An Ascochlorin Derivative, AS-6, Reduces Insulin Resistance in the Genetically Obese Diabetic Mouse, *db/db*

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SUMMARY

An ascochlorin derivative, AS-6, is a new hypoglycemic agent orally active in both obese hyperinsulinemic and insulin-deficient diabetic animal models. AS-6, when given as a 0.025–0.2% admixture in the diet, dose-dependently ameliorated polydipsia, polyuria, and glycosuria in the genetically obese diabetic mouse, C57BL/KsJ *db/db*, while neither insulin nor tolbutamide showed any beneficial effects. The amelioration by AS-6 was associated with a marked decrease in serum glucose and triglyceride. The effects persisted at least 10 wk, accompanied by a steady decrease in drinking water consumption. The chronic treatment prevented pancreatic islet degeneration, e.g., degranulation of the β-cells, basophilic appearance of the exocrine border around the islets, and small round cell infiltration. The isolated islets from AS-6-treated mice released much more insulin in response to glucose than those from untreated controls. A significant correlation between serum immunoreactive insulin and glucose/triglyceride from both treated and untreated mice suggests that AS-6 restores sensitivity and responsiveness to insulin to the mice.

In fact, the combined treatment with insulin synergistically decreased serum glucose by 50% below AS-6 treatment alone. Furthermore, the epididymal fat pad slices from AS-6-treated *db/db* mice increased CO_2 generation and lipogenesis over the untreated controls, and the glucose metabolic rate (CO_2 generation plus lipogenesis from U-[^{14}C]-glucose) in the slices and the serum glucose level inversely correlated at r = 0.8799. These facts indicate that AS-6 reduces insulin resistance in *db/db* mice. DIABETES 1985; 34:267–74.

In 1968, the present authors isolated ascochlorin from the culture broth of a fungus, *Ascochyta viciae*.[1,2] Since then, we have studied the effects of ascochlorin and its derivatives on carbohydrate and lipid metabolism in mammals.[3–6] Ascochlorin is poorly absorbed from the gastrointestinal tract because of its low polarity. Therefore, a new derivative, 4-O-carboxymethylated derivative (AS-6)[7] was synthesized to improve the physicochemical properties. Recent reports from our laboratory have shown that AS-6 potentiates insulin action both in normal and in streptozocin (STZ)-induced diabetic rodents.[8,9] The results prompted us to investigate whether or not AS-6 improves the metabolic defects in the genetically obese diabetic mouse C57BL/KsJ *db/db (db/db)* mouse,[10,11] as this mouse is a suitable model for studying the insulin resistance in diabetic state.

MATERIALS AND METHODS

Animals. The 12-wk-old *db/db* mice and their lean littermates (+/+ or *db*/+) were kindly supplied by the Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. The mice had free access to diet and water throughout the study.

Chemicals. AS-6 (Figure 1) was a kind gift from Dr. I. Matsuura, Research Laboratories, Chugai, Japan. The insulin used was Lente MC, purchased from Novo Industry (Copenhagen, Denmark), and tolbutamide was from Chugai. U-(^{14}C)-glucose was purchased from Amersham International, United Kingdom.

Dose-dependent amelioration of the diabetic syndrome by AS-6. Pharmacologic studies using diabetic animal models should be performed under conditions in which the diet intake is unaltered by the treatment. The *db/db* mice are so nervous that an individual housed in a metabolic cage loses its appetite. Therefore, groups of 3–6 mice were housed in a rat metabolic cage to collect 24-h urine. Also, the mice are so obese that drug administration by gastric intubation often stifles them, so AS-6 and the comparative control agent, tolbutamide, were given mixed with the standard commercial diet (CE-2, Nihon Clea Co., Ltd., Tokyo, Japan).

Twenty female *db/db* mice were randomly separated into four groups (N = 5), and each group was housed in a rat metabolic cage. The first week, they were fed the control

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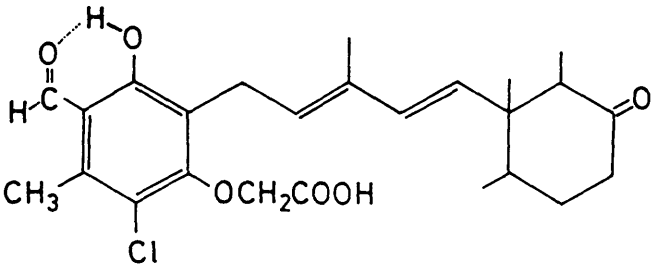

FIGURE 1. The structure of AS-6 (4-O-carboxymethylascochlorin). The dotted line indicates an intramolecular hydrogen bond.

diet, then AS-6 was added to the CE-2 diet for an additional week. During the entire period, the diet intake, water consumption, urine volume, urine glucose excretion, and body weight were measured every day, and averaged. Statistical significance of the difference between the pretreatment and treatment period was calculated by the unpaired Student's *t*-test.

