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Effects of Calcium Channel Blockers and Insulin on the Platelet Function in Patients with Diabetes Mellitus*

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Platelet aggregability was compared between platelets isolated from normal subjects and patients with diabetes mellitus in order to evaluate the effects of calcium channel blockers and insulin on the platelet function. The threshold aggregating concentration of adenosine diphosphate (ADP), which induces the second phase aggregation and reflects the platelet release reaction, was found to be significantly lower in diabetics than in normal subjects (1.8 μM vs 7.5 μM). It was observed that the second phase aggregation curve induced by ADP was inhibited by in vitro treatment of platelets with insulin (10-100 μU/ml), verapamil (1-10 μM), and diltiazem (1 μM) in diabetics. The result also shows that the inhibition was enhanced when insulin and calcium channel blockers were used together for in vitro treatment of diabetic platelets. Thus, the present study suggests that the use of calcium channel blockers combined with insulin would be more effective than the use of insulin alone in the prevention of diabetic vascular disease.

Key Words: Platelet hyperaggregability, threshold aggregating concentration, verapamil, diltiazem, insulin, diabetes mellitus.

Platelets obtained from diabetic patients show enhanced aggregation when stimulated by a variety of aggregating agents such as adenosine 5'-diphosphate (ADP), collagen or epinephrine (Sagel *et al.* 1975; Colwell *et al.* 1978) and the platelet aggregability is reported to be even further enhanced for patients with diabetic retinopathy, nephropathy and neuropathy (Heath *et al.* 1971; Bensoussan *et al.* 1975; O'Malley *et al.* 1975; Colwell *et al.* 1979, 1981). Platelet hyperaggregability of diabetic patients could be due to the alteration in the metabolism of arachidonic acid in platelets suggested by such observations as those of increased activity of phospholipase (Gerrard *et al.* 1980) as well as increased synthesis of prostaglandin E and thromboxane A2 in diabetes (Smith *et al.* 1973; Halushka *et al.* 1977, 1981; Takeda *et al.* 1981). Thromboxane A2 is a product of arachidonic acid metabolism in platelets (Hamberg *et al* 1975) and mobilizes Ca^{+2} from various storage sites which then inhibit adenylate cyclase and/or induce serotonin- and ADP-release from platelets, causing irreversible ADP-induced platelet aggregation (Gorman, 1979). Platelet aggregation can be clearly separated into first and second phase aggregation by certain concentrations of ADP, and the second phase aggregation can be increased by endogenous ADP which reflects the platelet release reactions (Sixma, 1972). The second phase platelet aggregation (release reaction) is inhibited by aspirin or other non-steroidal anti-inflammatory agents by blocking the activity of prostaglandin synthetase (Sagel *et al.* 1975; Smith and Willis, 1975).

There have been reports (Preston *et al.* 1978; Juhan *et al.* 1982) that insulin causes the reduction of whole blood aggregability and beta-thromboglobulin levels. Beta-thromboglobulin is a platelet specific protein which is liberated from platelets during the release reaction and, therefore, its level reflects in vivo platelet aggregations. On the other hand, platelet response to aggregating agents was also reported to be increased by in vitro insulin treatment (Hilsted *et al.* 1980; Janka *et al.* 1981).

The divalent cation, calcium, is also known to be involved in platelet release reaction, which is supported by an experiment demonstrating that a divalent cation ionophore, A23187, which increases the

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cytoplasmic level of calcium ions in platelets, triggers the release reaction and induces aggregation (Massini and Luscher, 1974; White *et al.* 1974). Calcium channel blockers, such as verapamil (α-isopropyl-α[N-methyl-N-homoveratry-γ-aminopropyl]-3,4-dimethoxyphenyl acetonitrile) and diltiazem (cis-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one) are being widely used clinically as coronary vasodilators and they are negatively inotropic, by failing to permit transmembrane calcium influx in coronary muscle (Braunwald, 1982). Recently, it has been suggested that calcium channel blockers inhibit some platelet functions (Mehta *et al.* 1983; Onoda *et al.* 1984).

