

Metformin Causes Reduction of Food Intake and Body Weight Gain and Improvement of Glucose Intolerance in Combination with Dipeptidyl Peptidase IV Inhibitor in Zucker *fa/fa* Rats

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Received February 2, 2004; accepted March 23, 2004

ABSTRACT

An incretin hormone, glucagon-like peptide-1 (GLP-1), has been shown to lower plasma glucose via glucose-dependent insulin secretion and to reduce appetite. We previously found that the biguanide metformin, an antidiabetic agent, causes a significant increase of plasma active GLP-1 level in the presence of dipeptidyl peptidase IV (DPP-IV) inhibitor in normal rats. This finding suggested that the combination treatment might produce a greater antidiabetic and anorectic effect, based on enhanced GLP-1 action. In this study, we assessed the effects of subchronic treatment with metformin and a DPP-IV inhibitor, valine-pyrrolidide (val-pyr), on glycemic control, food intake, and weight gain using Zucker *fa/fa* rats, a model of obesity and impaired glucose tolerance. The combination treatment caused a significant increase of GLP-1 level in Zucker *fa/fa* rats. In a subchronic study, val-pyr, metformin, or both compounds were

administered orally b.i.d. for 14 days. The combination treatment significantly decreased food intake and body weight gain, although neither metformin nor val-pyr treatment alone had any effect. In an oral glucose tolerance test on day 1, the coadministration caused a greater improvement of glucose tolerance and a prominent increase of plasma active GLP-1 without marked insulin secretion. The 14-day combination treatment produced a potent reduction of fasting blood glucose and plasma insulin levels. These results demonstrate that the combination therapy of metformin with DPP-IV inhibitor leads to reduced food intake and body weight gain, most likely through the significant increase of plasma GLP-1 level. The combination therapy seems to be a good candidate for treatment of type 2 diabetes with obesity.

Metformin (met) is widely used as an oral antidiabetic agent for the treatment of type 2 diabetes. It has multiple antidiabetic effects, such as inhibition of gluconeogenesis and delay of gastrointestinal absorption of glucose, and it reduces food intake or prevents body weight gain in obese patients with type 2 diabetes and in animal models of obesity (Bailey, 1992; Rouru et al., 1992; Bailey and Turner, 1996; Lee and Morley, 1998). However, its mechanism of action is not fully understood at the molecular level. Recently, it was indicated that metformin increases plasma active glucagon-like peptide-1 (GLP-1) in obese nondiabetic subjects (Mannucci et al., 2001).

GLP-1 is an incretin released from L cells in the intestine after oral ingestion of nutrients. This incretin has multiple actions, including stimulation of inhibition of glucagon secretion, increase of glycogen synthase activity, and slowing of

gastric emptying, in addition to promotion of satiety and inhibition of food intake (Drucker, 2001, 2002). Mannucci et al. (2001) proposed that the reduced food intake and body weight gain in subjects treated with metformin might be related to GLP-1 increase.

GLP-1 is rapidly degraded by dipeptidyl peptidase IV (DPP-IV or CD26, EC 3.4.14.5), resulting in a circulating half-life of only 1 to 2 min. Thus, inhibition of DPP-IV activity could be a useful strategy to enhance the activity of GLP-1. Many studies have confirmed the utility of DPP-IV inhibitors not only in the treatment of diabetes with obesity in animal models (Pederson et al., 1998; Balkan et al., 1999; Pospisilik et al., 2002; Reimer et al., 2002; Sudre et al., 2002), but also in humans (Ahrén et al., 2002). These data suggest that DPP-IV inhibitors would be of value in the treatment of obesity and diabetes.

Our previous study demonstrated that acute administration of metformin with valine-pyrrolidide, a DPP-IV inhibitor (Deacon et al., 1998; Ahrén et al., 2000), synergistically in-

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
DOI: 10.1124/jpet.103.064964.