Comparative efficacy of insulin, tolbutamide, and AS-6. Twenty-four male *db/db* mice were randomly allocated to four groups (N = 6) separately housed in metabolic cages. For the first 4 days, the mice were fed CE-2 only. For the succeeding 1 wk, one group was assigned as the untreated control, a second group as the insulin-treated control (15 U/kg, s.c., once daily), the third as the tolbutamide-treated control (0.2% in the CE-2), and the fourth as the AS-6-treated group (0.1% in the CE-2). During the study, the diet intake, water consumption, urine volume, urine glucose excretion, and body weight were measured daily. Statistical significance was calculated using analysis of variance.

Hypoglycemic response to AS-6. The studies were divided into two parts. For the 1-wk study, 14 male *db/db* mice and their 7 lean littermates were used. The *db/db* mice were randomly allocated to two groups (N = 7). One group, together with the lean mice, were fed the CE-2 diet. The other *db/db* group was fed the CE-2 diet containing 0.1% AS-6. On day 7 at 9:30 a.m., the food was removed from the cages, and the mice were killed 6 h later with ether anesthesia. Blood was withdrawn from the heart immediately after death. For the 10-wk study, 16 female *db/db* mice and their 8 lean littermates were treated according to the same experimental design as above. Once weekly, the mice were housed in metabolic cages for 24 h to measure the diet intake and water consumption. On day 70, the mice were killed, the blood was withdrawn from the heart, and the pancreas was removed as quickly as possible. The dissected pancreata were fixed with buffered 10% formalin.

Correlation between serum immunoreactive insulin (IRI) and serum glucose/triglyceride. Seventeen female *db/db* mice were randomly allocated to two groups, and the first group (N = 9) together with their lean group (N = 6) were fed the CE-2. The other *db/db* group (N = 8) was fed the CE-2 with 0.1% AS-6 added. After 7 days, the mice were killed and the serum glucose triglyceride, and IRI were measured.

Synergistic hypoglycemic effect of AS-6 with insulin. Twenty male *db/db* mice were randomly allocated to 4 groups (N = 5). The first and second *db/db* groups and their two lean-mice groups (each N = 5) were fed the CE-2 diet. The third and fourth *db/db* groups were fed the CE-2 with a 0.1% addition of AS-6. In addition, both the second and fourth *db/db* groups and the second lean group were injected with insulin (6 U/kg, s.c.) once daily for seven consecutive days. The others received saline. On day 7, the mice were killed 2 h after the final injection, and the serum glucose was determined.

Insulin release from the islets of Langerhans:[12] AS-6-treated db/db mice. Eight female *db/db* mice, 23 wk old, were randomly allocated to two groups (N = 4). One group was fed CE-2 and the other CE-2 containing 0.1% AS-6 for 1 wk. They were then killed and the pancreas was quickly removed. The pancreata of each group were digested with collagenase (Worthington, type IV) and the islets were collected. Forty-five islets of approximately the same size were suspended in Krebs-Ringer bicarbonate buffer (pH 7.3) supplemented with 2% bovine serum albumin, 5 mM each of Na-glutamate, Na-pyruvate, and Na-fumarate, and 2.8 mM of glucose, and packed into a perifusion chamber. The chamber was perifused at a constant rate; 20 min later, the glucose was increased to 20 mM. The perfusate was collected at 2-min intervals and the IRI released was determined with an RIA kit using porcine insulin as standard.

The effect of AS-6 on insulin release from isolated rat islets in vitro. Three male Wistar rats, weighing 340 g, were killed and the pancreata were removed. The islets were isolated as above, and 75 islets/chamber were perifused with or without 10 μM of AS-6.

Histopathologic examination of pancreas. For light microscopy, pancreas tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained. The staining intensity was graded as follows: ± represents a trace just above the lean mice, and + through + + + represent increasing degrees of histopathologic changes. All histopathologic sections were read without knowledge of the tissue's identity.

Metabolic activity of the adipose tissue in vitro.[13] Glucose metabolism was studied using epididymal fat pad slices from

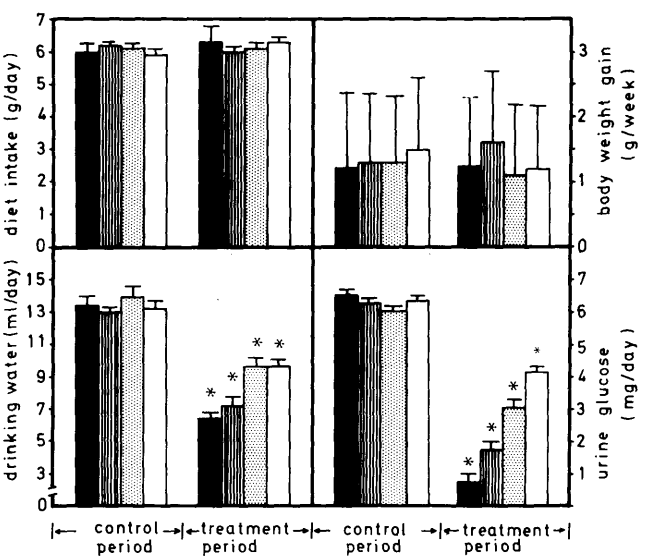

FIGURE 2. Dose-dependent amelioration of the diabetic syndrome by AS-6. (■), AS-6 0.2% in the diet during treatment; (▨), AS-6 0.1%; (▩), AS-6 0.05%; and (□), AS-6 0.025%. The bars represent mean ± SEM; *P < 0.05 and *P < 0.01.

