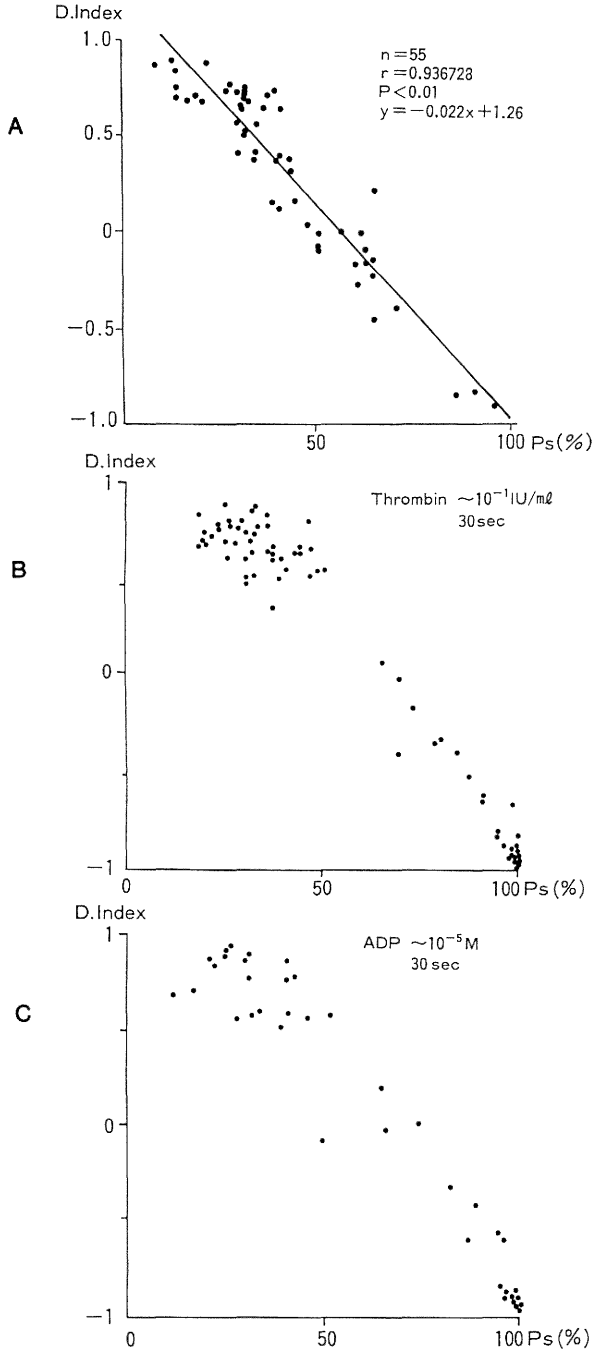

Fig. 4. Relationships between platelet parameters (D index and Ps) in various conditions. A: Exercise (at rest and during and after exercise in the pilot study). Note a narrow oblique zone of the points represented by the values of the parameters. B: *In vitro* activation of normal human platelets by thrombin ($0\sim10^{-1}$ IU/ml, 30 sec at 37° C). C: *In vitro* activation by ADP ($0\sim10^{-5}$ M, for 30 sec at 37°C). The graphs B and C were newly produced from the original data in our previous paper.[31] Note the similar relationship between the two parameters in A, B and C.

maximal activation, i.e., the discoidness index −1 and %Ps 100%. This close relationship between two parameters is quite similar to that in normal human platelets activated *in vitro* by ADP or thrombin (Fig. 4B, C: these have been recently drawn from original data in our previous paper).[31]

The effect of blood collecting and sampling methods on platelet shape and other parameters (Table 1)

In the second group of the exercise-free subjects (Table 1), the pre-exercise discoidness index was lower and the pre-exercise %Ps higher, than the corresponding values (p less than 0.05) in the first study group. This indicates that platelet activation, although moderate, occurred during the two syringe method of sampling, which was not performed in the first study. Both discoidness index and %Ps remained at almost the pre-exercise levels throughout the observation period, and the results on b-TG and TxB_2 showed a similar tendency. Thus, the retention of the needle and the two-syringe method in the second study caused statistically significant, though not prominent, activation, the extent of which did not change throughout the test.