ABBREVIATIONS: met, metformin; GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase IV; val-pyr, valine-pyrrolidide; OGTT, oral glucose tolerance test.

creases plasma active GLP-1 level in fasted normal rats, although neither metformin nor the DPPIV inhibitor alone affects the basal GLP-1 level (Yasuda et al., 2002). This result showed that DPPIV inhibition is necessary to maximize the efficacy of the GLP-1 increase induced by metformin. Consequently, we proposed that the combination treatment would be an effective new approach to elevate plasma active GLP-1 level.

Thus, we hypothesized that the combination treatment of metformin with a DPPIV inhibitor would provide greater benefit for the treatment of type 2 diabetes with obesity than treatment with either metformin or DPPIV inhibitor alone. The purposes of this study are therefore to evaluate the increase of plasma active GLP-1 in response to the combined treatment with metformin and DPPIV inhibitor and to investigate the effect of subchronic coadministration on glycemic control, food intake, and body weight gain in obese Zucker *fa/fa* rats, which display abnormalities characteristic of type 2 diabetes, including mild hyperglycemia, hyperinsulinemia, glucose intolerance, and impaired insulin secretion.

Materials and Methods

Chemicals. Metformin (1,1-dimethylbiguanide) hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Valine-pyrrolidide hydrochloride was synthesized in our laboratories. Metformin and val-pyr were dissolved in distilled water before administration.

Animals. Obese male Zucker *fa/fa* rats (5 weeks old) were purchased from Charles River Japan (Tokyo, Japan). Zucker *fa/fa* rats were individually housed under conventional conditions with controlled temperature, humidity, and lighting ($22 \pm 2^\circ\text{C}$, $55 \pm 5\%$, and a 12-h light/dark cycle with lights on at 7:00 AM), and provided with a commercial diet (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum. All procedures were conducted according to the Eisai Animal Care Committee's guidelines.

Biochemistry Determination. Blood glucose was measured with Glu CII-test (Wako Pure Chemicals, Osaka, Japan). Plasma immunoreactive intact GLP-1 was measured with a glucagon-like peptide (active) enzyme-linked immunosorbent assay kit (Linco Research, St. Charles, MO). Plasma immunoreactive insulin was determined with an insulin enzyme-linked immunosorbent assay kit using rat insulin as a standard (Morinaga, Yokohama, Japan).

Acute Administration Study. Fifteen-week-old Zucker *fa/fa* rats were used. In the nonfasted state, rats were randomly treated orally with metformin (300 mg/kg), val-pyr (30 mg/kg), a mixture of both (300 mg/kg metformin and 30 mg/kg val-pyr), or the vehicle (distilled water) via a gastric tube at 10:00 AM ($n = 7/\text{group}$). Blood (200 μl) was collected from the caudal vein into a heparinized capillary tube 0, 1, 3, 5, and 7 h after the administration and used for the measurement of plasma intact GLP-1 levels.

Subchronic Administration Study. Zucker *fa/fa* rats were divided into the following groups: vehicle (distilled water), val-pyr alone (30 mg/kg), metformin alone (300 mg/kg), and both val-pyr (30 mg/kg) and metformin (300 mg/kg); $n = 10$. These agents were administered by oral gavage twice daily (10:00 AM and 5:00 PM) from 13 weeks of age for 14 days.

Oral glucose tolerance test (OGTT) was performed on days 1 and 15 in the repeated administration study. The animals were deprived of food for 18 h and then the drugs were administered 0.5 h before glucose load (2 g/kg). Blood samples (250 μl) were collected 0.5 h before the glucose load, and 0, 0.5, 1, and 2 h after the glucose load from the caudal vein, using heparinized capillary tubes. They were centrifuged to obtain plasma, which was used for the measurement of insulin and active GLP-1 levels. Blood samples (10 μl) were also taken at -0.5, 0, 0.5, 1, 2, and 3 h from the caudal vein for blood glucose determination.

Food intake was measured every day (5:00 PM), and body weight was monitored under fed conditions on days 4, 7, 11, and 14 (5:00 PM). In addition, the rats were weighed in the fasted state on days 0 and 15 (10:00 AM).