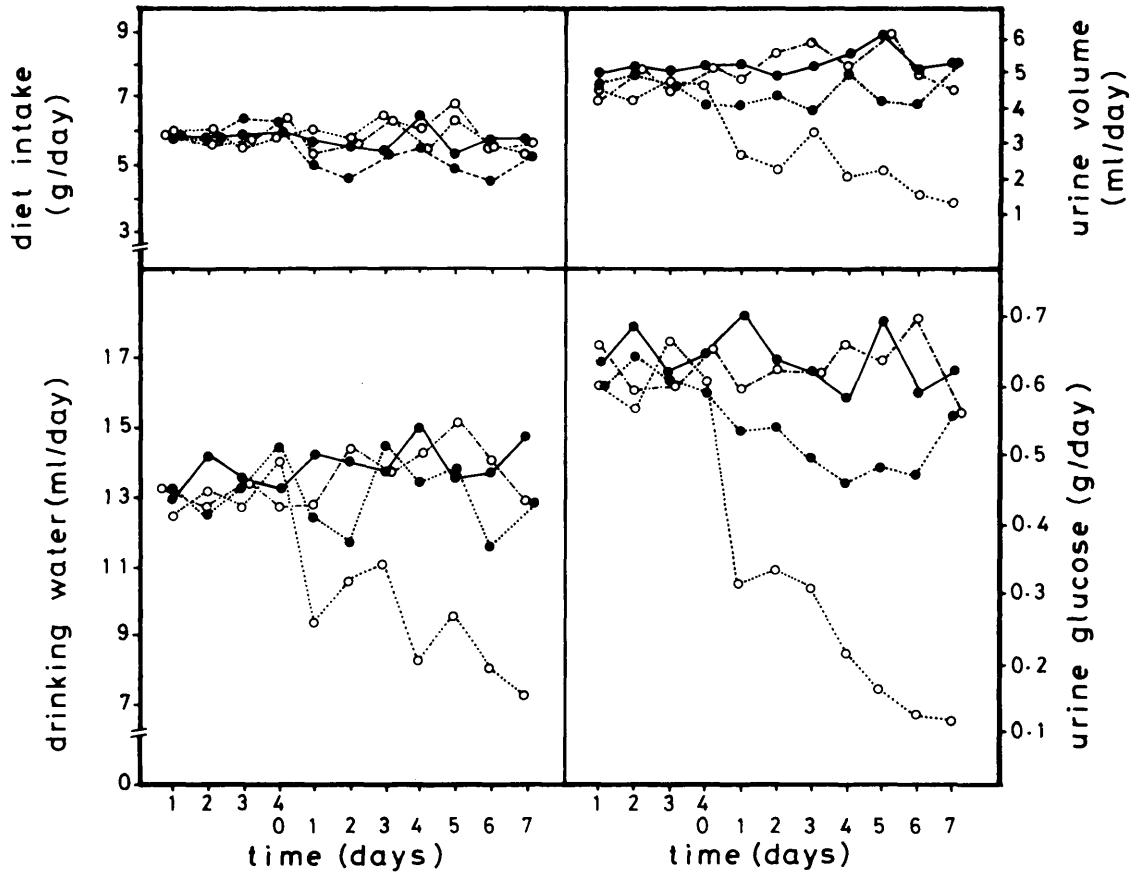

FIGURE 3. Comparison of efficacy between insulin, tolbutamide, and AS-6. (●——●), Untreated controls; (●·····●), treated with tolbutamide (0.2% in the diet); (○–·–·–○), treated with insulin (15 U/kg, once daily, s.c.); and (○·····○), treated with AS-6 (0.1% in the diet).

AS-6-treated and untreated *db/db* mice and their lean littermates in Krebs-Ringer bicarbonate buffer (pH 7.4) containing one-half the usual amount of calcium and 7.5 mM glucose. The control mice were fed the CE-2 diet and the treated *db/db* mice the CE-2 diet containing 0.1% AS-6. After 1 wk, the mice were killed, the blood was withdrawn from the heart, and the epididymal adipose tissues were removed. The tissue from *db/db* mice was cut into pieces approximately the same weight as that of an epididymal fat pad from a lean mouse to minimize differences in diffusion rates of substrates into the tissue. U-(^{14}C)-glucose was used at 1 μCi/ml. At the end of the incubation, after acidification of the medium, CO_2 was collected on pieces of filter paper moistened with 20% phenetylamine, which were subsequently dried and counted in toluene-based scintillation fluid. The tissue pieces were extracted according to Dole,[14] and radioactivity in acylglycerol was counted. The blood was centrifuged and the serum glucose was determined.

