Thiazolidinediones (AD-4833 and CS-045) Improve Hepatic Insulin Resistance in Streptozotocin-Induced Diabetic Rats

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Abstract. To investigate whether thiazolidinediones (AD-4833 and CS-045), new oral antidiabetic agents, are effective in insulin-dependent diabetes mellitus, the effect of thiazolidinediones on streptozotocin-induced diabetic rats was studied by the glucose clamp technique. Diabetic rats were divided into five groups: (1) intensively insulin treated group given a daily injection of 4–6 units Ultralente insulin, (2) AD-4833 group treated with a daily injection of 2 units Ultralente insulin, the minimal dose to make urinary ketones negative, and ingestion of 10 mg/kg of AD-4833 suspended in 5% gum arabic, (3) gum arabic group treated in the same way as the AD-4833 group except for the active drug, (4) CS-045 group treated with the same insulin injection and ingestion of 200 mg/kg CS-045 suspended in 0.5% chlormethyl cellulose, (5) chlormethyl cellulose group treated as the control for the CS-045 group. Seven days after these treatments, all five groups of diabetic rats and normal control rats were subjected to the glucose clamp study in which 3 mU·kg⁻¹·min⁻¹ porcine insulin was continuously infused. Glucose infusion rates (GIR) for the gum arabic and chlormethyl cellulose groups were significantly lower than in control rats, and the rates of hepatic glucose output (HGO) of these two groups were not suppressed, indicating the presence of hepatic insulin resistance. Intensive insulin treatment as well as administration of AD-4833 and CS-045 restored both GIR and HGO towards normal levels. It is concluded that thiazolidinediones improved hepatic insulin resistance in the presence of a minimal dose of insulin.

Key words: Thiazolidinediones, Streptozotocin, Insulin resistance, Glucose clamp.

IT HAS been reported that insulin resistance, one of the fundamental abnormalities seen in non-insulin dependent diabetes mellitus (NIDDM), was improved by thiazolidinediones, newly discovered antidiabetic agents. The use of these agents in NIDDM model animals such as KK mice, Zucker fatty rats, and Wistar fatty rats was effective in improving glycemic control [1, 2], and now clinical trials of these drugs have also been started [3]. However, whether these agents are also effective in insulin dependent diabetes mellitus (IDDM) model animals has not yet been confirmed. To elucidate this, we examined by the glucose clamp technique the effect of AD-4833 and CS-045 on insulin resistance in streptozotocin-induced diabetic rats, a model of IDDM.

Materials and Methods

Streptozotocin-induced diabetic rats and treatments

Male Wistar rats, purchased from Funabashi Farm (Shizuoka, Japan) and kept with free access to standard laboratory food and water in indi-
vidual metabolic cages in the Laboratory Animal Center of our university, were injected in the femoral vein with 50 mg/kg streptozotocin (Sigma, St. Louis, MO) dissolved in 0.05M citrate buffer, pH 4.5. Diabetic animals were allowed to recover for 2 days; after that, to avoid ketosis, which was confirmed by negative urinary test, 1–2 units/day of Ultralente insulin (Novo, Denmark) was injected subcutaneously between 1400–1600 h everyday for 2 weeks.

The rats were then divided into five groups of 5 each, which were treated for 7 days: (1) intensively insulin treated group, (2) AD-4833 group, (3) gum arabic group, (4) CS-045 group, (5) chlormethyl cellulose group. In the intensively insulin treated group, the doses of Ultralente insulin were increased to 4–6 units/day to minimize urinary glucose. Between 1400–1600 h on days 1, 4, and 7, urinary glucose and blood glucose levels were measured by the glucose oxidase method with the Glucose C test kit (Waco, Tokyo). In the AD-4833 group, the rats were treated with 2 units/day of Ultralente insulin and given via gastric tubes 10 mg/kg of AD-4833, the same agent called pioglitazone, provided by Takeda Chemical Industries (Osaka, Japan), suspended in 5% gum arabic (5 ml/kg). Urinary glucose and blood glucose levels were also measured by the same method on the same days as the intensively insulin treated group. In the gum arabic group as the control for the AD-4833 group, the rats were treated with 2 units of Ultralente insulin and given 5% gum arabic without AD-4833 via gastric tube. In the CS-045 group, treatment of the rats was combined with the subcutaneous injection of 2 units/day Ultralente insulin and, via the gastric tube injection of 200 mg/kg CS-045, a gift from Sankyo Company (Tokyo, Japan), suspended in 0.5% chlormethyl cellulose. In the chlormethyl cellulose group, treatment of the rats was the same as for the CS-045 group, except that CS-045 was not administered.