Statistical Analysis. Data are expressed as the mean \pm S.E.M. The statistical significance of differences among the vehicle, val-pyr, metformin, and combination groups was determined by one-way analysis of variance, followed by the Dunnett type multiple comparison test. A value of $p < 0.05$ (two-sided) was considered statistically significant. Statistical analysis was conducted using the software package SAS 8.1 (SAS Institute Japan Ltd., Tokyo, Japan).

Results

Effects of Acute Metformin and/or val-pyr Treatment on Plasma Active GLP-1 Levels in Fed Zucker *fa/fa* Rats. Zucker *fa/fa* rats were given metformin, val-pyr, or a mixture of both in the basal fed state. There were no statistically significant differences in basal plasma active GLP-1 levels among the four groups: vehicle, 5.5 ± 0.1 ; val-pyr, 5.3 ± 0.2 ; metformin, 5.7 ± 0.3 ; and combination, 5.4 ± 0.2 pM. Figure 1 shows that a significant increase of plasma active GLP-1 was observed only in the group given the combination treatment of metformin and val-pyr at 0, 1, 3, 5, and 7 h after the acute administration. Either metformin or val-pyr alone caused a slight elevation of plasma active GLP-1, but it was not statistically significant.

Baseline Body Weight, Food Intake, and Blood Glucose, Plasma Active GLP-1 and Plasma Insulin Levels in Zucker *fa/fa* Rats. Before the subchronic study, there were no significant differences of baseline body weight of the rats in the fed state or of food intake during 24 h among the

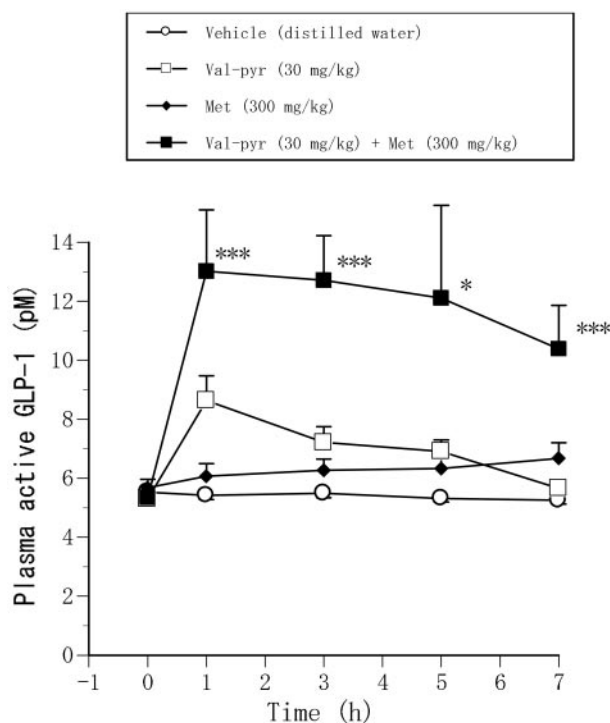


Fig. 1. Changes of plasma active GLP-1 levels in Zucker *fa/fa* rats treated with met and/or val-pyr, a DPPIV inhibitor. Rats were orally given met (300 mg/kg), val-pyr (30 mg/kg), or a mixture of both in the fed state. Significant increases of GLP-1 occurred only in rats treated with both met and val-pyr. * indicates significant differences versus the vehicle-treated group. Data are shown as means \pm S.E.M., $n = 7$ per group. One and three symbols indicate $p < 0.05$ and $p < 0.001$, respectively.

four groups: body weight (vehicle, 528.3 ± 5.5 ; val-pyr, 528.6 ± 2.2 ; metformin, 526.0 ± 6.3 ; and combination, 527.2 ± 6.9 g) and food intake (vehicle, 31.1 ± 1.1 ; val-pyr, 31.4 ± 1.1 ; metformin, 31.8 ± 1.1 ; and combination, 32.1 ± 1.2 g). There were also no statistically significant differences of basal fasting blood glucose, plasma insulin, or plasma active GLP-1 level among the four groups, indicated in Table 1.