TABLE 1
Effects of AS-6 on serum glucose and lipid levels in *db/db* mice (mean ± SEM)

Treatment period	Groups	N	Serum biochemistry (mg/dl)			
			Glucose	Triglyceride	Cholesterol	Free fatty acid
1 Wk	*db/db* Controls	7	710 ± 70	152 ± 16	111 ± 5	36 ± 2
	db/db Treated with AS-6	7	278 ± 19†	187 ± 18	53 ± 10†	31 ± 3
	Lean littermates	7	197 ± 10†	98 ± 7†	60 ± 8*	24 ± 1†
10 Wk	*db/db* Controls	9	582 ± 42	170 ± 8	149 ± 18	34 ± 5
	db/db Treated with AS-6	8	326 ± 37†	173 ± 7	105 ± 10	36 ± 5
	Lean littermates	6	194 ± 5†	88 ± 1†	91 ± 8†	24 ± 2†

In the 10-wk study, AS-6-treated *db/db* mice gained significantly more weight than the untreated controls; the body weight changes (g/mouse, mean ± SEM) were as follows: the untreated *db/db* controls, 49.2 ± 2.4 (g/mouse) to 52.4 ± 2.4; and the AS-6-treated *db/db* mice, 48.9 ± 0.7 to 57.6 ± 0.5.* The weight gain during 10 wk was significantly larger in the AS-6-treated group than in the untreated controls.
*$P < 0.05$ and †$P < 0.01$: significant difference from untreated *db/db* controls in the unpaired *t*-test.

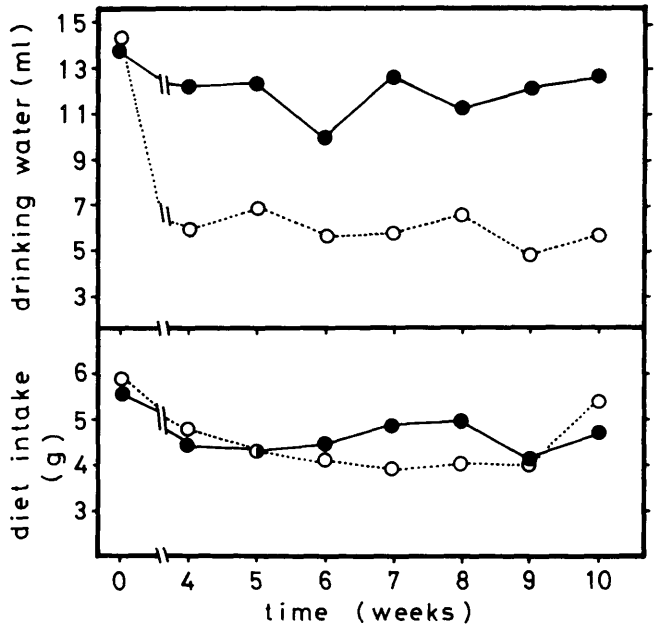

FIGURE 4. Diet intake and water consumption during the 10-wk experiment. (●——●), Untreated controls; and (○——○), treated with AS-6 (0.1% in the diet).

Biochemistry. Glucose was determined with a glucose-oxidase kit from Boehringer Mannheim, Japan, serum triglyceride by the method of van Handel,[15] free fatty acid by the method of Itaya[16] using palmitic acid as a standard, and serum total cholesterol by the method of Zurkowski.[17] Serum IRI was determined with an RIA kit from Dinabbott Laboratories, Osaka, Japan.

RESULTS

Dose-dependent amelioration of the diabetic syndrome by AS-6. The 12-wk-old *db/db* mice were markedly polydipsic, polyuric, and glycosuric (Figure 2). The diet intake and body weight gain were unaffected by AS-6 treatment in all groups, as compared with the preceding control period. However, the treatment dose-dependently ameliorated polydipsia and glycosuria; water intake was reduced 26% (0.025% in the diet) to 52% (0.2% in the diet), and urine glucose output was reduced 35–88%.

Comparison of AS-6 with insulin and tolbutamide. Treatment with large doses of insulin had no beneficial effects on the diabetic syndrome (Figure 3). Tolbutamide treatment significantly reduced the diet intake, probably due to its overdosage. Although consumption of drinking water is strictly proportional to diet intake in rodents, it was unaffected in the tolbutamide group regardless of the smaller diet intake during the treatment period. The slight, but significant, decrease in urine glucose output in this group may simply reflect reduction in caloric intake. In comparison, AS-6 decreased drinking water by 30%, urine volume by 55%, and urine glucose output by 72%. It is characteristic of AS-6 that these responses occurred simultaneously as early as 24 h after the treatment started.

