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Original Article

Anti-Platelet Action of Gliclazide, an Oral Hypoglycemic Agent, and its Possible Mechanism

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Abstract: Hyperaggregability of platelets observed in diabetic patients has been speculated as one of the causes of chronic diabetic complications. Gliclazide, an oral hypoglycemic agent, inhibited the platelet aggregation, ATP release, the phosphorylation of 40 K dalton protein and a thrombin-induced increase of intraplatelet calcium *in vitro*. Although a high concentration of gliclazide (500 μg/ml) was required to inhibit the increase of intraplatelet calcium induced by 0.1 U/ml thrombin, the inhibitory effect of gliclazide on platelet aggregation induced by 0.5 μg/ml collagen, was observed at a concentration as low as 1 μg/ml.

The results of our experiments demonstrated that gliclazide directly inhibits the platelet aggregation at a concentration attainable by the usual oral dose of gliclazide. Therefore, it is likely that gliclazide has a favorable action on diabetic complications, not only by reducing blood glucose level, but also by reducing platelet hyperaggregability.

Key words: Gliclazide, Oral hypoglycemic agent, Anti-platelet action, Diabetic complications

INTRODUCTION

Recently, increased polyol pathway metabolism and non-enzymatic glycation of various tissue proteins have been implicated in the pathogenesis of chronic diabetic complications[1)]. On the other hand, hyperaggregability of platelets in diabetic patients has also been speculated as a cause of chronic diabetic complications[1)]. So, it seems likely that normalization of hyperaggregability of platelets in diabetic patients may be beneficial for the prevention or treatment of chronic diabetic complications. Gliclazide, an oral hypoglycemic agent, was reported to inhibit the platelet aggregation, not only *in vivo*, but also *in vitro* at a very high concentration[2)]. But it is not clear whether the drug has any direct effect on the platelet aggregation at the usual oral dose. Therefore, we conducted experiments to elucidate whether gliclazide directly inhibits platelet aggregation *in vitro* at a therapeutic concentration and also to clarify its mechanism.

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MATERIALS AND METHODS

Materials

Gliclazide was a generous gift from Dainippon Pharmaceutical Co., Ltd. Aequorin, collagen and Chrono-lume were purchased from Chrono-log Japan. Thrombin was purchased from Mochida Pharmaceutical Co., Ltd. Prostaglandin E_1 (PGE_1) was a generous gift from Ono Pharmaceutical Co., Ltd. Sodium dodecyl sulfate (SDS), acrylamide and bisacrylamide were purchased from Bio Rad Co., Ltd.

X-ray films (X-Omat) were purchased from Kodak. ^{32}P-Orthophosphate was purchased from Amersham Japan.