Effects of Subchronic Metformin and/or val-pyr Treatment on Food Intake and Body Weight Gain. A significant reduction in food consumption had already occurred on day 1 in the combination treatment group ($p < 0.01$), and this reduction continued throughout the experiment (Fig. 2). In the metformin-treated rats, a significant decrease of food intake was observed only on day 1. On day 14, the values of cumulative food intake were as follows: vehicle, 478.1 ± 14.8 ; val-pyr, 491.8 ± 11.1 ; metformin, 484.4 ± 13.3 ; and metformin plus val-pyr, 417.3 ± 11.1 g.

Reflecting the continuous reduction in food consumption, body weight gain in the combination group was significantly smaller than those in the other groups on days 4, 7, 11, and 14 ($p < 0.05$) (Fig. 3). Body weight gains in the fed state on day 14 were 66.0 ± 4.2 , 60.9 ± 4.4 , 62.6 ± 3.7 , and 39.5 ± 4.7 g in the vehicle, val-pyr, metformin, and combination groups, respectively.

Effects of Metformin and/or val-pyr on Blood Glucose, Plasma Insulin, and Plasma Active GLP-1 in OGTT. On day 1, metformin did not affect plasma active GLP-1 level at any time point after glucose load (Fig. 4C). Val-pyr increased plasma active GLP-1 level at 1 h ($p < 0.01$), compared with the control, but GLP-1 returned to the control level at 2 h (Fig. 4C). In the combination group, a marked increase of active GLP-1 was observed at 0, 0.5, 1, and 2 h, in comparison with the vehicle group ($p < 0.001$, $p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively) (Fig. 4C). The combination treatment of metformin with val-pyr improved glucose tolerance more effectively than either metformin or val-pyr treatment alone (Fig. 4A). Insulin secretion in response to the oral glucose load was greater in the val-pyr-treated rats at both 0.5 and 1 h compared with the vehicle-treated rats ($p < 0.05$) (Fig. 4B). On the other hand, no significant elevation was observed in plasma insulin level in either the metformin or combination group. After the subchronic treatment, similar responses of blood glucose, insulin, and active GLP-1 were observed in OGTT (Fig. 4, D–F).

Effects of Subchronic Treatment with Metformin and/or val-pyr on Fasting Blood Glucose, Plasma Insulin, and Plasma Active GLP-1. After the 14-day adminis-

tration, the subchronic combination treatment showed a great reduction of fasting blood glucose and insulin levels versus metformin or DPPIV inhibitor treatment alone (Table 1). No significant difference of basal plasma GLP-1 level was found among the four groups after the 14-day administration.

Discussion

Our previous study showed that metformin causes a clear elevation of plasma active GLP-1 level in the presence of DPPIV inhibitor in normal fasted rats and increases plasma active GLP-1 in a dose-dependent manner in DPPIV-negative rats (Yasuda et al., 2002). These results suggest that metformin increases plasma active GLP-1 in a DPPIV-independent manner possibly through direct GLP-1 secretion.

In this study, we confirmed that the combination treatment of metformin with val-pyr led to a marked and lasting increase of plasma active GLP-1 level in fed Zucker *fa/fa* rats, an animal model of obesity and mild type 2 diabetes, although neither metformin nor val-pyr alone caused any significant change of the basal active GLP-1 level. Although the detailed mechanism of the enormous impact of the combination on active GLP-1 is unclear, we hypothesize that GLP-1 secretion by metformin plays a major role in the great increase of GLP-1 as mentioned in our previous report (Yasuda et al., 2002). However, possible other factor(s) may contribute to the lasting existence of GLP-1 in plasma; for example, metformin might inhibit renal GLP-1 excretion or increase transcription/translation of the proglucagon gene.