Hypoglycemic response to AS-6. Treatment with AS-6 for 1 and 10 wk decreased serum glucose and triglyceride (Table 1); the treatment for 1 wk decreased serum glucose by 61% and triglyceride by 50% compared with the untreated controls. These beneficial effects persisted as long as 10 wk accompanied by a greater weight gain in the treated group than in the untreated group. However, treatments for both 1 and 10 wk did not alter serum levels of total cholesterol and free fatty acid.

Figure 4 shows the diet and drinking water intake during the 10-wk study. The data from 1 to 3 wk are omitted, since the trend was the same as in the succeeding weeks. The AS-6 treatment resulted in a steady decrease in drinking water below the untreated control levels without affecting the diet intake.

Histopathologic findings in the pancreatic islets. The pancreatic islets of the untreated, 23-wk-old *db/db* mice increased in number and size compared with those of the lean, and marked degranulation of β-cells occurred suggesting the overproduction and exhaustive release of insulin (Table 2). Furthermore, the exocrine border around the islets became basophilic, acinar cells collected, and small round cells interstitially infiltrated into the islets, with fat necrosis occurring around the pancreas. The treatment with AS-6 did

TABLE 2
Histopathologic findings of pancreata in AS-6-treated *db/db* mice and their lean littermates

Findings	Untreated *db/db*					AS-6 Treated					Lean littermates				
	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Increase in size and numbers of islets	0	2	5	1	0	0	1	3	3	1	8	0	0	0	0
Degranulation of β-cells	0	0	0	4	4	0	0	6	2	0	8	0	0	0	0
Collection of acinar cells in islets	1	2	4	1	0	3	4	1	0	0	8	0	0	0	0
Basophilic appearance in exocrine border around islets	0	0	6	2	0	5	3	0	0	0	8	0	0	0	0
Interstitial small round cell infiltration	5	1	1	1	0	8	0	0	0	0	8	0	0	0	0
Fat necrosis around pancreas	5	0	3	0	0	5	0	3	0	0	8	0	0	0	0

The staining intensity was graded as follows: ± represents a level just above that of lean littermates, and + through +++ represent increasing degrees of histopathologic changes.

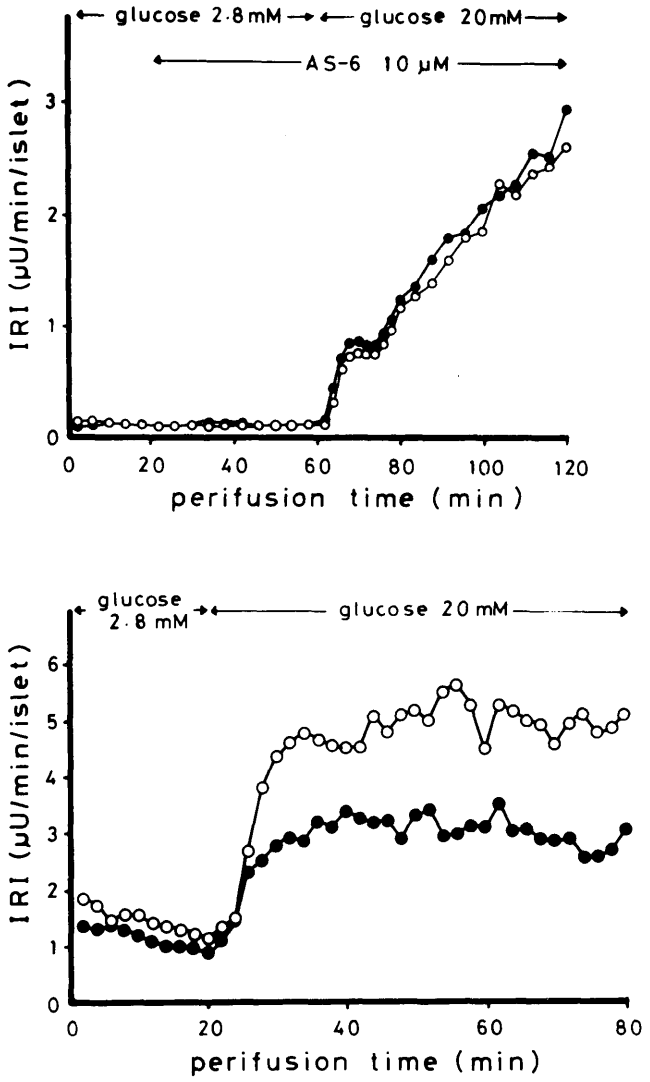

FIGURE 5. Insulin release from isolated pancreatic islets. Top: release from rat islets; bottom: release from *db/db* mouse islets. (●——●), Untreated controls; and (○——○), treated with AS-6 (0.1% in the diet).

not attenuate the increase in size and number of islets, but all other degenerative changes of islets were significantly attenuated. The α-cells were, however, unaffected by the treatment as compared with the untreated controls.