The present study was, therefore, undertaken in order to investigate and clarify the effect of calcium channel blockers (verapamil and diltiazem) and insulin on platelet aggregation in patients with diabetes.

SUBJECTS AND METHODS

Diabetic patients (9) admitted to Severance Hospital at the Yonsei University College of Medicine and apparently healthy control subjects (3) were available for this study. The control subjects had no history of diabetes mellitus or any other diseases that might alter platelet aggregation. Neither controls nor diabetics had taken aspirin, any other antiplatelet agents, or other medications, including anti-inflammatory agents, for at least two weeks prior to the study. Blood was collected from subjects 4 hrs after a meal, in vacuum tubes, using 3.8% sodium citrate as an anticoagulant.

Platelet-rich plasma (PRP) was prepared by immediately centrifuging the blood at 160×g for 7 min at 22°C. The supernatant PRP was carefully removed with a Pasteur pipette. Continued centrifugation at 2,500×g for 20 min yielded a clear platelet-poor plasma (PPP). The platelet count of PRP was adjusted to $3\text{-}4\times10^5/mm^3$ with PPP.

Platelet aggregation was measured using a Lumi-aggrometer (Chrono-Log Corp., Pa., USA) by the method of Born (1962). A threshold aggregating concentration (TAC) of ADP was taken as the minimum amount of ADP required to produce the biphasic response, and the effect of drugs on the second phase aggregation was observed as the change of optical density caused by the drugs during the aggregation. Platelet aggregation was induced and measured by adding a predetermined threshold concentration of ADP after treating PRP with distilled water, insulin, verapamil, diltiazem, insulin with verapamil or insulin with diltiazem by stirring (1,000 rpm) at 37°C for 1 min. All measurements were made within 3 hrs from the time of blood withdrawal.

RESULTS

The threshold aggregating concentration (TAC) of ADP is the lowest concentration of ADP required to produce the biphasic aggregation curve (Fig. 1). The TAC of ADP was 0.5-2.5μM (average: 1.8 μM) for 9 diabetic patients which was significantly less than that of controls (2.5-15 μM, average: 7.5 μM) measured from 3 normal subjects (Table 1).

The effect of insulin treatment in vitro on the platelet aggregation was tested. The addition of insulin to the final concentration of 10-100 μU/ml caused a reduction of the second phase aggregation induced by ADP in 3 diabetic patients (Fig. 4-1, Table 1), whereas no change was observed for 2 normal subjects and one with diabetes. The reduction of the second phase aggregation induced by ADP was more apparent by the calcium channel blockers, and verapamil (1-10 μM) and diltiazem (1 μM) showed the effect in 7 out of 8 diabetics and 8 of 9 diabetics,

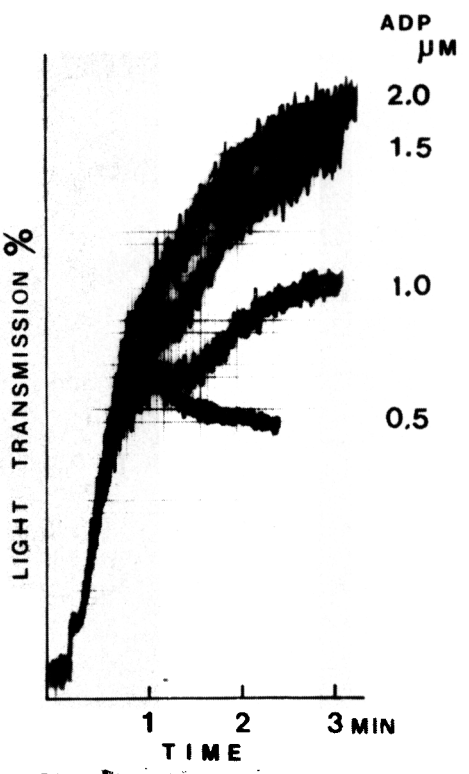

Fig. 1. Representative aggregation curves for determining the threshold-aggregating concentration (TAC) of ADP in diabetic platelets.