Furthermore, the present study revealed that the combination treatment, but not the metformin or val-pyr treatment, resulted in a significantly reduced body weight gain during 14-day subchronic administration, presumably due to the significant decrease of food intake, in Zucker *fa/fa* rats. This phenomenon could be explained by the remarkable increase of plasma active GLP-1 level in the animals receiving the combination treatment, because it is well known that GLP-1 enhances satiety and causes inhibition of food intake and body weight gain in animal models and type 2 diabetes patients (Tang-Christensen et al., 1996; Flint et al., 1998; Kalra et al., 1999; Zander et al., 2002).

We speculate that the alteration of appetite in the combination treatment group could have been caused through both peripheral and central actions. As a peripheral action, GLP-1 delays gastric emptying, which would be expected to induce a sensation of fullness (Willms et al., 1996; Flint et al., 1998). Furthermore, a central effect of GLP-1 on satiety in the

TABLE 1

Blood glucose, plasma insulin, and active GLP-1 levels of fasted Zucker *fa/fa* rats treated with met (300 mg/kg) and/or val-pyr (30 mg/kg), a DPPIV inhibitor, b.i.d. for 14 days

The values are those just before the drug treatment on day 1 and day 15. Data are shown as means \pm S.E.M. $n = 10$ per group.

	Day 1			Day 15		
	Glucose	Insulin	Active GLP-1	Glucose	Insulin	Active GLP-1
	mg/dl	ng/ml	pM	mg/dl	ng/ml	pM
Vehicle	101.4 ± 3.4	9.8 ± 1.1	4.8 ± 0.2	$116.9 \pm 4.3^{\#}$	$17.9 \pm 1.6^{\#\#}$	4.7 ± 0.1
met (300 mg/kg)	102.6 ± 3.0	8.8 ± 1.1	5.4 ± 0.3	107.4 ± 1.9	$10.7 \pm 1.1^{**}$	5.8 ± 0.8
val-pyr (30 mg/kg)	108.9 ± 5.6	11.9 ± 1.1	5.1 ± 0.3	$105.4 \pm 1.7^*$	$16.0 \pm 1.6^{\#\#}$	5.0 ± 0.3
val-pyr (30 mg/kg) + met (300 mg/kg)	99.0 ± 4.6	9.3 ± 1.3	5.1 ± 0.5	$97.3 \pm 3.8^{**}$	$8.4 \pm 0.7^{**}$	7.4 ± 2.5

* $p < 0.05$ and ** $p < 0.01$ compared with those of the vehicle group on the same day.

$^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with those on day 1.

