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EFFECT OF ORAL TREATMENT WITH A NEW HYPOGLYCEMIC AGENT, AS-6, ON THE METABOLIC ACTIVITIES OF ADIPOCYTES IN DB/DB MICE: A comparative study

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Summary: The mechanism of a new hypoglycemic agent, AS-6, was comparatively studied using the adipocytes from AS-6 treated and untreated genetically obese diabetic mice, db/db. The db/db mice were treated for 1 week with a diet admixture of AS-6 (0.1%). The treatment resulted in the following alterations in metabolic activities; (1) AS-6 treatment increased ^{125}I-insulin binding by 1.4-3.3 fold over the insulin range of 1-1000 μU/ml,(2) the treatment increased the basal activities in 2-deoxyglucose uptake, and in CO_2 generation and lipogenesis from U-(^{14}C)-glucose compared with the db/db controls,(3) the treatment partially restored insulin responsiveness in 2-DG uptake and CO_2 generation, and (4) 1 mU/ml of insulin greatly stimulated lipogenesis by 5.6 fold above the basal in the control adipocytes while AS-6 treatment changed the lipogenic response less stimulative to the insulin. The results suggest that AS-6 treatment significantly increases insulin binding to the adipocytes associating with an enhancement in glucose metabolism under basal and physiological concentrations of insulin.

The genetically obese diabetic mouse, C57BL/KsJ db/db (1,2), is an excellent model of insulin-independent adult-onset diabetes (type II). The mouse is hyperinsulinemic, and treatment with large doses of insulin shows no beneficial effects on the diabetic syndrome (3). Oral treatment with the pharmacological dose of an ascochlorin derivative, AS-6, ameliorated within 24 h the diabetic syndrome not only in db/db mice but also in insulin-deficient streptozotocin-diabetic animals (4-6). This amelioration was accompanied by reduction in serum immunoreactive insulin. Therefore, we consider that the common mechanism underlies in the beneficial action of AS-6 in the two-types diabetic models. The present study aims to evaluate how oral treatment with AS-6 affects the carbohydrate metabolism in the cellular level of db/db mice.

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MATERIALS AND METHODS

Animals: Male 12 weeks old db/db mice and their lean littermates used were supplied from the Research Laboratories, Chugai Pharmaceutical CO., Tokyo. They were allowed free access to food and water throughout the study.
Chemicals: U-(^{14}C)-glucose and 1-(^{14}C)-2-deoxyglucose (2-DG), were purchased from Amersham International, England. ^{125}I-insulin was from Dinabbott Laboratories, Osaka, collagenase type IV from Worthington Chemicals, and bovine serum albumin type V (BSA) from Armour.
AS-6 treatment: The mice was given AS-6 as an admixture (0.1%) of diet (CE-2, a standard diet for mouse, Nihon CLEA, Tokyo), since the mice are so fatty that it is difficult to give the drug by gastric intubation. The db/db mice were randomly allocated to 2 groups soon after arrival and the treatment was initiated simultaneous with the allocation. Duration of the treatment was 1 week. In the insulin binding study, the lean littermate controls were fed the CE-2. On day 7 all of the mice were sacrificed by ether anesthesia, and the epididymal fat pads were removed.
Adipocytes: Epididymal adipocytes were prepared according to the method of Rodbell (7). The fat pads from each group were combined, cut into 3 mm pieces, and digested in Krebs-Ringer Bicarbonate buffer (KRB, pH 7.4) containing 5 mM glucose, 2% BSA and 2 mg/ml of collagenase . After 1 h the cells were passed through 250 µm mesh, washed twice with KRB, and counted with a hemocytometer. All of the assay were carried out quadruplicate, and the difference from db/db control was calculated according to the Student unpaired t-test.
Insulin binding (8): Isolated adipocytes were suspended in a buffer containing 35 mM Tris, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, and 1% BSA (pH 7.6), and incubated for 1 h with ^{125}I-insulin and native insulin in plastic flasks in 24 °C shaking water bath. The incubation was terminated by removing 200 µl portions from the flasks and rapidly centrifuging the cells in plastic microfuge tubes with silicone oil (9). The top layer was then removed and the radioactivity was determined. Specific insulin binding was calculated by subtracting the amounts of ^{125}I-insulin nonspecifically bound at each insulin concentration. Nonspecific binding is defined as the amount of ^{125}I-insulin remaining bound in the presence of a large excess of native insulin.
2-DG uptake(10): The adipocytes (2 x 10^5 cells/ml) were incubated in KRB containing labeled 2-DG (0.5 µci/ml) and 2% BSA at 24 °C for 20 min. Four 200 µl aliquots of the cell suspensions were passed through membrane filters, the filters were washed with KRB, dried, and counted in toluene based scintilation fluid.
CO_2 generation and lipogeneis from U-(^{14}C)-glucose (7): The ability of adipocytes to metabolize glucose was determined according to the method of Rodbell with a slight modification (11). The cells were incubated in a total volume of 1 ml in a scintilation vial at 37 °C for 45 min in KRB supplemented with U-(^{14}C)-glucose (0.5 µci/ml), 5 mM glucose, and 2% BSA with or without insulin. Quadruplicate vials were used for each group. After the reaction was terminated by adding 5 N surfuric acid, CO_2 generated was trapped with a filter paper tip moistened with 20% phenetilamine. The radiolabeled lipids synthesized during incubation were extracted by the method of Dole (12). Radioactivity was determined with a liquid scintilation counter.

