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EFFECTS OF ORAL HYPOGLYCAEMIC AGENTS ON PLATELET FUNCTIONS

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Abstract—The *in vitro* effects of three oral hypoglycaemic agents, gliclazide (1-(4-methylbenzensulfonyl)-3-[3-azabicylo(3,3,0)octyl]urea), glibenclamide (1-[4-[2-(chloro-2-methoxybenzamide)-ethyl]-phenyl-sulfonyl]-3-cyclohexyl-urea) and glimepiride (1-[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-carboxamide)-ethyl]-phenylsulphonyl]3-(4-methylcyclohexyl)-urea), on functions of human platelets were evaluated. None of these agents up to a concentration of 40 μM inhibited platelet aggregation induced by thrombin. Glibenclamide and glimepiride in the range of 20–40 μM suppressed Ca^{2+} release from internal Ca^{2+} stores induced by thrombin. Gliclazide showed no effect on arachidonic acid metabolism of human platelets. Glimepiride selectively inhibited the cyclooxygenase pathway, while the activities of 12-lipoxygenase and phospholipase A_2 were unaffected. Glibenclamide inhibited both the cyclooxygenase and 12-lipoxygenase pathways. It also attenuated arachidonic acid release from phospholipase A_2. Oral hypoglycaemic agents with inhibitory effects on arachidonic acid metabolism may prove useful for the treatment of diabetic patients with enhanced platelet functions.

Atherosclerosis and thrombosis are frequent complications of diabetes mellitus, which often exacerbate the disease course. A number of factors have been implicated in the pathogenesis of these complications including enhanced platelet sensitivity to agonists and/or vessel wall changes [1–3].

Oral hypoglycaemic agents have been widely used for the therapy of diabetes mellitus. If they modified the hyperactive behaviour of platelets in diabetic patients, the therapeutic benefits would be increased, by control of blood sugar levels as well as prevention of vascular complications. In view of these potential benefits, there has been extensive exploration into the effects of oral hypoglycaemic agents on platelet function. A large body of evidence has been accumulated to suggest that gliclazide and glibenclamide, two of the most widely used of these agents, have platelet-suppressing effects. Gliclazide inhibits platelet adhesion and inhibits platelet aggregation induced by ADP and other agonists [4–6]. Glibenclamide competitively inhibits thromboxane A_2 receptors on platelets, thromboxane A_2 being one of the most potent stimulators of platelets [7].

Glimepiride is a new hypoglycaemic agent. Its structure resembles closely that of glibenclamide [8] but it has a higher potency, long-lasting effects and fewer adverse effects on healthy individuals than glibenclamide. In a preliminary experiment, we found that glimepiride inhibits ADP- and collagen-induced aggregation of platelets *in vitro* as potently as glibenclamide. We here present findings which suggest that glimepiride as well as glibenclamide modifies platelet activation through suppression of arachidonic acid (AA) metabolism, and that the mode of inhibitory effect differs between these two agents.

MATERIALS AND METHODS

Agents. Fura 2-acetoxymethyl ester was obtained from Dojin Laboratories (Kumamoto, Japan). The three sulphonylureas used in the present study, gliclazide (1-(4-methylbenzensulfonyl)-3-[3-azabicyclo(3,3,0)octyl]urea), glibenclamide (1-[4-[2-(chloro- 2- methoxybenzamide)- ethyl]- phenyl- sulfonyl]-3-cyclohexyl-urea) and glimepiride (1-[4-[2-(3-ethyl- 4- methyl- 2- oxo- 3- pyrroline- carboxamide)-ethyl]-phenylsulphonyl]3 - (4 - methylcyclohexyl) - urea), were dissolved in dimethylsulfoxide at 50 mM and stored at −60° until use. The solutions were diluted just prior to experiments, and platelets were preincubated with them for 5 min at 37° before agonist stimulation. Modified Hepes/Tyrode's buffer containing 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM Hepes pH 7.15 and 5.5 mM glucose, was passed through 0.45-μm Millipore filters, and stored at 4° until use.

