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Effects of Mitiglinide on Glucose-Induced Insulin Release into the Portal Vein and Fat-Induced Triglyceride Elevation in Prediabetic and Diabetic OLETF Rats

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Objective: The effect of single-dose mitiglinide on glucose and lipid metabolism was examined in OLETF rats with spontaneous type 2 diabetes in which the early insulin response following glucose challenge is known to diminish over time and become lost with aging. Methods: (1) With catheters inserted into the portal veins, 12-wk-old prediabetic OLETF rats were given an OGTT of 1 g/kg after 17 h of fasting. Eight rats each were orally given mitiglinide 1 mg/kg, nateglinide 50 mg/kg, or glibenclamide 1 mg/kg, vs 0.5% carboxymethylcellulose (CMC) as control, and were given an OGTT immediately afterward. Following oral administration of mitiglinide, nateglinide, glibenclamide, or 0.5% CMC, the 24-wk-old overt-diabetic OLETF rats were immediately given an OGTT of 1g/kg. (2) After 17 h of fasting, 24-wk-old OLETF rats were subjected to a fat-loading test. Eight rats each were given mitiglinide 3 mg/kg, glibenclamide 1 mg/kg, or glimepiride 1 mg/kg, vs 0.5% CMC, and were given soy oil 2 g/kg immediately afterward. They were also given mitiglinide orally and examined for LPL mRNA expression in their adipose tissue. Results: (1) After OGTT, mitiglinide produced a significant increase in portal insulin levels 15 min after its administration, as well as a significant decrease in peripheral glucose levels 15–120 min after its administration in the OLETF rats. Likewise, nateglinide produced an increase in portal insulin levels and a decrease in peripheral glucose levels shortly after its administration in these rats. Glibenclamide increased portal insulin levels for an extended time after its administration, and significantly decreased peripheral glucose levels in the rats 120–300 min after its administration in the rats. In contrast, as in the 12-wk-old rats, a precipitous rise in insulin secretion was seen in the portal vein of 24-wk-old rats given mitiglinide, which peaked 15 min after mitiglinide administration, but the insulin levels continued to increase for 120 min or longer in the 24-wk-old rats given glibenclamide. In addition, as in the 12-wk-old rats, a significant decrease in glucose levels in peripheral blood was noted 30 and 60 min after mitiglinide administration and 300 min after glibenclamide administration in the 24-wk-old rats. (2) Mitiglinide increased LPL mRNA expression 120 min after its administration, and significantly decreased peripheral TG and chylomicron-TG levels after fat challenge in the 24-wk-old OLETF rats. Conclusion: Mitiglinide exhibited fast-onset and short-acting insulin-secretagogic effects, inhibiting post-glucose challenge increases in glucose levels and post-fat challenge increases in TG levels.

Key Words: Mitiglinide; fast-acting insulin secretagogue; early insulin secretion; postprandial hyperglycemia; postprandial hyperlipidemia.

Introduction

In patients with type 2 diabetes, the rapid pulsatile secretion of insulin that occurs in response to a rise in blood glucose levels is impaired and usually results in postprandial hyperglycemia *(1,2)*. The second-generation sulfonylurea drug glibenclamide, a strong insulin secretagogue used over the last 30 yr as an oral hypoglycemic drug, increases insulin secretion for an extended time. However, it cannot stimulate a rapid rise in insulin levels that normally occurs in response to a glucose challenge. Glimepiride, a third-generation sulfonylurea with extra-pancreatic effects, has antiglycemic activity equivalent to that of glibenclamide, although its insulin-secretagogic activity is weaker than that of glibenclamide *(3)*. On the other hand, while glinides promote insulin secretion similarly to sulfonylureas by binding to the