Table 1. Effects of insulin and calcium channel blockers on the second-phase aggregation curve induced by ADP

	AGE/SEX	TAC (μM) of ADP	Inhibitors (Concentrations)				
			I (μU/ml)	V (μM)	D (μM)	I (μM)+V (μM)	I (μM)+D(μM)
Control	24/M	2.5		1*,10*			
	52/F	5.0	10,100		1,10		10+10
	21/F	15.0	10,100	10,100*	10,100*		10+100**
Diabetes	52/F	0.5	10,100		1, 10		10+10*
mellitus	59/F	1.0		1,10,100*	1*		
	70/M	1.5	10*,100*	1*,10*	1*	10+1**,10+10**	10+1**
	54/F	1.5	10, 100*	1*	1*	10+1**	10+1**
	54/M	2.0		1 ,10*	1*		
	51/M	2.0		1*,10*	1*		
	17/F	2.0		1*	1*		
	64/M	2.0		1*	1*		
	53/M	2.5	10*	10*	1*,10*	10+10*	10+**

I, insulin; V, verapamil; D, diltiazem

* The second phase aggregation curve was inhibited.

**The second phase aggregation curve was further inhibited.

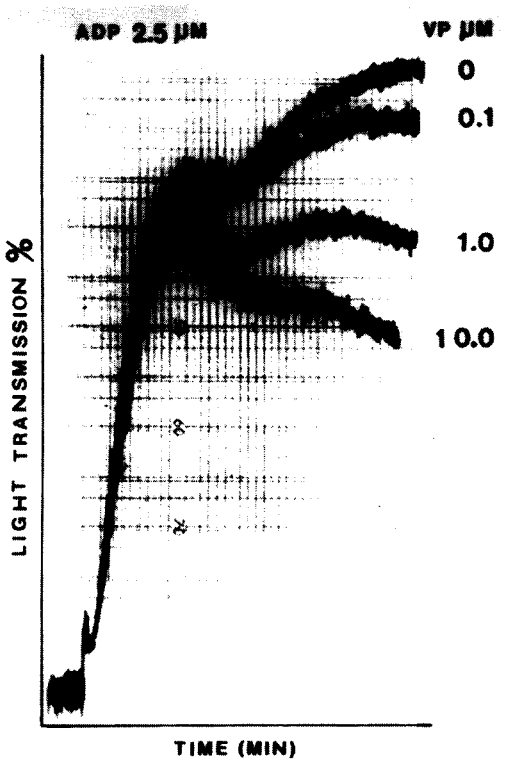

Fig. 2. *Effect of verapamil at various concentrations on normal platelet aggregation in vitro. PRP was incubated with verapamil for 1 min and subjected to platelet aggregation induced by TAC (2.5 μM) of ADP. VP, verapamil.*

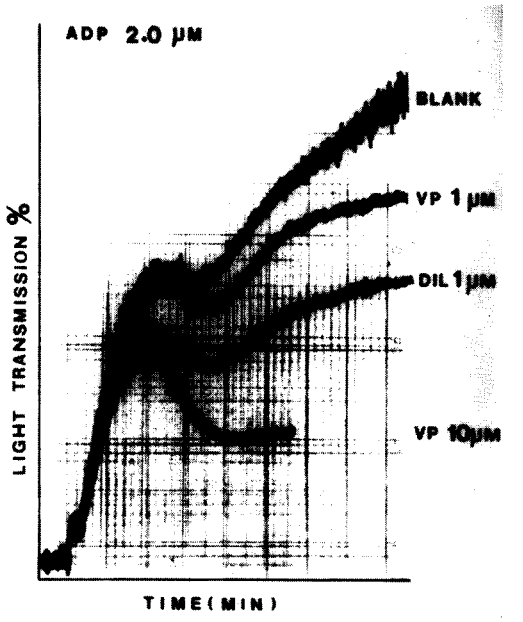

Fig. 3. *Effects of verapamil and diltiazem on diabetic platelet aggregation induced by TAC (2.0 μM) of ADP in vitro. VP, verapamil; DIL, diltiazem.*

respectively (Table 1). There was one case of diabetes which, while it showed no change as a result of the treatment with 10 μM of verapamil, did show a reduction in the second phase aggregation when the con-