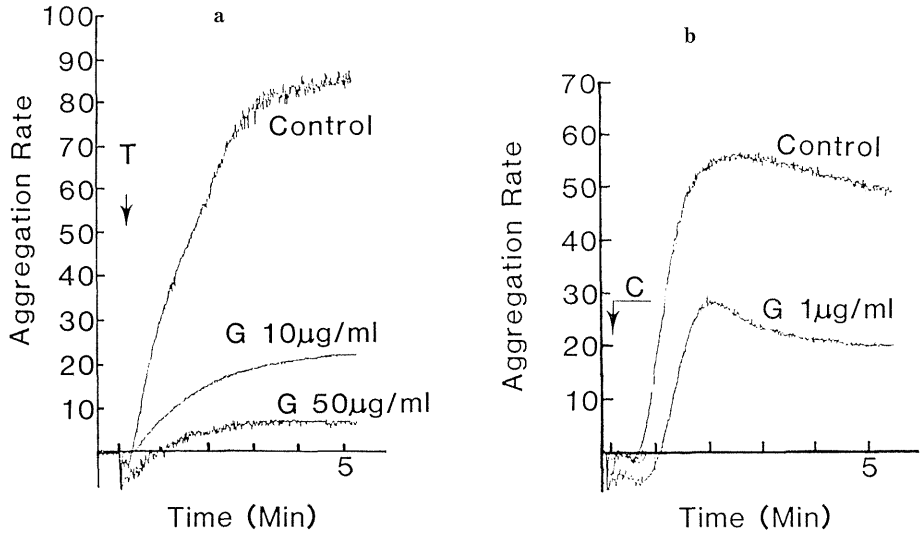

Fig. 1. The effects of gliclazide (G) on platelet aggregation rate.
Platelets were stimulated either by thrombin (a) or collagen (b) at the time indicated by the arrows. The concentrations of thrombin (T) and collagen (C) were 0.01 U/ml and 0.5 μg/ml, respectively.

Methods

1) Preparation of washed platelets

Washed platelets were prepared according to the method described by Ware *et al.*[3]. Blood was obtained from healthy volunteers in the presence of 1/10 volume of 3.8% sodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation (150 ×*g*, 15 min) at room temperature. PGE_1 was added to a final concentration of 1 μM, and followed by centrifugation (400×*g*, 15 min) at room temperature. Precipitated platelets were suspended in Hepes-Tyrodes buffer (NaCl 129 mM, $NaHCO_3$ 8.9 mM, KH_2PO_4 0.8 mM, $MgCl_2$ 0.8 mM, glucose 5.6 mM, Hepes 10 mM, pH 7.4) containing 1 μM of PGE_1, and then centrifuged again (400×*g*, 15 min) at room temperature. Precipitated platelets were then resuspended in Hepes-Tyrodes buffer, and gel filtrated at room temperature through Sepharose 2B which had been equilibrated with the same buffer.

The number of platelets was adjusted to $4\times10^5/mm^3$ with the same buffer, and then diluted by the addition of equal amounts of Hepes-Tyrodes buffer containing 0.2 mM $CaCl_2$ and various concentrations of gliclazide which was dissolved in distilled water. The mixture was preincubated for 10 min at room temperature before the initiation of experiments.

2) Measurements of platelet aggregation

The platelet aggregation was determined by Hematracer (NBS-Japan).

3) Phosphorylation of platelet proteins

Ten mililiters of PRP were incubated with 1 mCi of ^{32}P-orthophosphate at 37°C for 90 min[4]. Then, ^{32}P-labelled washed platelets were obtained as described in the article 1). After 10 min of pre-exposure to gliclazide, the assay was started by the addition of thrombin or collagen, and terminated by the addition of an equal amounts of SDS containing buffer[5]. The samples were then boiled for 3 min and used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of