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sulfonylurea receptors of the pancreatic β cell K_{ATP} channels where they inhibit the activity of the K_{ATP} channels thereby promoting the inflow of extracellular Ca^{2+} ions into pancreatic cells, they have shorter durations of action and more rapid onset than sulfonylureas, thus rapidly lowering glucose levels during their short duration of action. Glinides in current clinical use include nateglinide, repaglinide, and mitiglilnide, but exactly how they differ in their ability to promote insulin secretion remains to be elucidated. Of note, mitiglinide, a derivative of benzylsuccinic acid in structure and a non-sulfonylurea agent devoid of the sulfonylurea base, is a fast-acting insulin secretagogue with a short duration of action *(4,5)*, and reportedly promotes early insulin secretion in normal rats, where its action is seen to peak as early as 15 min after its administration *(6)*. However, the majority of studies investigating the insulin-secretagogic effect of mitiglinide have been conducted in normal rats, with no reports available that investigated the effect of single-dose mitiglinide in spontaneously type 2 diabetic rats in which the early insulin response following glucose loading is known to diminish over time and become lost with aging. Furthermore, the insulin-secretagogic effect of mitiglinide was investigated in the peripheral blood in all reports, despite the fact that peripheral insulin levels do not accurately reflect the actual amount of insulin secreted, as 50% of the insulin secreted is immediately taken up by hepatic cells via the portal vein, leaving only 50% available for peripheral circulation, and insulin becomes quickly degraded.

To clarify the role of early insulin secretion during postprandial hyperglycemia and postprandial hyperlipidemia in type 2 diabetes, we therefore produced various insulin-secreting states in portal vein using mitiglinide, nateglinide, glibenclamide, and glimepiride, and investigated their comparative effects on blood glucose elevations following glucose challenge in peripheral blood and on triglyceride (TG) elevations following fat challenge in Otsuka Long Evans Tokushima Fatty (OLETF) rats *(7)* that are characterized by insulin resistance and intraabdominal fat accumulation in which the early insulin response to glucose challenge is first reduced and then eliminated with aging *(8)*, as they progress from IGT to type 2 diabetes over time.

Results

Effects of Mitiglinide on Early Insulin Secretion and Blood Glucose Levels After Glucose Loading

Mitiglinide produced a sharp increase in portal insulin levels, which peaked 15 min after mitiglinide administration and returned to basal levels within 60 min, in 12-wk-old, prediabetic rats. In contrast, elevated insulin levels persisted for an extended time in the glibenclamide group (Fig. 1). Peripheral glucose levels were significantly lower in the rats given mitiglinide 15, 30, 60, and 120 min after mitiglinide administration and in the glibenclamide group 120 min or longer following glibenclamide administration compared to the control group (Fig. 1). An increase in portal insulin levels as well as a decrease in peripheral glucose levels was noted in the nateglinide group shortly after nateglinide administration, as well as in the mitiglinide group. On the other hand, as in the 12-wk-old rats, a precipitous rise in insulin secretion peaked 15 min after mitiglinide administration in the portal vein of 24-wk-old, overt-diabetic rats, but the insulin levels continued to increase for 120 min or longer in the glibenclamide group (Fig. 2). In addition, as in the 12-wk-old rats, a significant decrease in glucose levels in peripheral blood was noted 30 and 60 min after mitiglinide administration and 300 min after glibenclamide administration in the 24-wk-old rats (Fig. 2). An increase in portal insulin levels as well as a decrease in peripheral glucose levels was noted in the nateglinide group shortly after nateglinide administration, as well as in the mitiglinide group.

Effects of Mitiglinide on TG Levels After Fat Loading

Mitiglinide promoted insulin secretion that peaked in peripheral blood 30 min after its administration (data not shown). Compared to the control animals given vehicle, the animals treated with mitiglinide showed lower blood glucose levels (AUC of 0 to 120 min: mitiglinide group, 311.4 ± 19.0 mg·h/dL; control group, 406.6 ± 21.9 mg·h/dL; $p < 0.01$) and lower FFA levels (AUC of 0 to 120 min: mitiglinide group, 3.12 ± 0.18 mEq·h/L; control group, 3.98 ± 0.26 mEq·h/L; $p < 0.05$). The mitiglinide group showed significantly lower TG levels 180 min after mitiglinide administration compared to the control group (Fig. 3). Under the sustained insulin-secretion dynamics promoted by glimepiride or glibenclamide, reductions in blood glucose levels were noted 120, 180, 240, and 300 min (data not shown), but no significant change in TG was observed (Fig. 3). AUC for $TG_{0–300}$ min was significantly ($p < 0.05$) lower in the mitiglinide group than the control group, while no significant differences were observed in AUC for $TG_{0–300}$ min between the glibenclamide or glimepiride group and the control group (AUC of 0 to 300 min: mitiglinide group, 1474.8 ± 80.7 mg·h/dL; glibenclamide group, 1694.5 ± 240.2 mg·h/dL; glimepiride group, 2025.0 ± 198.4 mg·h/dL; control group, 2138.4 ± 190.3 mg·h/dL). The mitiglinide group also showed significantly lower chylomicron-TG levels 180 min after mitiglinide administration compared to the control group, while there was no significant difference in very-low-density lipoprotein (VLDL)–TG levels between these groups (Fig. 3).