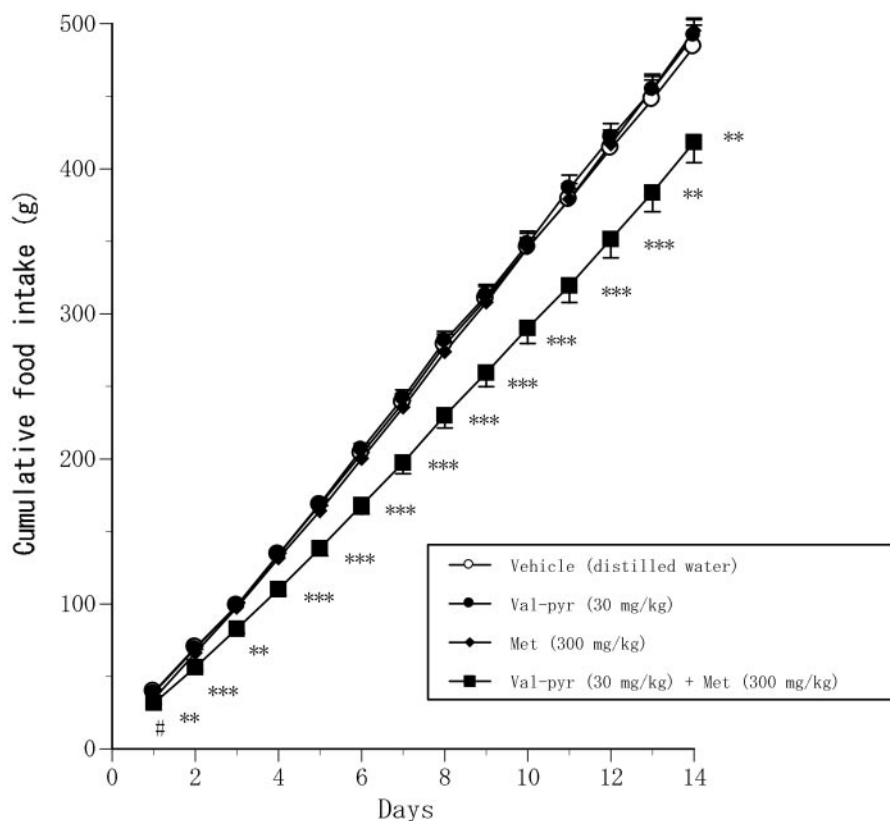


Fig. 2. Cumulative food intake in male obese *fa/fa* Zucker rats treated with met (300 mg/kg) and/or val-pyr (30 mg/kg), a DPPIV inhibitor, b.i.d. (10:00 AM and 5:00 PM) for 14 days. Cumulative food intake is calculated from the beginning of the study at 13 weeks of age. Food intake was determined by weighing the remaining food every day before the treatment at 5:00 PM. # and * indicate significant differences in the met- and both val-pyr and met-treated groups, respectively, compared with the vehicle-treated group. Data are shown as means \pm S.E.M., $n = 10$ per group. One, two, and three symbols indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

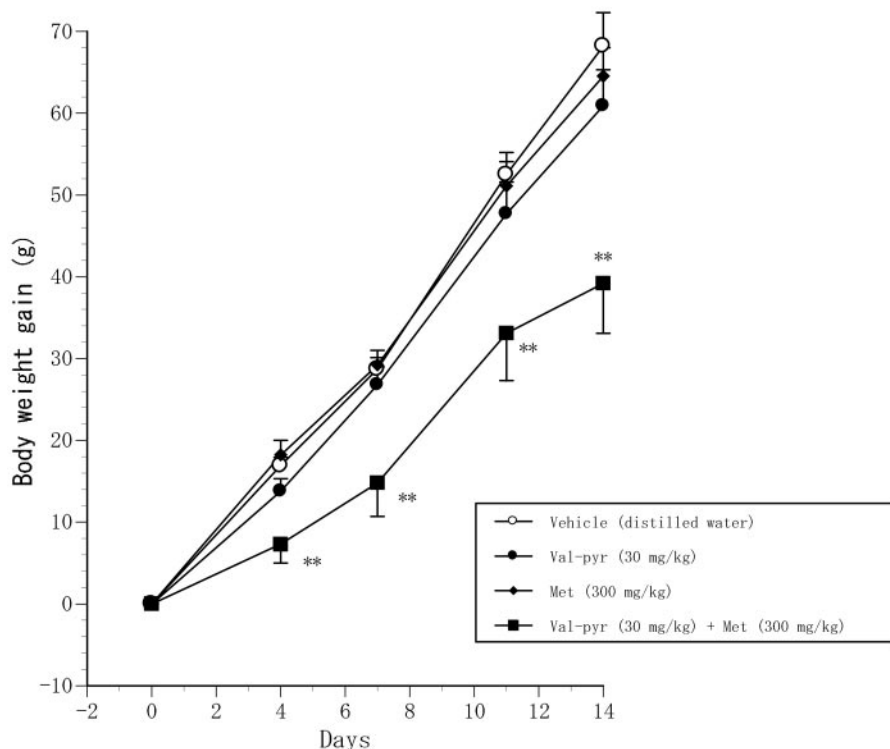


Fig. 3. Changes of body weight gain in male obese *fa/fa* Zucker rats treated with met (300 mg/kg) and/or val-pyr (30 mg/kg), a DPPIV inhibitor, b.i.d. (10:00 AM and 5:00 PM) for 14 days. Body weight gain is the change in body weight from the beginning of the study at 13 weeks of age. Body weight was monitored under fed conditions on days 4, 7, 11, and 14 before the administration at 5:00 PM. There are significant differences between the vehicle control and the val-pyr and met-group (**, $p < 0.01$). Data are shown as means \pm S.E.M., $n = 10$ per group.