Insulin release from isolated islets. Insulin release from isolated rat islets was slightly affected in the presence of 10 μM AS-6 under the basal glucose concentration of 2.8 mM (Figure 5, upper part). When glucose concentration was increased to 20 mM, insulin release was suppressed by AS-6, but the difference was not statistically significant. The rate of insulin release appears to be much faster and greater in *db/db* mice than in rats (Figure 5, lower part). The islets from AS-6-treated *db/db* mice released significantly more insulin than did those from untreated controls under the stimulus of increased glucose. A similar result occurred with 12-wk-old male *db/db* mice.

Statistical correlation of serum IRI with serum glucose/triglyceride. If treatment restores peripheral sensitivity and responsiveness to insulin, it should decrease the serum IRI after a decrease in insulin requirement in the body. In fact, AS-6 treatment did decrease serum IRI, and when the serum IRI and glucose/triglyceride were plotted, there was a significant correlation between the two sets of parameters (Figure 6).

Synergistic hypoglycemic activity of AS-6 and insulin. The results shown above strongly suggest that AS-6 potentiates peripheral sensitivity and responsiveness to insulin; if so, the treatment should restore the hypoglycemic response to insulin. As shown in Figure 7, daily insulin treatment decreased serum glucose by 50% in the lean mice compared with the untreated controls, while the insulin treatment produced no significant decrease in *db/db* mice. AS-6 treatment alone for 1 wk decreased serum glucose by 30% compared with the untreated *db/db* controls. Moreover, the combined treatment with insulin decreased serum glucose by 50% below the value for AS-6 treatment without insulin.

Glucose metabolism in epididymal fat pad slices. As shown in Figure 8, the fat pad slices from *db/db* controls metabolized less glucose than did those from the lean littermates. AS-6 treatment recovered the metabolic activity of

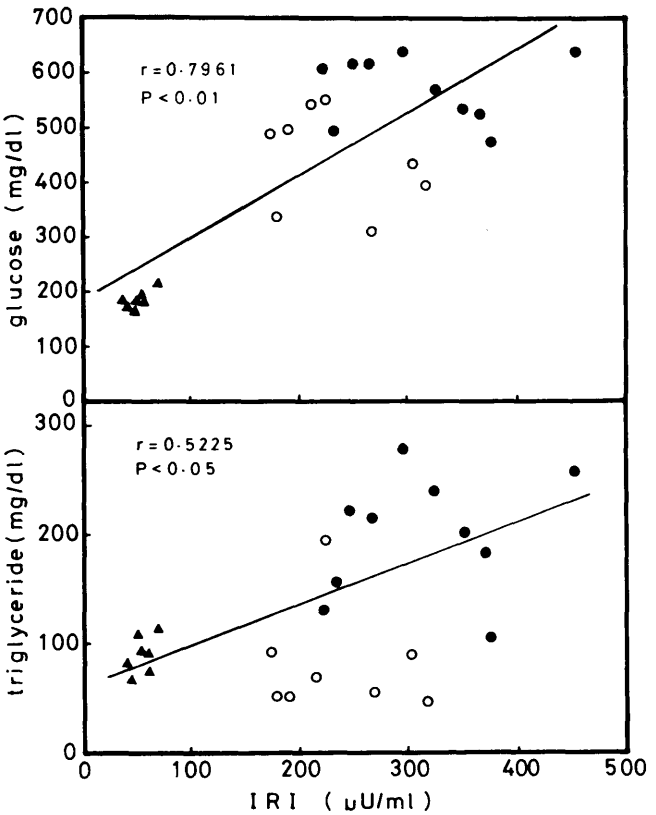

FIGURE 6. Correlation of serum immunoreactive insulin with serum glucose/triglyceride. Seventeen female *db/db* mice were randomly allocated to two groups, and the first group (N = 9) together with their lean littermates (N = 6) were fed the control diet, CE-2. The second *db/db* group (N = 8) was fed the CE-2 containing 0.1% AS-6. After 7 days, all the mice were killed, the blood was withdrawn from the heart, and serum was obtained by centrifugation. The serum glucose, triglyceride, and IRI were then determined, and the individual amounts were plotted for analyzing statistical significance between serum IRI and glucose/triglyceride. The upper figure shows the relation between serum IRI and glucose, and the lower shows the relation between serum IRI and triglyceride. (▲), Lean littermate controls; (●), untreated *db/db* controls; and (○), *db/db* mice treated with AS-6 (0.1% in the diet).