RESULTS

Insulin binding to adipocytes: Frechet (13) and Kahn (14) have shown that the db/db mice have decreased insulin binding capacity in the plasma membrane of adipose tissue and liver. In the present study the decrease in insulin binding capacity was evident in the adipocytes of the control db/db mice, as

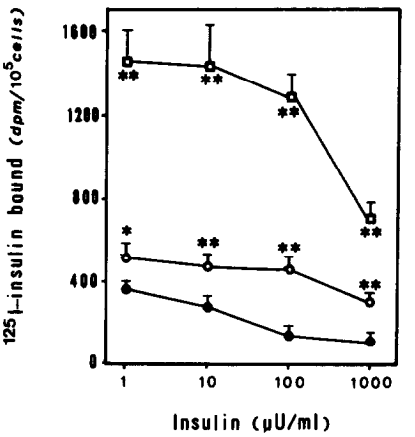


Figure 1. ^{125}I-Insulin binding to the adipocytes.
*P < 0.05 and **P < 0.01, in t-test. Filled circles represent the db/db controls, the open circles the adipocytes from AS-6 treated db/db mice, and the open square their lean littermate controls. The bars indicate SE. (n =4)

shown in Fig.1. The adipocytes from AS-6 treated group bound much more ^{125}I-insulin than those from control db/db mice; those from the treated group bound 1.4 times as much ^{125}I-insulin at 1 μU/ml of native insulin, 1.7 times as much at 10 μU/ml, 3.3 times as much at 100 μU/ml, and 2.8 times as much at 1000 μU/ml. However, exact comparison is difficult on a cellular basis between db/db and their lean littermates, since the diameter of db/db adipocytes enlarged by 2-3 fold compared with their lean littermates (15,16). For this reason the metabolic activities were compared between AS-6 treated and untreated db/db groups.

2-DG uptake in adipocytes: AS-6 treatment increased basal 2-DG uptake (+ 17.5%, $P < 0.05$) in the adipocytes compared with db/db controls (Table 1). In addition, 1 mU/ml of insulin stimulated the uptake in the adipocytes of the treated group, while the hormone had no effect on it in the controls.

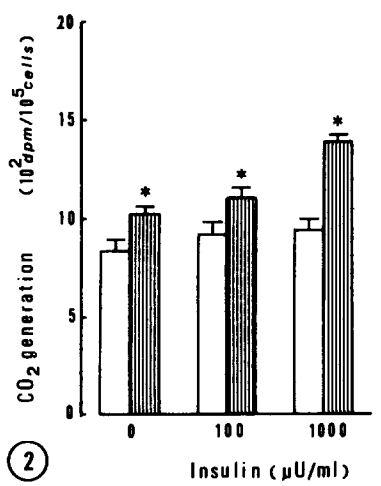
CO_2 generation: As shown in Fig 2, the control adipocytes generated extremely low CO_2 from U-(^{14}C)-glucose in the absence of insulin, and were unresponsive to the hormone (1 mU/ml). AS-6 treatment increased the basal CO_2 generation by 21%, and restored the responsiveness to insulin (1 mU/ml), though the rate of increase was small (+ 35% over the basal, $P < 0.05$).