paraventricular nucleus of the hypothalamus has been reported (Turton et al., 1996; Kalra et al., 1999). However, further studies will be needed to clarify the mechanisms involved, including the possibility that other factors besides GLP-1 play a role.

To examine the effect of the combination treatment with

metformin and val-pyr on oral glucose tolerance in Zucker *fa/fa* rats, OGTT was conducted before the subchronic administration in Zucker *fa/fa* rats. This study indicated that a marked increase of active GLP-1 level was induced by the combination treatment in fasted Zucker *fa/fa* rats. In addition, the improvement of glucose tolerance caused by the

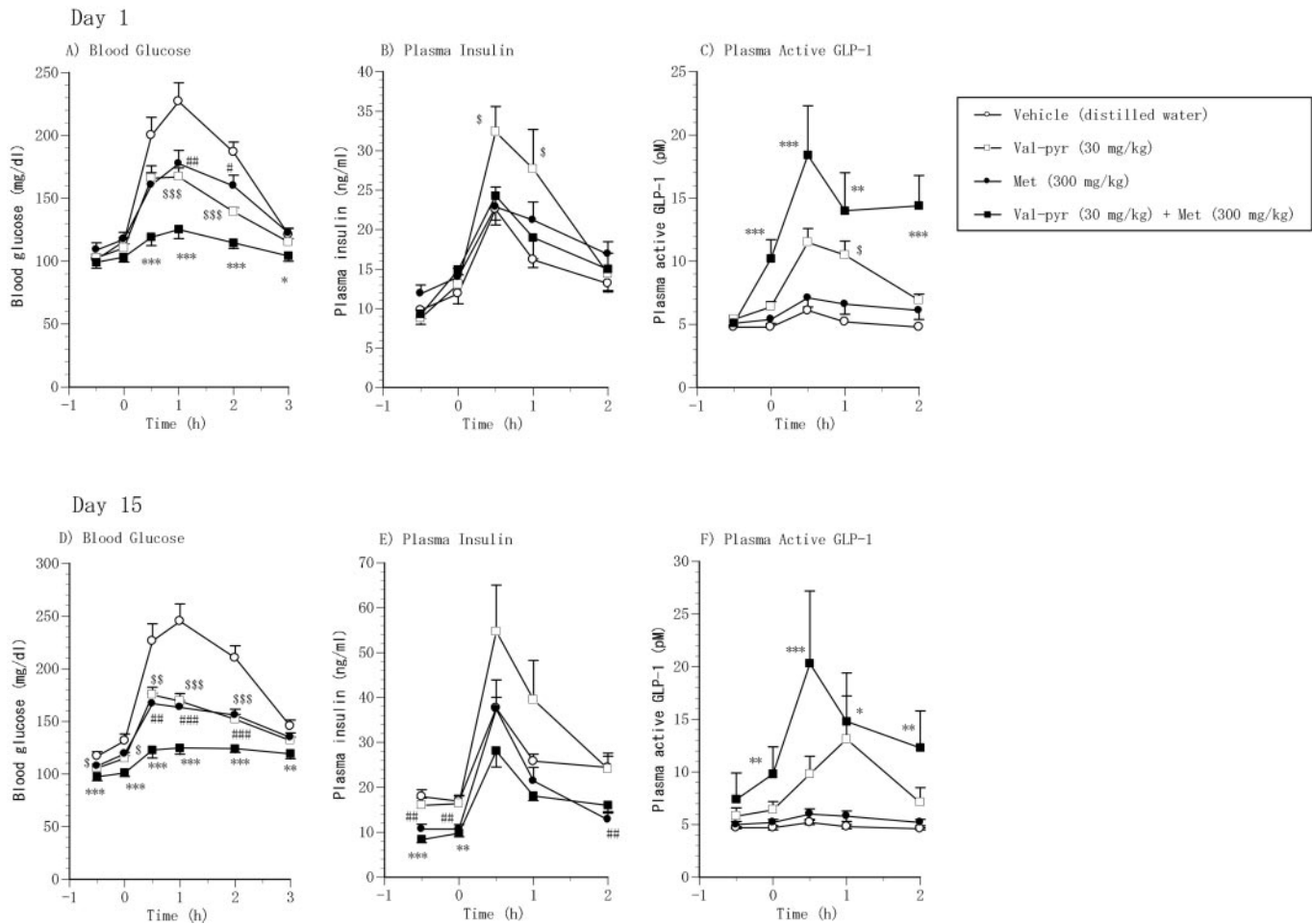


Fig. 4. Changes of blood glucose (A and D), plasma insulin (B and E), and plasma active GLP-1 excursion (C and F) in oral glucose tolerance test in male obese *fa/fa* Zucker rats treated with met (300 mg/kg) and/or val-pyr (30 mg/kg), a DPPIV inhibitor, on day 1 (A–C) or 15 (D–F). \$, #, and * indicate significant differences in the val-pyr-, met-, and both val-pyr and met-treated groups, respectively, compared with the vehicle-treated group. Data are shown as means \pm S.E.M., $n = 10$ per group. One, two, and three symbols indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

combination treatment was greater than that produced by monotherapy with metformin or val-pyr in the OGTT. Val-pyr increased active GLP-1 level after glucose loading through the inhibition of active GLP-1 degradation by DPPIV and caused an enhancement of glucose-dependent insulin secretion with a consequent attendant improvement in glucose tolerance. In contrast, metformin improved glucose tolerance, without affecting the peripheral plasma insulin or GLP-1 level, suggesting that the antidiabetic effect of metformin may occur through the potentiation of peripheral insulin action and inhibition of gluconeogenesis (Bailey, 1992; Bailey and Turner, 1996). In the combination treatment group, no increase of plasma insulin level was seen in response to the oral glucose load, in