Effects of Mitiglinide on LPL mRNA Expression in Adipose Tissues

Lipoprotein lipase (LPL) mRNA expression in the adipose tissue increased significantly 120 min after mitiglinide administration compared to baseline (Fig. 4). No change in LPL mRNA expression was noted at the other measurement time points (30 and 60 min after mitiglinide administration).

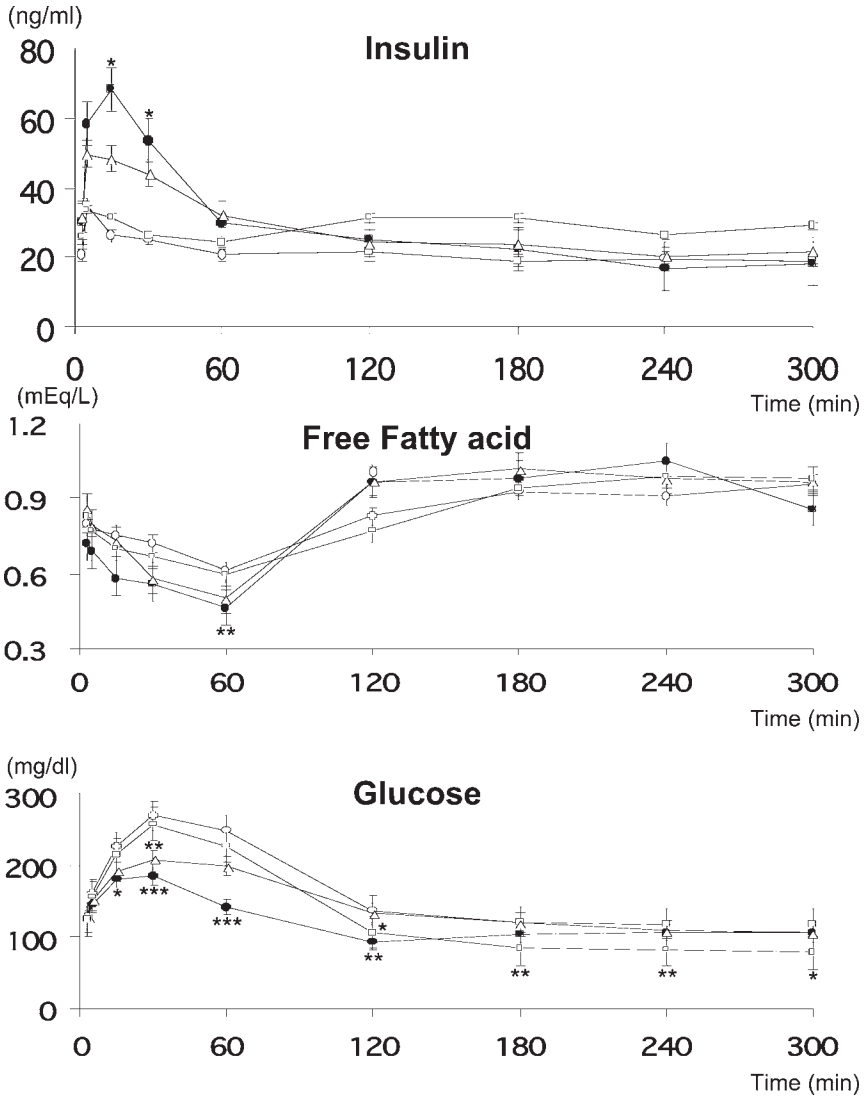

Fig. 1. Insulin and free fatty acid levels in portal blood and glucose levels in peripheral blood of 12-wk-old prediabetic Otsuka Long Evans Tokushima Fatty (OLETF) rats. -●-, mitiglinide 1 mg/kg ($n = 8$); -△-, nateglinide 50 mg/kg ($n = 8$); -□-, glibenclamide 1 mg/kg ($n = 8$); -○-, control ($n = 8$) (mean ± SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control; no significant differences in portal insulin and FFA levels were observed at all time points ($p > 0.05$) between the mitiglinide and nateglinide groups and between the mitiglinide and glibenclamide groups.

Discussion

Our present study investigated the effect of single-dose mitiglinide on reducing short-term blood glucose after glucose challenge, and the study results strongly indicated a close correlation between postprandial hyperglycemia and the dynamics of insulin secretion into the portal vein. While mitiglinide produced a sharp spike of insulin secretion that led to an immediate reduction in blood glucose after glucose challenge not only in 12-wk-old prediabetic rats whose early insulin secretion was reduced, but also in 24-wk-old overt-diabetic rats whose early insulin secretion was eliminated, glibenclamide produced sustained insulin secretion that did not immediately lower blood glucose after glucose challenge. We assumed that this difference could be attributable at least in part to the "sharp spike" dynamics of insulin secretion into the portal vein that mitiglinide produced, which we assumed had a major impact on the insulin-induced suppression of hepatic glucose output, as well as on the insulin-induced acceleration of hepatic glucose uptake. At the same time, while nearly all early insulin secretion was eliminated, and the insulin levels were seen to increase 2 h after glucose challenge in the portal vein of 24-wk-old OLETF rats, glibenclamide could further promote this delayed excess insulin secretion. Of the two fast-acting insulin secretagogues used, mitiglinide tended to show a more sustained glucose-lowering effect on the peripheral blood than nateglinide, while these drugs similarly promoted early insulin secretion in the portal vein, and exhibited the similar dynamics of insulin secretion.

LPL, an enzyme produced by adipose and muscle cells, is present on the surface of vascular endothelial cells, and is responsible for the intravascular hydrolysis of TG, and