Platelet separation. Citrate anti-coagulated venous blood was obtained from healthy human donors who had not received any medication for a minimum of 2 weeks preceding the experiment. The blood was centrifuged at 60 *g* for 15 min to obtain platelet-rich plasma. Platelets were washed twice with Hepes/Tyrode's buffer witn 0.2 μM prostaglandin I_2 and resuspended in Hepes/Tyrode's buffer containing 100 μM Ca^{2+} at a concentration of 2×10^5 cells/μL, unless otherwise stated.

Preparation of fura 2-loaded platelets. To platelet-rich plasma obtained as described above, fura 2-acetoxymethylester at a final concentration of 3 μM

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† Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; AA, arachidonic acid.

was added, and the mixture was incubated at 37° for 30 min. After incubation, platelets were washed twice with Hepes/Tyrode's buffer and resuspended in the same buffer at a concentration of 1×10^5 cells/μL.

Measurement of fura 2-detected intracellular calcium ion ($[Ca^{2+}]_i$) changes. Fura 2 fluorescence was measured with a Hitachi F-2000 fluorescence spectrophotometer, with the excitation wavelength alternating every 0.5 sec between 340 and 380 nm, and the emission wavelength set at 510 nm. A fura 2-loaded platelet suspension (1×10^5 cells/μL) was kept at 37° with constant stirring throughout measurement. $[Ca^{2+}]_i$ values were determined from the ratio of fura 2 fluorescence intensity at 340 nm excitation and 380 nm excitation, as described by Grynkiewicz *et al.* [9].

Aggregation. Platelet aggregation was measured as a change in optical density of platelet suspensions. Briefly, 1 mL of a platelet suspension (2×10^5 cells/μL) was added into a cuvette, and the change in optical density after agonist stimulation was continuously monitored with a Sysmex A-100 platelet aggregometer (Kobe, Japan). The data are presented as the maximum per cent decrease in optical density over the control.

Measurement of AA metabolites by HPLC. Platelet samples used for the measurement of aggregation were used for the assessment of AA metabolite production. Briefly, 4 mL ethyl acetate were added to a platelet suspension that had been stimulated with thrombin, ionomycin or AA. Prostaglandin B_2 (450 ng) was also added to the suspension as an internal standard. Extracted lipids were evaporated under a nitrogen stream, and the evaporated residue was redissolved in 100 μL methanol. AA metabolites contained in this methanol solution were then subjected to reversed-phase HPLC using TSK-Gel ODS-80T_m (4.6 × 150 mm; Toyo Soda, Tokyo, Japan). The mobile phase consisted of methanol/water/acetic acid (75:25:0.01, v/v/v) at a flow rate of 1 mL/min. Column effluents were monitored at 275 nm for prostaglanding B_2, and at 235 nm for 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). HHT and 12-HETE were identified by the chromatographic behaviours of authentic samples; the amounts were quantified by comparing the peak area with that of the internal standard.

Statistical analysis. Statistical analysis was performed using Student's *t*-test for paired data.

RESULTS

Thrombin induced platelet aggregation at concentrations as low as 0.02 U/mL. In preliminary experiments, however, AA metabolite production was sometimes below detectable levels in our system and $[Ca^{2+}]_i$ changes were variable when thrombin below the concentration of 0.1 U/mL was used as an agonist. Thrombin stimulation was performed with 0.2 U/mL thrombin throughout this study, since this agonist concentration allowed reproducible $[Ca^{2+}]_i$ rise and AA metabolism.

$[Ca^{2+}]_i$ elevation

$[Ca^{2+}]_i$ elevation induced by 0.2 U/mL thrombin was biphasic, consisting of an initial rapid peak and a subsequent slower phase which gradually returned to the resting level within a few minutes (Fig. 1a). Glibenclamide and glimepiride at concentrations higher than 40 μM appeared to suppress the first peak of $[Ca^{2+}]_i$ elevation that occurred immediately after thrombin stimulation, while they had virtually no effect on the second $[Ca^{2+}]_i$ peak (Fig. 1c, d). Gliclazide, on the other hand, had no effect on $[Ca^{2+}]_i$ changes at any of the concentrations tested (Fig. 1b). Figure 2 illustrates the inhibitory effects of these agents on the peak $[Ca^{2+}]_i$ elevation induced by thrombin. The maximum inhibition of $[Ca^{2+}]_i$ elevation by glibenclamide or glimepiride was in the range of 30–40%.