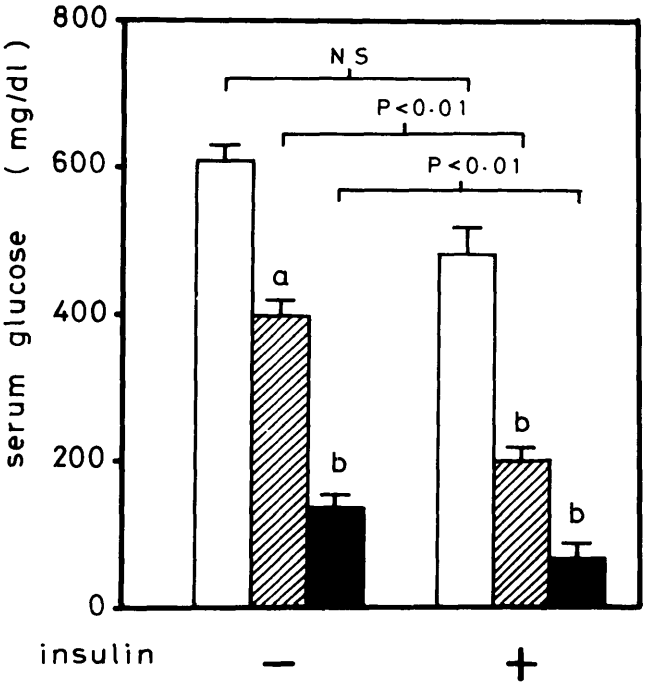

FIGURE 7. Synergistic hypoglycemic activity of AS-6 with insulin. The bars represent mean ± SEM. The open bars represent *db/db* mice treated with AS-6 (0.1% in the diet), the shaded bars are untreated *db/db* controls, and black bars are untreated lean littermate controls. $^{a}P < 0.05$ and $^{b}P < 0.01$.

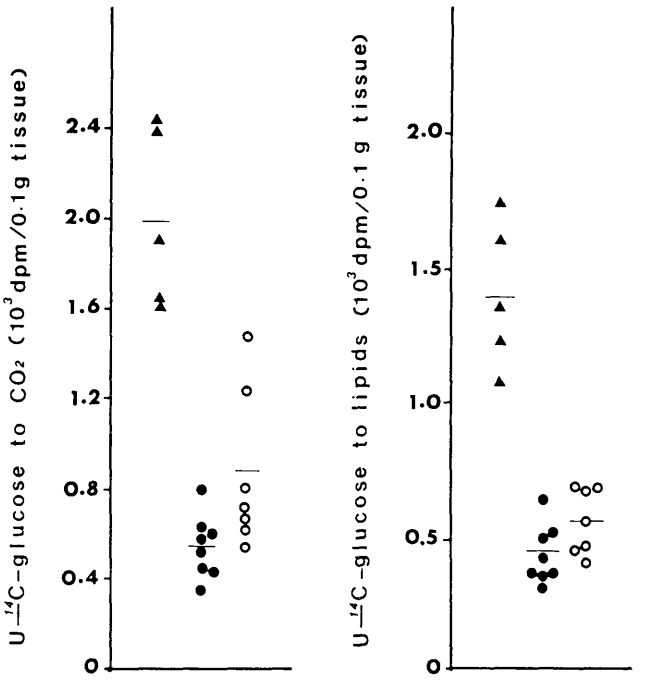

FIGURE 8. Glucose metabolism in the epididymal fat pads from AS-6-treated and untreated *db/db* mice and their lean littermates. Metabolic activity of the epididymal fat pads (conversion of U-[^{14}C]-glucose to CO_2 and lipids/0.1 g tissue/2 h) is shown. The open circles represent the activity of *db/db* mice treated with AS-6 (0.1% in the diet for 1 wk), the filled circles that of untreated *db/db* controls, and the filled triangles that of untreated lean littermates. Horizontal bars represent the mean of each group.

glucose in the epididymal fat pad slices; in the treated group, the CO_2 generation and lipogenesis were 60% and 20% larger than in the untreated controls, respectively.

If AS-6 relieves peripheral resistance to insulin in the *db/db* mice, the serum glucose should be inversely correlated with glucose metabolic rate in the tissues sensitive to insulin. We regarded the epididymal fat pad slices as representative of the sensitive tissues, and examined the statistical relation between these two. As hypothesized, there was a striking correlation ($r = -0.8799$) between the serum glucose and the glucose metabolic rate (CO_2 generation plus lipogenesis from labeled glucose), as shown in Figure 9. This result indicates that serum glucose lowering by AS-6 (42% decrease in this study) is at least partly attributable to an increase in glucose metabolism in the adipose tissue.

DISCUSSION

Pathologic animal models may present a clue to human disease study, and this holds for insulin-resistant diabetes mellitus. Our recent studies have shown the possibility that potentiation of insulin action results in amelioration of the diabetic syndrome in insulin-deficient animal models.[8]

Briefly, AS-6, when given mixed with diet for a week to both normal and STZ-diabetic mice, significantly decreased plasma levels of glucose, lipids, and IRI below the corresponding control levels without affecting the diet intake. The glucose tolerance test in 24-h-fasted normal mice also showed that AS-6 treatment accelerated plasma glucose disposal and IRI decay, although the basal IRI level was significantly lower in the AS-6-treated group than in the untreated controls. In STZ-diabetic mice, AS-6 restored hepatic lipogenesis from acetate in vivo to the normal control level. The inverse relation between serum glucose and CO_2 generation rate from the epididymal fat pad slices derived from both AS-6-treated and untreated STZ-diabetic rats indicates that the treatment relieves the diabetic syndrome through