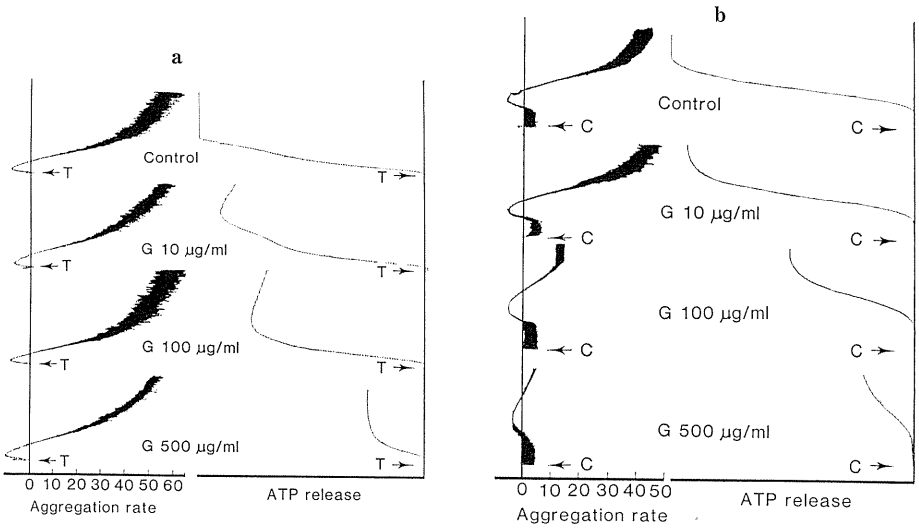

Fig. 2. The effects of gliclazide (G) on ATP release.
Platelets were stimulated either by thrombin (a) or collagen (b) at the time indicated by the arrows. The left half indicates platelet aggregation rate and the right half indicates ATP release. Relative ATP release was compared by the height of Chrono-lume signal. The concentrations of thrombin (T) and collagen (C) were 0.02 U/ml and 2 μg/ml, respectively.

Laemmli[5]. The gel was stained, destained and dried, and followed by autoradiography.

4) Platelet ATP release

Platelet ATP release was detected by Lumi-aggregometer (AHS, Japan) in the presence of Chrono-lume.

5) Measurements of intraplatelet calcium concentration

Aequorin was loaded into platelets according to the method of Johnson *et al.*[6], and intracellular calcium was measured by Lumi-aggregometer (AHS, Japan). Intracellular calcium concentrations were calculated according to the specification attached to aequorin.

Results

As shown in Fig. 1, platelet aggregation induced either by 0.01 U/ml thrombin (Fig. 1a), or 0.5 μg/ml collagen (Fig. 1b) were reduced by gliclazide. The inhibitory effect of gliclazide was observed at a concentration as low as 1 μg/ml (Fig. 1b).

Fig. 2 shows the effect of gliclazide on platelet ATP release and aggregation observed simultaneously by Lumi-aggregometer.

ATP release and aggregation stimulated by 2 μg/ml collagen were progressively reduced as the concentration of gliclazide increased (Fig. 2b). ATP release induced by 0.02 U/ml thrombin was also progressively inhibited as the concentration of gliclazide increased (Fig. 2a). Although the final aggregation rate was not significantly lower as compared to Fig. 1, aggregation velocity was slightly retarded as the concentration of gliclazide increased (Fig. 2a).

The results of experiment on the phosphorylation of platelet proteins is shown in Fig. 3. Gliclazide, at a concentration of 20 μg/ml, inhibited the phosphorylation of 40 K dalton protein induced by 0.02 U/ml thrombin. Similarly, the phosphorylation of 40 K dalton protein induced by collagen

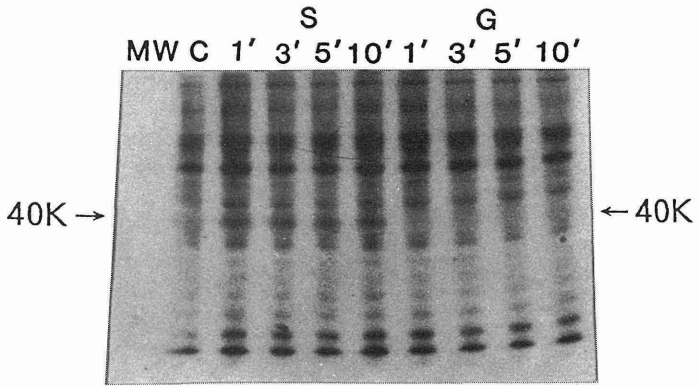

Fig. 3. The effect of gliclazide (G) on the phosphorylation of the platelet proteins.

Platelets were stimulated by 0.02 U/ml thrombin. MW indicates molecular weight markers and 40 K indicates the 40 K dalton protein. Lane C indicates a sample without thrombin stimulation. S indicates samples without gliclazide and G indicates samples in the presence of 20 μg/ml of gliclazide. The numbers indicate time in min after thrombin stimulation.