Effect of trapidil

In the control experiment of the third study group (Table 2), both the shape parameters (discoidness index and %Ps) showed changes at the end of exercise and 30 min later they were close to those found in the first study. The discoidness index decreased from 0.51±0.13 to −0.47±0.41, and %Ps increased

Table 1. Platelet shape parameters and other parameters in normal subjects during saline infusion without exercise in the second study

Time (min)*	0	3	6	12	42
D. Index	0.29±0.20**	0.25±0.20	0.29±0.22	0.28±0.22	0.27±0.19
Ps (%)	49.7±8.3	49.7±10.4	51.4±9.0	48.5±12.7	50.3±11.2
β-TG (pg/ml)	55±22	42±14	56±11	86±52	74±37#
TxA_2 (pg/ml)	41±15	48±27	46±31	58±28	39±13

*: time 0, 3, 6, 12 and 42 min correspond to before exercise, 50 W 75 W, 125W, and 30 min after exercise, respectively in Fig 1 and 2. **: values are mean ±SD (n=5). #: $p<0.05$ vs 0 min value. D. index: discoidness index. Ps: platelets with pseudopods. β-TG: β thromboglobulin. TxB_2: thromboxane B_2. The shape parameters did not significantly change during this study, suggesting those changes in the first study (Fig. 1) were the effect of exercise. However, decrease in D. index and increase in Ps at rest in this study, in comparison with those in the first study, indicate that platelet activation was induced somewhat by the blood collecting procedures in the second study.

Table 2. The results of the third study on the effect of trapidil

		Before Ex	Maximum Ex	After EX
D. index	C†	0.51±0.13††	−0.13±0.48	−0.47±0.41
	T†	0.51±0.15	0.06±0.40*	0.06±0.21*
Ps(%)	C	34.8±12.1	59.0±25.0	72.4±17.9
	T	40.8±8.6	52.6±18.2	46.1±12.5**
β-TG	C	43±12	62±18	72±28
(ng/ml)	C T	55±19	57±25	62±39

†C: control group, T: trapidil group. ††Values are mean±SD (n=12).
*$p<0.05$, **$p<0.01$ vs control group. Ex: exercise. See Fig. 1 for other abbreviations. Changes in the platelet shape parameters were significantly suppressed by trapidil, whereas plasma β-TG was unchanged.

Table 3. Platelet function during exercise without or with trapidil ingestion

	Control			Trapidil		
	At Rest	Max Ex	After Ex	At Rest	Max Ex	After Ex
Retention (%)	65.01 ±10.64	60.15±18.79	58.90±17.10	73.29± 6.71	72.54±15.05	71.64± 9.38
ADP (1 μM)	9.00*± 5.17	8.75±31.19	11.00±41.00	13.22±11.93	15.22±10.34	16.44±15.37
ADP (10 μM)	57.63*±11.97	54.25±21.99	59.14±15.53	58.11±16.09	62.56±16.99	59.11±17.80
A.A. (2 mM)	23.63*±19.74	38.25±30.19	34.00±33.42	37.00±28.20	43.22±26.46	39.78±29.69

No significant changes were observed in values between the control and trapidil groups. Retention (%): retention rate examined by Hellem II method. ADP, AA: agonists and the final concentrations. For details see the description. Ex: exercise. At Rest, Max. Ex, After Ex correspond to before exercise, at 125 W exercise, and 30 min after exercise, respectively, in Fig 1. *: Maximum aggregation rate (%). Values are mean ±SD (n=12).

from 34.8±12.1% to 72.4±17.9%. These changes were significantly suppressed after ingestion of trapidil. In contrast, the increase in plasma b-TG levels was not significantly affected by the drug. Aggregability (as expressed by MAR) induced by ADP or arachidonate, and platelet retention were not also affected during and after exercise (Table 3).

DISCUSSION

The shape change is one of the earliest responses in platelets to various stimuli including aggregating agents. Our previous *in vitro* study, the present semi-quantitative analysis,[29] has shown that the shape change is induced by ADP at more than 10^{-8} M, thrombin at over 10^{-2} IU/ml, and by arachidonate at over 1.25×10^{-4} M, and that it is reversible after weak activation, but irreversible after stronger stimulation; it is not caused by adrenalin.[34,35] The result of shape analysis depends considerably on the method of blood drawing or bleeding and the procedures before fixation.[30] This suggests a possible activation of the platelets *ex vivo*. In the present study, it was shown that the activation may partly be ascribed to the retention of the needle, as reported previously,[30] and partly to the two-syringe method.