Aggregation

The magnitude of aggregation induced by 0.2 U/mL thrombin was affected by none of the sulphonylurea agents at any of the concentrations tested in this study (Fig. 3).

AA Metabolism

We evaluated the effects of the sulphonylurea agents on AA metabolism induced by thrombin. The analysis of AA metabolites by HPLC provides information on two major pathways of AA metabolism in human platelets, the cyclooxygenase and the 12-lipoxygenase pathways. HHT represents a product of the cyclooxygenase pathway, and 12-HETE of the 12-lipoxygenase pathway. Thrombin at a concentration of 0.2 U/mL induced HHT production of 67.5 ± 21.6 ng/2×10^8 cells and 12-HETE production of 31.8 ± 16.3 ng/2×10^8 cells. Gliclazide up to a concentration of 40 μM had no significant effect on AA metabolism induced by 0.2 U/mL thrombin. In contrast, glimepiride in a dose-dependent manner inhibited HHT production, but not that of 12-HETE. Glibenclamide inhibited the production of both HHT and 12-HETE dose dependently. Figure 4 summarizes the effects of the sulphonylurea agents on AA metabolism induced by thrombin.

To address these issues more clearly, we sought to evaluate the effects of the sulphonylurea agents on Ca^{2+}-ionophore-induced AA metabolism (Fig. 5) and also on the production of AA metabolites when exogenous AA served as substrate (Fig. 6). Ionomycin, a Ca^{2+}-ionophore, directly elevates $[Ca^{2+}]_i$ which then stimulates phospholipase A_2 with resultant AA release. If there is a discrepancy between results obtained with ionomycin and those with addition of exogenous AA, it implies that the activity of phospholipase A_2 may be altered.

In our system, 2 μM ionomycin induced HHT production of 73.7 ± 35.8 ng/2×10^8 cells and 12-HETE production of 131 ± 67.1 ng/2×10^8 cells. With 10 μM exogenous AA, HHT production was 113 ± 11.2 ng/2×10^8 cells and 12-HETE, 320 ± 96.3 ng/2×10^8 cells. Gliclazide, as may be expected from the results with thrombin stimulation, had no effects on ionomycin-induced AA metabolism or that with addition of exogenous AA. Glimepiride inhibited the production of HHT induced both by ionomycin activation and by direct AA addition, while 12-HETE production was unaffected at all