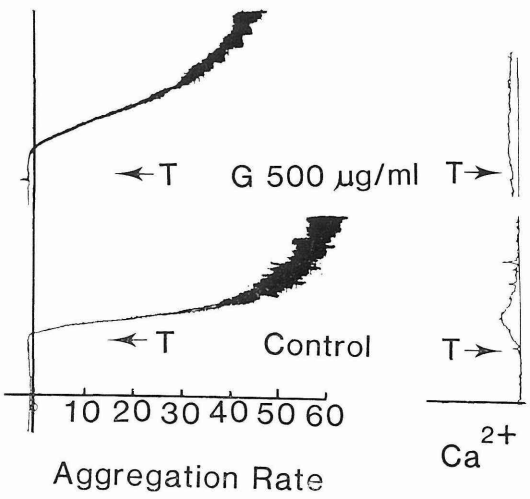

Fig. 4. The effects of gliclazide (G) on intraplatelet calcium.

Platelets were stimulated by 0.1 U/ml thrombin (T) at the time indicated by the arrows. The left half indicates the aggregation and the right half indicates calcium signal by aequorin.

was also inhibited by gliclazide (data not shown).

The effects of gliclazide on intraplatelet calcium concentration and on platelet aggregation are shown in Fig. 4. Thrombin, at a concentration of 0.1 U/ml, increased the intraplatelet calcium concentration from 2.23 μM to 3.98 μM in the absence of gliclazide. Gliclazide, at a concentration of 500 μg/ml, inhibited the increase of intraplatelet calcium induced by 0.1 U/ml thrombin. Gliclazide also reduced the platelet aggregation rate and velocity, and increased the latent time of aggregation after thrombin stimulation.

Discussion

The present study has revealed that gliclazide reduces the platelet aggregation, ATP release, the phosphorylation of 40 K dalton protein and the intraplatelet calcium increase in human platelets *in vitro*.

It was previously reported that the intravenous administration of 12 mg/kg or oral administration of 20 mg/kg of gliclazide for 7 days, reduced platelet aggregability in rabbits[2]. In the same study, it was reported that gliclazide, at a concentration

of 2×10^{-3} M (646 μg/ml), directly inhibits platelet aggregation induced either by 0.5 U/ml thrombin or 5 μM epinephrine *in vitro*. Recently, Kajinuma *et al.* reported that the serum concentration of gliclazide after a single 40 mg oral dose can reach 2.6 μg/ml with a half life of 8.6 hours[7]. Since the usual oral dose of gliclazide administered to diabetic patients ranges from 40 mg to 120 mg per day, a serum concentration of 2×10^{-3} M should not be attainable by the usual oral dose.

We intended to clarify whether gliclazide can directly reduce the platelet aggregation at a concentration attainable by the usual oral dose. Our data demonstrated that gliclazide directly inhibits the platelet aggregation induced by 0.5 μg/ml collagen at a concentration as low as 1 μg/ml. We believe that our results demonstrate that one can indeed expect a direct anti-platelet action of gliclazide at the usual oral dose.

In addition, we have found that 500 μg/ml gliclazide was needed to inhibit the intraplatelet calcium increase induced by 0.1 U/ml thrombin. The fact that the inhibition of three platelet parameters, (aggregation, ATP release and intraplatelet calcium increase), required different concentrations of glicalzide probably reflects the requirement for different concentrations of thrombin needed to elicit them. In our experiments, 0.1 U/ml thrombin was required to detect the aequorin signal, while 0.01 U/ml thrombin was enough to observe the platelet aggregation.

Although our data demonstrate a direct anti-platelet action of gliclazide, the precise mechanism of this inhibitory action still remains to be elucidated.

However, since the hyperaggregability of platelets in diabetic patients is a well-known fact, it appears reasonable to use gliclazide as an oral hypoglycemic agent, considering its possible amelioration of diabetic complications, not only by reducing the blood glucose level, but also by reducing the platelet hyperaggregability.

Further investigations are necessary to clarify whether gliclazide actually has long-term favorable effects on chronic diabetic complications.

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