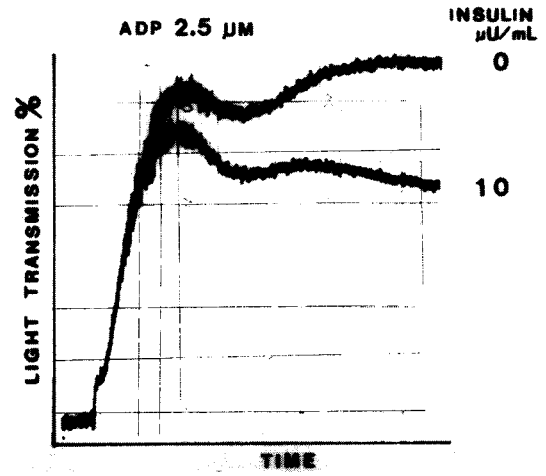

Fig. 4-1. Effects of insulin on diabetic platelet aggregation induced by TAC (2.5 μM) of ADP.

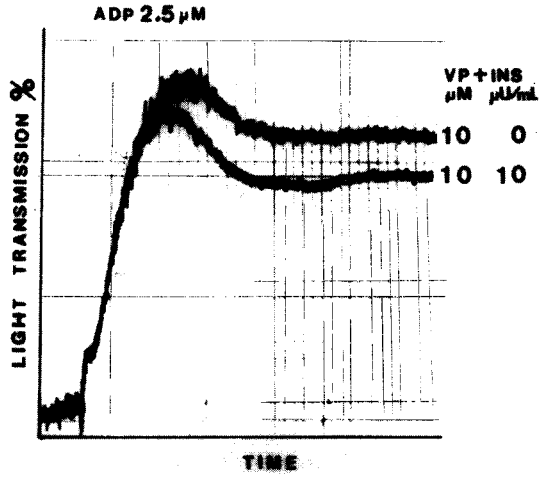

Fig. 4-3. Synergism of insulin and verapamil on diabetic platelet aggregation induced by TAC (2.5 μM) of ADP. VP, verapamil; INS, insulin.

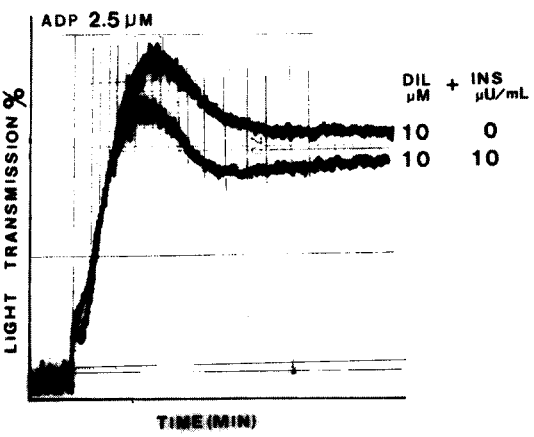

Fig. 4-2. Synergism of insulin and diltiazem on diabetic platelet aggregation induced by TAC (2.5 μM) of ADP. DIL, diltiazem; INS, insulin.

centration was increased to 100 μM. In addition, we observed a similar reduction in one of three normal subjects. The results (Fig. 2,3) also showed that the degree of reduction in the second phase platelet aggregation was increased in linear relationship with the concentration of verapamil, and the reduction was observed for the first phase aggregation as well as the second phase aggregation at 1 μM and 10 μM of verapamil.

Insulin also caused the reduction of both first and second phase aggregation at 10 μU/ml (Fig. 4-1). Moreover, the combination of 10 μU/ml of insulin with 10 μM of verapamil or diltiazem was found to reduce further both the first and second phase aggregation compared to the individual treatment (Fig. 4-1,2,3 and Table 1).

DISCUSSION

Both macrovascular and microvascular complications are major causes of morbidity and mortality resulting from diabetes mellitus (Colwell *et al.* 1979; Ganda, 1980). Hyperactive platelets are believed to be involved in the etiology of diabetic atherosclerotic complications (Ganda, 1980) as well as microangiopathy and microthromboses (Waitzman, 1979).