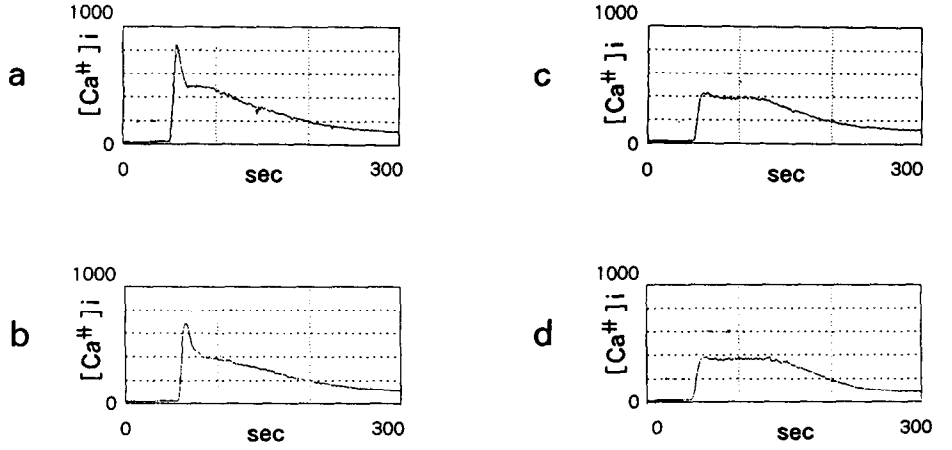

Fig. 1. Effect of three hypoglycaemic agents on the time course of $[Ca^{2+}]_i$ changes induced by thrombin. Fura 2-loaded platelets were incubated with gliclazide, glibenclamide or glimepiride at 40 μM or the equivalent volume of saline for 5 min. After incubation, fura 2 fluorescence of the cell suspension was measured by a fluorescence spectrophotometer with constant stirring. Thrombin (0.2 U/mL) was added to the cell suspension 50 sec after initiation of the measurement. (a) Control, (b) 40 μM gliclazide, (c) 40 μM glibenclamide, (d) 40 μM glimepiride. The traces are representative of three experiments. The ordinate represents $[Ca^{2+}]_i$ in nanomoles calculated from fura 2 fluorescence.

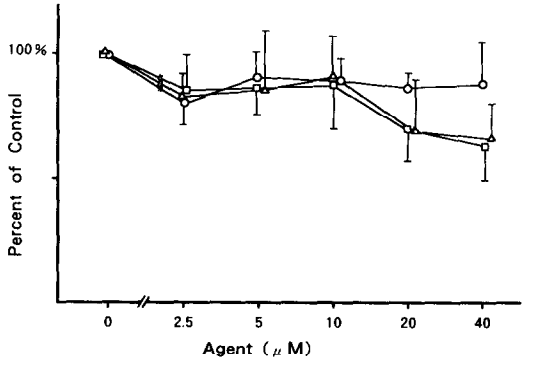

Fig. 2. Effects of three hypoglycaemic agents on thrombin-induced $[Ca^{2+}]_i$ changes. Fura 2-loaded platelets were incubated with various concentrations of each agent for 5 min. After the addition of 0.2 U/mL thrombin, fura 2 fluorescence of the cell suspension was continuously measured by a fluorescence spectrophotometer. The maximum level of $[Ca^{2+}]_i$ calculated from fura 2 fluorescence was recorded for each set of experiments. The data are presented as the means ± SD of three experiments. (○) Gliclazide, (△) glibenclamide, (□) glimepiride.

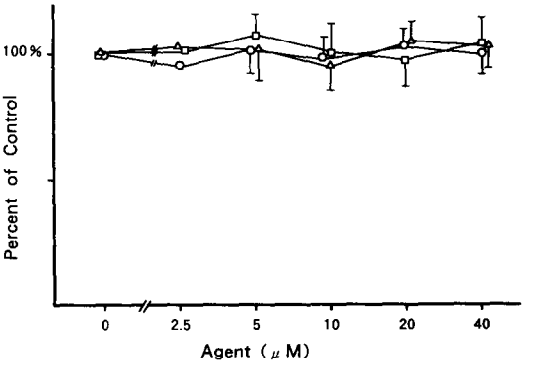

Fig. 3. Effects of three hypoglycaemic agents on thrombin-induced platelet aggregation. The maximum changes in optical density induced by 0.2 U/mL thrombin were measured with a platelet aggregometer. The data are the means ± SD of three experiments. (○) Gliclazide, (△) glibenclamide, (□) glimepiride.

the concentrations tested. On the other hand, glibenclamide in a dose-dependent manner suppressed both the production of HHT and that of 12-HETE induced by ionomycin, with a preference for HHT. While glibenclamide also inhibited the production of both AA metabolites derived from exogenously administered AA, the reduction in 12-HETE production was of a smaller magnitude than that with ionomycin-induced activation.