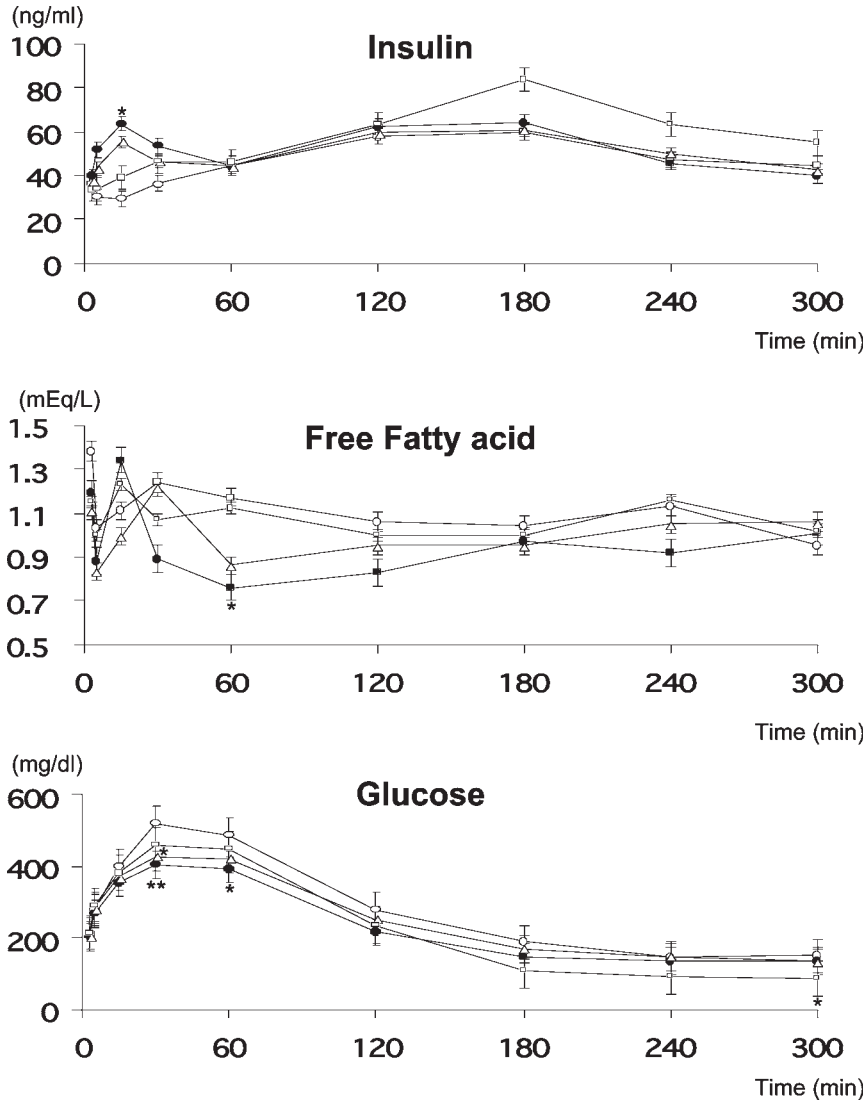

Fig. 2. Insulin and free-fatty-acid levels in portal blood and glucose levels in peripheral blood of 24-wk-old overt-diabetic Otsuka Long Evans Tokushima Fatty (OLETF) rats. -●-, mitiglinide 1 mg/kg ($n = 10$); -△-, nateglinide 50 mg/kg ($n = 10$); -□-, glibenclamide 1 mg/kg ($n = 10$); -○-, control ($n = 10$) (mean ± SEM). *$p < 0.05$, **$p < 0.01$, vs control; no significant differences in portal insulin and FFA levels were observed at all time points ($p > 0.05$) between the mitiglinide and nateglinide groups and between the mitiglinide and glibenclamide groups.

impaired LPL function is known to result in the onset of hypertriglyceridemia. LPL is one of the many enzymes regulated by insulin, and its plasma activity reflects insulin sensitivity *(9)*. Plasma LPL activity has been shown to be decreased in insulin-resistant subjects without diabetes *(10)*, and recently, overall plasma LPL activity was shown to be inversely correlated with insulin resistance in patients with type 2 diabetes *(11)*. Our current study also investigated the effect of single-dose mitiglinide on reducing TG levels following fat loading, and the results strongly suggested that the sharp spike of insulin secretion into the portal vein produced by mitiglinide played a major role in postprandial hyperlipidemia, as the rat model used in our study is reported to show significantly reduced LPL expression in the subcutaneous adipose tissue and skeletal muscles, along with delayed TG metabolism *(12)*. We thus hypothesized that the reduced LPL levels associated with insulin resistance in these rats had contributed considerably to the outcomes reported here. In fat-loading tests using Zucker fatty rats, Mine and colleagues *(13)* noted that the rapid insulin secretion that occurred following administration of the fast-acting insulin secretagogue nateglinide led to a reduction in TG after fat loading, in agreement with the results of our present study. Furthermore, in a clinical study using challenge diet *(14)*, nateglinide has been shown to suppress post-challenge increases in TG and remnant-like particle (RLP)–TG levels. In our present study, it was confirmed that mitiglinide promotes early insulin secretion thereby decreasing the level of chylomicron, an exogenous TG-rich lipoprotein. This has led us to hypothesize that the mechanism likely involved is mitiglinide-induced early insulin secretion, which may have affected TG-rich lipoprotein metabolism