Douste-Blazy et al.,[4] who used, for single venipunctures, a slightly thinner (19G) needle than that used in our study, reported that the platelet shape change after exercise occurred in patients, but not in normal subjects. The present study first demonstrates that a marked platelet shape change occurs during exercise in normal subjects. This change is so striking and equivocal that the effects of *ex vivo* treatment of blood can be omitted from consideration. Furthermore, the platelet change persisted after exercise, indicating a kind of platelet activation duration during this stage. The discrepancy between our results and those of Douste-Blazy et al.[4] may be ascribed to one or more of the differences in blood sampling techniques, shape change classification, but more likely to the kind of exercise, our maximum load being heavier than theirs.

The mechanisms of platelet activation during exercise are not clearly understood, although perturbed blood flow (increased shear rate),[36,37] cardiac ischemia, pathological vessels[6-8] in myocardium or increased blood levels of catecholamines have been speculated to be responsible. Among these, adrenalin, may be responsible for the platelet shape change since adrenalin, not noradrenalin, can activate platelets. However, the effect of adrenalin must be indirect, probably through ADP release or thromboxane formation or others, because adrenalin itself could not directly cause the shape change.[34,35]

A close relationship was found between the discoidness index and %Ps in this study. That essentially the same relationship was found *in vitro* activation of platelet by ADP or thrombin, however, does not necessarily indicate that the platelet deformation in this study was caused by these agonists, since platelets were found to transform similarly also in responses to arachidonate, STA_2 (a thromboxane analog), and A23187.[39] Interestingly, we have found that such a relationship between the two shape parameters was not observed in disseminated intravascular coagulation (DIC) in which platelets are strongly activated. In DIC, many platelets were swollen but had no, or rather few, pseudopods.[9] The discrepancy indicates a difference in the mechanism causing platelet shape change in exercise and DIC.

The shape change of platelets was suppressed considerably by trapidil. In addition to the effects on the circulatory system[25,27,40-42] trapidil causes a direct inhibition of platelet aggregation,[23,24]

spreading[23,24] and production of thromboxane A_2[26] as well as an increasing production of prostacyclin by stimulating synthetase.[22] It also inhibits *in vitro* thrombus formation in Chandler tubes[21] and in arteries.[43] Our previous study[22] *in vitro* showed that trapidil inhibited the shape change induced by 10 μM ADP or 2 mM arachidonate, although the precise mechanism has remained obscure.

Some attempts have been made previously to suppress exercise-induced platelet and/or coagulation activation: diltiazem hydrochloride[16] was found to have no effect, but beta-adrenoreceptor blockade suppressed several platelet or coagulation parameters.[5,11,44] The clinical implications of the latter result remain obscure.

In this study, trapidil suppressed the shape change of platelets but not the elevation of β-TG. This discrepancy is not precisely understood. Our preliminary data[45] show that β-TG and ATP releases occur at 2-4 times the concentrations of ADP which cause, a minimum shape change. Therefore, a speculation may be made that β-TG might be released by the relatively few platelets which were strongly activated at some sites of vessels or tubes *in vitro*, whereas the majority of the platelets might not release even if activated to transform. Another possibility is that the shape change may be ascribed to an increased fraction (not measured in this study) of the circulating platelets which have been speculated to be larger forms released from the spleen.[46,47] This possibility seems, however, to be unlikely because of the characteristic relationship between the discoidness index and pseudopod formation, as found in the acute activation of the platelets by agonists (Fig. 3B, C). In any event, the present study indicates the necessity of re-evaluation of the results of elevated plasma levels of alpha granule contents in many clinical conditions.

In conclusion, the platelet shape is considered to be a sensitive parameter for exercise-induced platelet activation, under the condition that the methods of blood drawing or collection, and quantitative analysis are carefully conducted. Trapidil may have some benefit in suppressing platelet activation during and after exercise.

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