DISCUSSION

Glibenclamide and glimepiride at concentrations higher than 40 μM suppressed the first peak of thrombin-induced $[Ca^{2+}]_i$ elevation while gliclazide was without effect. Since we and others have confirmed that the first rapid peak of $[Ca^{2+}]_i$ is mainly due to Ca^{2+} release from the $Ca^{2+}{}_i$ storage sites and that the second phase is largely attributed to Ca^{2+} influx from the extracellular fluid [10, 11], these findings suggest that glibenclamide or glimepiride acts on step(s) involved in Ca^{2+} release induced by thrombin with virtually no inhibition of Ca^{2+} influx. The inhibitory effects of these

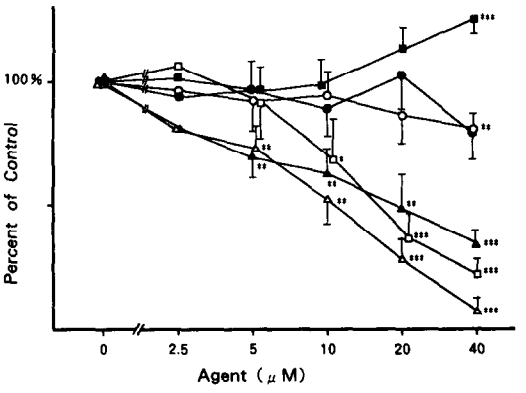

Fig. 4. Effects of three hypoglycaemic agents on thrombin-induced AA metabolism. Platelets were first incubated with various concentrations of the hypoglycaemic agents for 5 min. The cells were then activated by 0.2 U/mL thrombin for 10 min. Lipids were extracted, and analysed with HPLC for the production of HHT and 12-HETE. The data in each experiment were calibrated with controls. (○) HHT gliclazide, (●) 12-HETE gliclazide, (△) HHT glibenclamide, (▲) 12-HETE glibenclamide, (□) HHT glimepiride, (■) 12-HETE glimepiride. The data are the means ± SD of five experiments. The P values for significant differences were expressed as * 0.02, ** 0.01 and *** 0.001.

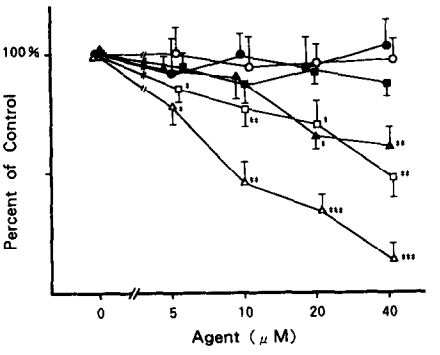

Fig. 6. Effects of three hypoglycaemic agents on platelet metabolism of exogenous AA. Platelets were first incubated with various concentrations of the hypoglycaemic agents for 5 min. AA at a concentration of 10 μM was then added to a platelet suspension, and the mixture was incubated for another 10 min. Lipids were extracted, and analysed with HPLC for the production of HHT and 12-HETE. The data in each experiment were calibrated with controls. (○) HHT gliclazide, (●) 12-HETE gliclazide, (△) HHT glibenclamide, (▲) 12-HETE glibenclamide, (□) HHT glimepiride, (■) 12-HETE glimepiride. The data are the means ± SD of three experiments. The P values for significant differences are expressed as * 0.02, ** 0.01 and *** 0.001.

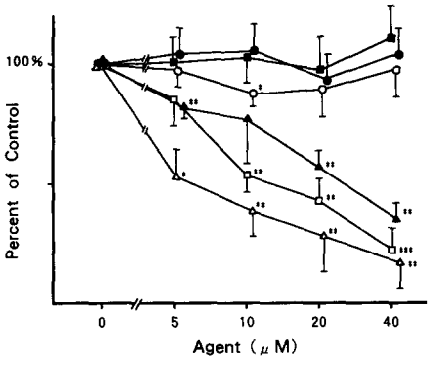

Fig. 5. Effects of three hypoglycaemic agents on AA metabolism induced by a Ca^{2+}-ionophore. Platelets were first incubated with various concentrations of the hypoglycaemic agents for 5 min. The cells were then activated by 2 μM ionomycin for 10 min. Lipids were extracted, and analysed with HPLC for the production of HHT and 12-HETE. The data in each experiment were calibrated with the control HHT and 12-HETE production. (○) HHT gliclazide, (●) 12-HETE gliclazide, (△) HHT glibenclamide, (▲) 12-HETE glibenclamide, (□) HHT glimepiride, (■) 12-HETE glimepiride. The data are the means ± SD of three experiments. The P values for significant differences are expressed as * 0.02, ** 0.01 and *** 0.001.