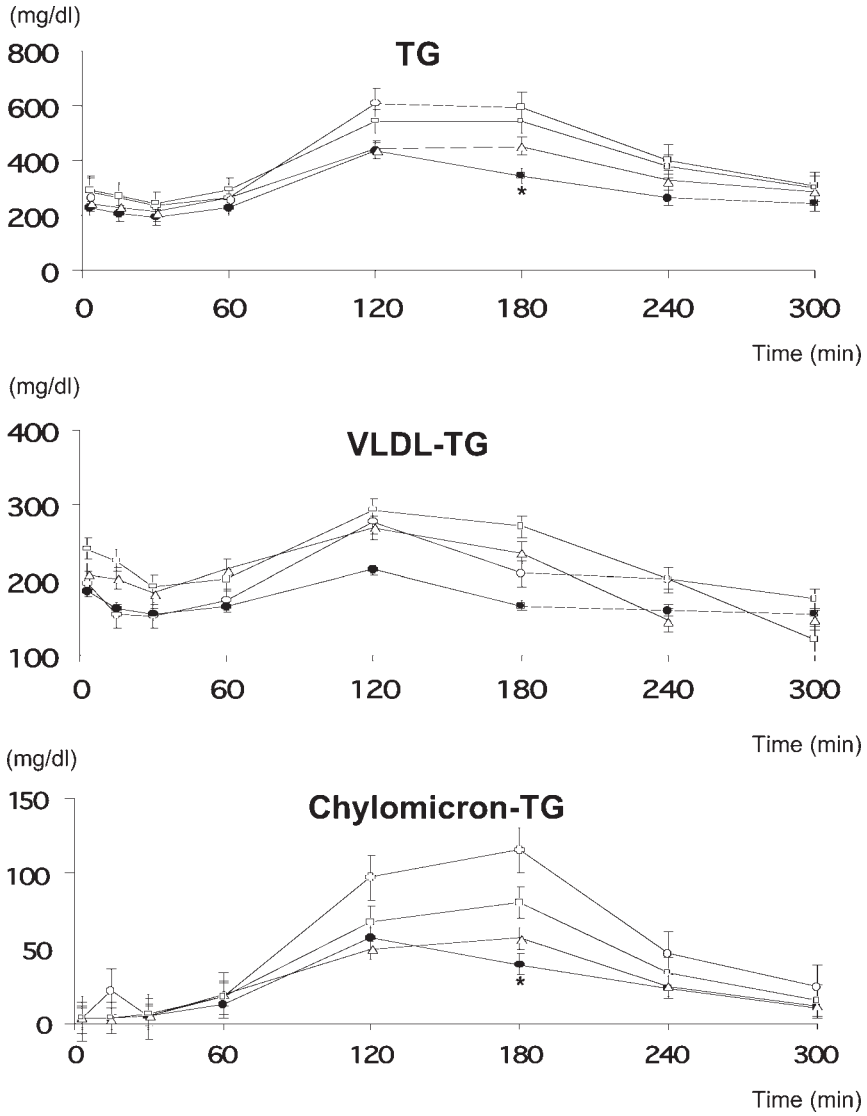

Fig. 3. TG, VLDL-TG, and chylomicron-TG levels in peripheral blood of 24-wk-old overt-diabetic Otsuka Long Evans Tokushima Fatty (OLETF) rats. -●-, mitiglinide 3 mg/kg ($n = 8$); -△-, glibenclamide 1 mg/kg ($n = 8$); -□-, glimepiride 1 mg/kg ($n = 8$); -○-, control (mean ± SEM). *$p < 0.05$ vs control; no significant differences in peripheral TG, VLDL-TG, and chylomicron-TG levels were observed at all time points ($p > 0.05$) between the mitiglinide and nateglinide groups and between the mitiglinide and glibenclamide groups.

by increasing the level of LPL. Furthermore, as we reported earlier that LPL mRNA expression in adipose tissue was significantly increased 2 h after administration of single-dose nateglinide in these rats compared to baseline, while this was not the case with single-dose glibenclamide *(15)*, the increase in LPL mRNA expression in adipose tissue shown in our current study was assumed to be mediated through the effect of mitiglinide on early insulin secretion, as was the case with nateglinide.