spite of the greater increase of plasma active GLP-1 level. The reason for this is not clear. As a possible explanation, we speculate that an increase of portal insulin may be involved in this phenomenon. Because plasma active GLP-1 was already sufficiently high to enhance glucose-stimulated insulin secretion before the glucose load, enhancement of portal insulin level by GLP-1 could occur immediately after the glucose administration and could promote the metformin-induced inhibition of glucose production in the liver, leading to the suppression of acute elevation of blood glucose. This could explain why no increase

of insulin level was observed in the peripheral caudal region. Alternative interpretation is that the enhanced GLP-1 levels per se directly contributes to the improvement of glucose tolerance, because GLP-1 itself has antidiabetic effects, such as inhibition of glucose production from liver (Ikezawa et al., 2003) and increase of glycogen synthase activity (Alcántara et al., 1997).

On day 15, responses of blood glucose, insulin, and active GLP-1 were basically similar to those on day 1 in OGTT. But it is noteworthy that the subchronic coadministration of metformin and val-pyr caused significant reductions of fasting blood glucose and fasting plasma insulin level (namely, the values at -0.5 h in the OGTT), compared with the vehicle treatment group. In this study, the changes were not significant between the combination group and the metformin or val-pyr alone group. Zucker *fa/fa* rats are obese and insulin resistant, with normal or slightly elevated glucose concentrations in the basal state, reflecting glucose intolerance. To demonstrate effects of the combination treatment on the improvement of insulin resistance and glycemic control more clearly, a further study will be needed using type 2 diabetic animal models with hyperglycemia and marked insulin resistance, such as Zucker diabetic fatty rats and *db/db* mice.

Obesity, which leads to hyperlipidemia, hyperinsulinemia,

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