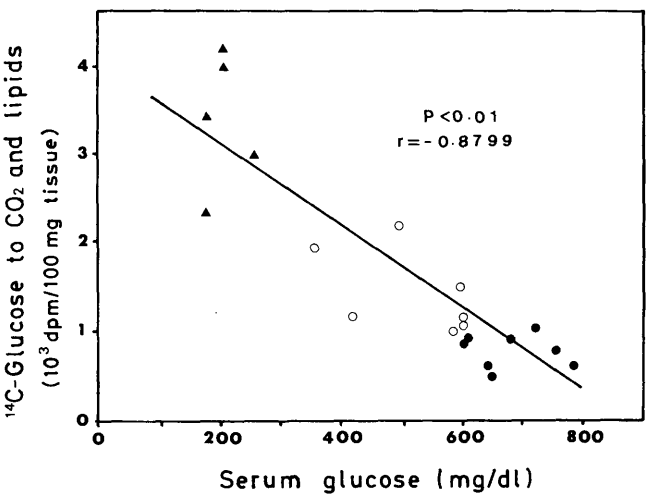

FIGURE 9. Correlation between the serum glucose and glucose metabolic rates (CO_2 generation plus lipogenesis from U-[^{14}C]-glucose) in the epididymal fat pads. The open circles represent *db/db* mice treated with AS-6 (0.1% in the diet), the filled circles are untreated *db/db* controls, and the filled triangles are untreated lean littermates. $r = 0.8799$ ($P < 0.01$).

potentiation of peripheral insulin action. This finding provoked our interest in studying the way AS-6 acts in insulin-resistant animal models.

In the present study, AS-6 markedly improved the diabetic syndrome of *db/db* mice; the improvement was noted as early as 24 h after initiating the treatment and persisted for at least 10 wk. Several mechanisms have been suggested to explain how AS-6 ameliorates the syndrome. The result shown in Figure 2 completely ruled out the possibility that the improvement is due to decrease in diet intake. It is unlikely that AS-6 acts on renal tubules, thus unusually enhancing the reabsorbing capacity of glucose, electrolytes, and water, since AS-6 not only ameliorated polydipsia and glycosuria, but also produced both hypocaloremic and hypoinsulinemic responses in the *db/db* mice. The hepatic mechanism, by which AS-6 inhibits gluconeogenesis after hepatotoxicity, is also unlikely because the treatment for 10 wk showed no toxic signs in liver either biochemically or histopathologically. That AS-6, like tolbutamide, stimulates insulin release from islets is highly improbable, because *db/db* mice are hyperinsulinemic, being unresponsive to exogenous insulin,[18] and tolbutamide showed no beneficial effects.

The most plausible mechanism is that AS-6 releases peripheral resistance to insulin in *db/db* mice. Genuth et al.[19] have shown that the serum glucose level is correlated with serum IRI in obese hyperglycemic mice. If AS-6 treatment would restore the sensitivity and responsiveness to insulin in the peripheral tissues of *db/db* mice, the resulting down-regulation would reduce the insulin requirement in association with a fall in serum concentrations of IRI and metabolic fuels. As expected, the hypoglycemic and hypotriglyceridemic activities of AS-6 were correlated with the hypoinsulinemic activity, and the combined treatment with insulin synergistically decreased serum glucose compared with AS-6 treatment alone. The assumption was further supported by histopathologic findings of pancreas and insulin release from the isolated islets. The islets of untreated 23-wk-old *db/db* mice showed degenerative changes that were probably induced by overproduction and exhaustive release of insulin.[20–22] Such degenerative changes were significantly less in the treated group than in untreated controls, indicating that the insulin requirement became smaller in the AS-6-treated group than in untreated controls. Reduction of insulin requirement by AS-6 treatment may protect the β-cells from overproduction and exhaustive release of insulin. The perifusion study proved that the islets of treated *db/db* mice stored much larger amounts of insulin than those of the untreated controls; these findings were inconsistent with the histopathologic findings. Therefore, the mechanism of AS-6 is different from dehydroepiandrosterone (DHEA), since DHEA treatment produces hyperinsulinemia.[23] Also, the activity of AS-6 is distinguishable from that of ciglytazone,[24,25] since AS-6 produces hypoglycemia in insulinopenic diabetic animal models. That AS-6 treatment improves glucose metabolism in peripheral tissue was confirmed by enhanced metabolic activity of fat pads derived from the AS-6-treated *db/db* mice. The slices from the treated mice metabolized significantly more glucose in vitro, and the metabolic activity (CO_2 generation plus lipogenesis) inversely correlated with the serum glucose level ($r = -0.8799$), indicating that the hypoglycemic activity of AS-6 is due to relief from peripheral insensitivity and unresponsiveness to insulin in *db/db* mice.

Therefore, the evidence presented here unequivocally indicates that AS-6 restores the in vivo sensitivity and responsiveness to insulin in the insulin-resistant, diabetic animal model. Studies of the mechanism of in vitro action will be presented in a forthcoming paper.

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