Recent research indicates that impaired glucose tolerance (IGT) is a risk factor for the development of arteriosclerotic conditions *(16,17)*, and there is considerable interest in the relationship between postprandial hyperglycemia and arteriosclerotic changes. While this relationship still remains poorly understood at present, correlation has been reported between postprandial hyperglycemia and factors such as impaired vascular endothelial function *(18)*, changes in platelet activity *(19)*, and reduced fibrinolytic activity *(20)*. More recently, it has become clear that postprandial hyperglycemia and postprandial hyperlipidemia act independently or additively to induce oxidative stress, thereby impairing vascular endothelial function *(21)*. This hints at a possible mechanism in which reduced early insulin secretion gives rise to postprandial hyperglycemia and postprandial hyperlipidemia, and in which these two postprandial conditions act additively to impair vascular endothelial function. Published reports consistently associate early insulin secretion and atherosclerosis, including the finding that mitiglinide works to correct postprandial oxidative stress production and inflammation in patients with type 2 diabetes *(22)*; that the improvement of postprandial glucose elevations with nateglinide goes hand in hand with that of vascular endo-

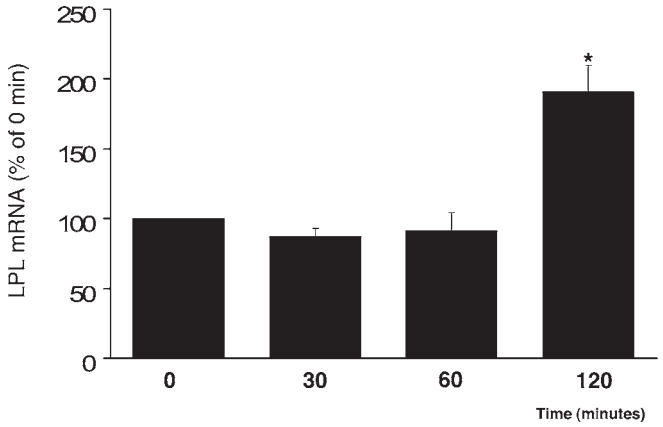

Fig. 4. The expression of lipoprotein lipase (LPL) mRNA in adipose tissue before and 30, 60, and 120 min after mitiglinide administration (mean ± SEM). *$p < 0.001$ vs 0 min.

thelial dysfunction *(23)*; and that repaglinide inhibits thickening of the carotid intima-media in patients with type 2 diabetes *(24)*. Thus, restoration of early insulin secretion with mitiglinide may not only contribute toward improving postprandial hyperglycemia and hyperlipidemia, but also toward improving vascular endothelial dysfunction, which leads to the development of atherosclerosis, thus eventually arresting the development of atherosclerosis.

Our study results thus demonstrate that mitiglinide induces a quick-onset but short-lasting release of insulin, which limits the increases in blood glucose and TG levels following oral glucose and fat loading. It is also confirmed that mitiglinide is a more effective drug for type 2 diabetes.

Materials and Methods

Animals

Male OLETF rats *(7,8)* were obtained at 4 wk of age from the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). The animals were housed in plastic cages (320 × 270 × 175 mm) in an animal room at a controlled temperature (23 ± 2°C) and a relative humidity (55 ± 15%) and kept in a 12-h light/12-h dark cycle (lights on at 07:00 AM). They were supplied with a rat chow (CE-2; Clea Japan, Tokyo, Japan) and tap water *ad libitum* until 12 and 24 wk of age. The Guidelines for Laboratory Animal Facilities of the Jikei University School of Medicine were followed for the care and use of the animals in this study, and the protocol for the animal experiments in this study was approved by the institutional ethics committee.

Drugs

Mitiglinide, nateglinide, and glimepiride were synthesized at the Kissei Pharmaceutical Co., Ltd. (Nagano, Japan). Glibenclamide was purchased from Sigma (St. Louis, MO, USA). Mitiglinide (1 mg/kg and 3 mg/kg) and glibenclamide (1 mg/kg) were suspended in 0.5% carboxymethylcellulose (0.5% CMC), and nateglinide (50 mg/kg) and glimepiride (1 mg/kg) were suspended in 0.5% and 0.75% methylcellulose, respectively, and they were given orally to the rats in doses of 5 mL/kg. These doses of the drugs were chosen on the basis of previous studies *(4,6)*. Control rats were given 0.5% CMC (vehicle control) alone.