ADP is a platelet aggregating agent which induces platelet aggregation followed by the release reaction. Platelet aggregation can be clearly separated into the first and second phases by certain concentrations of ADP, and the second phase aggregation reflecting the release reaction causes irreversible aggregation (Sixma, 1972). In the present study, the second phase aggregation curve was induced by a considerably lower TAC of ADP in diabetics compared to that in normal subjects. The result is consistent with observations of other investigators in which it has been seen that platelets from diabetic patients are hypersensitive to ADP, a situation that might contribute to the development of diabetic angiopathy (Sagel *et al.* 1975; Col-

well *et al.* 1978).

The effect of in vitro insulin pretreatment on the platelet aggregation in diabetics was an inhibition of the second phase aggregation curve induced by TAC of ADP. This result agrees with the reports (Preston *et al.* 1978; Juhan *et al.* 1982) showing that insulin lowered the level of beta-thromboglobulin and the whole blood aggregability which reflects in vivo platelet aggregation and release reaction. Moreover, it has also been found in diabetics that phospholipase A2, the rate-limiting enzyme in the synthesis of prostaglandins and thromboxanes, returns to normal activity with insulin treatment (Gerrard *et al.*1980). On the other hand, the present result contradicts that of other studies (Hilsted *et al.* 1980; Janka *et al.* 1981) which have reported the increased platelet aggregability by in vitro insulin treatment for aggregating agents, including ADP. In view of the association between insulin and atherosclerosis, considerable controversy exists regarding the role of insulin, and evidences for both the protecting and promoting influences of insulin in the development of atherosclerosis have appeared in both clinical and experimental situations. Therefore, further studies are necessary to clarify the true effects of insulin on platelet function and diabetic angiopathic complications.

Mehta *et al.* (1983) have examined the effects of verapamil on platelet function. They used 0.5 μg/ml (1.11 μM) of verapamil, which is similar to the concentration we used, and reported that this concentration of verapamil inhibited the platelet aggregation induced by threshold amounts of ADP. Their results also showed a dose-dependent inhibition of aggregation by verapamil at a higher concentration of ADP, but verapamil had no effect on the first phase curve induced by TAC of ADP. In the present study, we observed a similar inhibitory effect on aggregation by all the calcium channel blockers used; diltiazem having a stronger inhibitory effect on aggregation than verapamil, results which are similar to the results of Onoda *et al.* (1984).

When both calcium channel blockers and insulin were used together, the first and second phase aggregation curves were more inhibited than when only one or the other was used. When they were used separately, the inhibition of the first phase aggregation curve appeared to be caused mainly by insulin.

Therapy based on the concept of pharmacologic inhibition of platelet hyperaggregability has been proposed (Genton *et al.* 1975). While some studies have shown benefits resulting from the use of anti-platelet agents in some forms of macrovascular disease, such data are not yet available for the diabetic population. Since ADP is capable of inducing only aggregation-dependent secretion (Charo *et al.* 1977), and in the present study the combination of calcium channel blockers and insulin inhibited the diabetic platelet aggregation induced by ADP in a synergistic manner, further investigations on clinical basis are needed in order to establish an effective therapy for the prevention and treatment of diabetic angiopathic complications.

CONCLUSIONS

1. The threshold aggregating concentrations of ADP inducing the second phase aggregation curve which reflects the platelet release reaction were determined as 0.5-2.5 μM (average: 1.8 μM) for 9 diabetic patients and 2.5-15 μM (average: 7.5 μM) for 3 normal subjects.

2. The effect of in vitro insulin treatment on the platelet aggregation was examined, and insulin caused the inhibition of the second phase aggregation curve induced by TAC of ADP at concentrations of 10-100 μU/ml in 2 of 3 diabetic patients.

3. In vitro treatment with the calcium channel blockers, verapamil (1-10 μM) and diltiazem (1 μM) caused the inhibition of the second phase aggregation curve in 7 of 8 diabetics and 8 of 9 diabetics, respectively.

4. At TAC of ADP, the combined treatment of insulin 10 μU/ml and verapamil 10 μM or diltiazem 10 μM caused a further inhibition of both the first and second phase aggregation curves, compared to treatment with either one or the other.

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