agents cannot be attributed to the suppression of thromboxane A_2 formation, since 1 mM aspirin which is a potent blocker of cyclooxygenase did not induce a change in $[Ca^{2+}]_i$ similar to these agents (data not shown).

In the present study, we investigated the effects of three oral hypoglycemic agents on AA metabolism of human platelets. Three activators with different activation pathways were utilized to identify the sites of actions on AA metabolism. Thrombin stimulation represents a receptor-mediated signal transduction pathway which leads to phospholipase A_2 activation through $[Ca^{2+}]_i$ elevation, most probably by the involvement of a GTP-binding protein(s) [12]. Ionomycin, a Ca^{2+}-ionophore, mechanically introduces Ca^{2+} into the cytoplasm which directly elicits phospholipase A_2 activation, bypassing most of the intracellular pathways involved in receptor-mediated activation. Exogenous AA administration is useful for identifying inhibitory effects on AA metabolism at a site distal to phospholipase A_2.

Gliclazide had no effect under any of the experimental conditions used. Our findings suggest that this agent lacks an inhibitory effect on three major enzymes involved in AA metabolism in platelets, phospholipase A_2, cyclooxygenase and 12-lipoxygenase. An *in vitro* study on AA metabolism of guinea pig platelets, with which our findings are in agreement, showed that gliclazide at a millimolar concentration range inhibited AA release but had no effect on cyclooxygenase [13]. Most of the previous reports on the inhibitory effects of this agent on platelets and AA metabolism dealt with *ex vivo* experiments [14]. It may be that gliclazide may indirectly modify platelet AA metabolism *in vivo*.

The results with glimepiride were consistent with its having an inhibitory effect on the cyclooxygenase pathway. This agent neither suppressed thrombin-induced production of 12-HETE nor that induced by ionomycin, suggesting that phospholipase A_2 activation itself was not affected. Thus, in terms of AA metabolism, glimepiride appears to be a specific inhibitor of cyclooxygenase. Since thromboxane A_2, the most potent endogenous platelet activator, is a product of this pathway, glimepiride is expected to modulate through suppressed production of thromboxane A_2 those platelet responses which are triggered by thromboxane A_2. In accord with this assumption, we found in a preliminary study that glimepiride inhibited in a dose-dependent manner platelet aggregation induced by ADP and collagen, whose activation pathway largely depends upon thromboxane A_2 production [15]. Our failure to demonstrate an inhibitory effect on thrombin aggregation is not contradictory, since platelet aggregation induced by thrombin at the concentration tested in the present study is known to be independent of thromboxane A_2 formation [16–19]. In agreement with this concept, we confirmed that pretreatment with 1 mM aspirin had no effect on thrombin-induced aggregation in our system (data not shown).

Glibenclamide appears to have multiple sites of action on AA metabolism in platelets. This agent inhibits both cyclooxygenase and 12-lipoxygenase, the former being suppressed more markedly. It also has an inhibitory effect on phospholipase A_2, suggested by a greater reduction in thrombin- or ionomycin-induced production of AA metabolites than that with exogenous AA administration.

Oral hypoglycaemic agents with suppressive effects on platelets may be beneficial because hypersensitivity and enhanced function of platelets in diabetic patients is linked to various complications. Glibenclamide which has multiple sites of action on AA metabolism should reduce both the production of thromboxane A_2 and 12-lipoxygenase-related products. On the other hand, glimepiride which specifically inhibits cyclooxygenase should affect the production of thromboxane A_2 without changing the level of 12-lipoxygenase-related products, but the role of 12-lipoxygenase-related products in platelet functions remains to be elucidated.

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