Experimental Design

Effects of Mitiglinide on Early Insulin Secretion and Blood Glucose Levels After Glucose Challenge

Indwelling catheters were placed in the portal vein in 12- and 24-wk-old OLETF rats with the exposed end of the catheter positioned behind the head. On the next day, after being made to fast for 17 h, the rats were subjected to oral glucose tolerance testing (OGTT, 1 g/kg) under no anesthesia or restraint. Following oral administration of mitiglinide ($n = 8$), nateglinide ($n = 8$), glibenclamide ($n = 8$), or 0.5% CMC (control) ($n = 8$), the 12-wk-old OLETF rats were immediately given an OGTT of 1 g/kg. Following oral administration of mitiglinide ($n = 10$), nateglinide ($n = 10$), glibenclamide ($n = 10$), or 0.5% CMC (control) ($n = 10$), the 24-wk-old OLETF rats were immediately given an OGTT of 1 g/kg. Blood samples were drawn from the rats before glucose and drug administration and 5, 15, 30, 60, 120, 180, 240, and 300 min after glucose challenge. Portal blood samples were taken via the catheter in the portal vein, and peripheral samples were drawn from the caudal vein.

Effects of Mitiglinide on TG Levels After Fat Loading

The 24-wk-old OLETF rats were fasted for 17 h, after which they were subjected to an oral fat-loading test under no anesthesia or restraint. The rats were orally given mitiglinide ($n = 8$), glimepiride ($n = 8$), glibenclamide ($n = 8$), or 0.5% CMC (control) ($n = 8$), followed immediately by oral administration of fat emulsion (Intralipos, Welfide, Osaka, Japan) at a dose of 10 mL/kg (containing 2 g soybean oil). Samples of the peripheral blood were drawn from the caudal vein at regular intervals (0, 15, 30, 60, 120, 180, 240, and 300 min after fat loading), and blood glucose, insulin, free fatty acids (FFA), TG, and lipoprotein fractions were measured. Area under the curve (AUC) was calculated for glucose and FFA.

Effects of Mitiglinide on LPL mRNA Expression in Adipose Tissues

A similar fat-loading test was performed separately in 24-wk-old OLETF rats, and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of lipoprotein lipase (LPL) mRNA expression in adipose tissue was performed. The epididymal fat pad was excised 0, 30, 60, or 120 min after drug administration ($n = 5$ for each time point) in OLETF rats, then frozen in liquid nitrogen and stored at −80°C.

Biochemical Analysis

Blood samples were immediately centrifuged at 4°C, and the plasma was separated, which was then stored at –20°C until they were subjected to assay. Plasma glucose levels were determined by the glucose oxidase method using a commercial kit (Glucose C2; Wako Pure Chemical, Osaka, Japan). Plasma FFA and TG levels were measured enzymatically using enzyme reagents (NAFA C; Wako Pure Chemical, Osaka, Japan; Triglyceride E; Wako Pure Chemicals, Osaka, Japan). Plasma insulin levels were determined with a commercial enzyme-linked immunoassay kit (Insulin ELISA kit, Seikagaku, Tokyo, Japan) using rat insulin as the standard. The plasma TG levels in lipoprotein fractions were determined by agarose gel electrophoresis, followed by TG-specific staining using the Chol/Trig Combo system (Helena Laboratories, Saitama, Japan).

Measurements of LPL mRNA Expression

Total RNA was prepared using an RNA extraction kit (ISOGEN; Nippon Gene, Tokyo, Japan). Superscript II Rnasa H Reverse Transcriptase and an oligo (dT) primer (Invitorogen, Tokyo, Japan) were used to synthesize copy DNAs (cDNAs) from 500 ng of total RNA. The resulting cDNAs were amplified using the SYBER Green PGR kit. Quantitative PCR was performed on the ABI PRISM 7700 sequence detection system (Applied Biosystems Japan, Tokyo, Japan). The primer sequence for the LPL was 5'-tat ggc aca gtg gct gaa ag-3' (sense); and 5'-ctg acc agc gga agt agg ag-3' (antisense). LPL mRNA expression data were normalized to the cyclophilin B mRNA expression in the corresponding sample. The magnitude of LPL mRNA expression was assessed as percentage of baseline values.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed with SAS System Version 8.2 (SAS Institute Inc.). To detect any significant differences among the groups, comparisons were performed using one-way analysis of variance, followed by Dunnett's test. A value of $p < 0.05$ was considered statistically significant.

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