Table 1. Uptake of 2-DG by the adipocytes from AS-6 treated and untreated db/db mice (Mean ± SE)

Groups	2-DG uptake (dpm/10^5 cells)		P
	Insulin = 0	Insulin = 1 mU/ml	Insulin(-) vs. (+)
Controls	3753 ± 168	3977 ± 351	ns.
AS-6 treated	4410 ± 253*	6282 ± 685**	+ 42.4% *
	(+ 17.5%)	(+ 58.0%)	

*P < 0.05 and **P < 0.01 in the unpaired t-test. (n = 4)

Male db/db mice 12 weeks old were randomly allocated to 2 groups (n = 6), and the treatment was initiated immediately after the allocation. One group together with their lean littermates (n = 6) were fed the control diet (CE-2, Nihon CLEA, Tokyo) and the other group was fed a diet admixture of AS-6 (0.1% in CE-2). On day 7 in the morning all of the mice were killed by ether anesthesia, blood was drawn from the heart, and the epididymal fat pads were removed. The adipocytes were prepared as described in the text according to the method of Rodbell (7). Under this condition, AS-6 treatment does not affect the diet intake in the db/db mice, resulting in decrease in the serum glucose by 25-50%, the triglyceride by 40-60% and the immunoreactive insulin (IRI) by 20-40%. The body weight gain is slightly greater in the treated group than in the controls. Significant statistical correaltions were always noted either between the serum glucose and serum IRI (a positive correlation), or between the serum glucose and basal glucose metabolic rate in the fat pad slices in vitro (an inverse correation) in AS-6 treated and untreated db/db mice and their lean littermates (5).



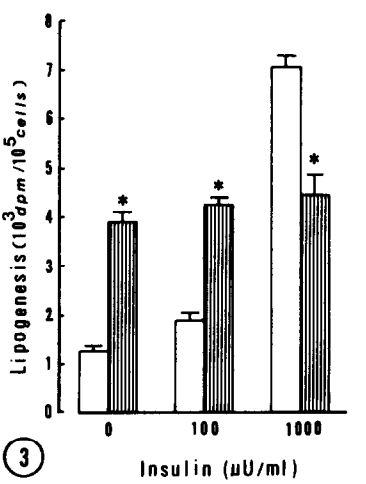


Fig. 2: CO_2 generation from U-(^{14}C)-glucose in the db/db adipocytes. The bars represent the mean + SE (n=4). The open bars are the metabolic activity of the control adipocytes, and the striped bars, that from AS-6 treated db/db mice. The asterisks indicate the statistical significance ($P<0.01$) between AS-6 treated and untreated groups.

Fig. 3: Lipid synthesis from U-(^{14}C)-glucose in the db/db adipocytes. The bars represent the mean + SE (n=4). The symbols are the same as in Fig. 2.

Lipogenesis: In the absence of insulin the adipocytes from AS-6 treated group synthesized more lipids (3.4 fold) from U-(^{14}C)-glucose than the controls (Fig 3). In the presence of 100 μU/ml insulin, the lipogenesis was still higher in AS-6 treated group (2.2 fold) than the controls. However, 1 mU/ml of insulin greatly stimulated the lipogenesis over the basal by 5.6 fold in the control adipocytes, and the control group exceeded the AS-6 treated group($P < 0.01$).

DISCUSSION

This is the first demonstration of metabolic alteration in the adipocytes from db/db mice. The rat adipocyte is a useful tool for studying hormonal effects on the metabolism in vitro. However, the mouse adipocyte has not yet been used for metabolic study, since the adipocytes, even though prepared by the same method as the rat (unpublished data), are less responsive to insulin. It is not clear why the mouse adipocytes respond poorly to insulin. The db/db mice are highly resistant to insulin action and repeated therapy with insulin shows without any beneficial effects on the syndrome. We have proven that AS-6 relieves insulin resistance of db/db mice in animal and at tissue levels thus ameliorating the syndrome (5). Therefore, it is of interest to know how AS-6 treatment alters the carbohydrate metabolism and insulin binding at the adipocyte level.

The present study revealed that the treatment increased insulin binding to db/db adipocytes. We expected that this increase would accomany a relief from insulin resistance at the cellular level, since Kahn et al.(14) have shown that the changes in the binding inversely correlate with the insulin resistance in ob/ob mice. However, the concept is not generally accepted, because insulinopenic diabetes dissociates stimulus-response coupling between insulin binding to its receptors and the response distal to the binding, and an increase in the binding capacity not always associates with restoration in the responsiveness (17,18). AS expected, AS-6 treatment increased all of the basal metabolic activities studied in db/db adipocytes. And this increase

associated with the partial restoration in insulin responsivenesse of 2-DG uptake and CO_2 generation.

In contrast, the control adipocytes responded to high concentrations of insulin selectively enhancing lipid synthesis from glucose. The enhancement was apparently unusual, since it occurred without any increase in CO_2 generation. This metabolic alteration is consistent with the hyperinsulinemia and the resulting obesity in db/db mice. The evidence presented here indicates that db/db adipocytes are not unresponsive to insulin but have an altered responsiveness which results in selective enhancemnt of lipogenesis in adipose tissues leading to obesity.

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