Five other normal male Wistar rats were also used in the glucose clamp study as a control group.

Glucose clamp technique

Seven days after these treatments, rats which had fasted for 20–24 h were subjected to the euglycemic glucose clamp by a modified method [4, 5] of Andres and DeFronzo [6, 7]. Under 0.35 g/kg ip chloral hydrate (Nakarai Chemical, Kyoto, Japan) anesthesia, two Silastic catheters (Dow-Corning, Corp., Midland, MI) were cannulated into the left femoral vein for $^3$H-3-glucose (New England Nuclear, Boston, MA) and 20% glucose infusion, and another catheter into the right femoral vein for infusion of porcine insulin (Actrapid MC insulin, Novo, Denmark) diluted with saline containing 0.25% bovine albumin (Sigma, St. Louis, MO). A double lumen catheter was inserted into the right jugular vein for the sampling of heparinized blood to measure glucose levels by Glucose Monitor (Kyoto Daiichi Kagaku, Kyoto, Japan). Another Silastic catheter was cannulated into the left carotid artery for blood sampling to determine the specific activity of $^3$H-3-glucose as well as the insulin level. One more Silastic catheter was inserted directly into the urinary bladder through a hole in the abdominal wall to measure the urinary glucose excretion.

After surgical procedure, while the rats were still under the same anesthesia with chloral hydrate, 5 $\mu$Ci $^3$H-3-glucose was infused as a bolus and then followed a continuous infusion at a rate of 0.05 $\mu$Ci/min using a peristaltic roller pump (Minipuls 2, Gilson, France) until the entire experiment was over. To measure glucose disappearance during the basal steady state, at 55 and 60 min after $^3$H-3-glucose infusion was started, blood was sampled for determination of the specific activity of $^3$H-3-glucose, and urine was collected for measurement of urinary glucose loss. Then 3 mU·kg$^{-1}$·min$^{-1}$ porcine insulin was infused in the priming mode to obtain suspended hyperinsulinemia. In cases of hyperglycemia, the infusion of 20% glucose was postponed until the blood glucose level fell to 140–150 mg/dl. To maintain euglycemia for 60 min, a variable infusion rate of 20% non-tritiated glucose was infused according to an algorithm described previously [5]. At 55 and 60 min after 20% glucose infusion was started, blood was sampled for determination of the specific activity of $^3$H-3-glucose, and at the end of the clamp blood was taken for determination of the plasma insulin level.

Measurement and calculation

One parameter of insulin action, the glucose infusion rate (GIR), was calculated as mean of all 2 min glucose infusions during the last 20 min of the
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Blood sampled at 55 and 60 min during the basal period (before porcine insulin infusion) and also at 55 and 60 min of the clamp was mixed with ZnSO₄ and Ba(OH)₂ and centrifuged. The specific activity of ³H-3-glucose in the deproteinated supernatant was measured with a liquid-scintillation counter. Mean rates of glucose disappearance (Gd) during both the basal and the clamp steady state were calculated by Steele’s equation [8].

In the basal state before the porcine insulin infusion, basal hepatic glucose output (HGObasal) was estimated to be the same as the basal Gd, because the liver must be the only source of glucose in the blood pool. Glucose uptake (Gu) by peripheral tissue in the basal state was calculated by subtracting urinary glucose excretion from the total basal Gd which was obtained by Steele’s method described above, since glucose entry into the blood pool (HGO) must be equal to glucose outflow from the blood pool which is composed of Gu plus urinary glucose loss during the basal steady state.

On the other hand, HGO during the clamp (HGOnclamp) was determined by subtracting GIR from the clamp total Gd. If HGO was found to be a minus datum, HGOnclamp was estimated as zero. Percent suppression of HGO by insulin infusion was calculated according to Moxley III et al. [9] as follows: % suppression of HGO = (1−HGOnclamp/ HGObasal) × 100. During the clamp steady state, Gu was estimated to be the same as clamp Gd, since during this clamp the blood glucose level was maintained at around 140 mg/dl without any urinary glucose loss. If HGO was calculated as a minus datum, Gu was estimated to be the same as GIR.

Insulin levels in the blood sampled at the end of the clamp were determined by a radioimmunoassay [10], using porcine insulin as a standard.

Data are the mean ± SD unless otherwise indicated, and statistical significance was assessed by Student’s t-test.

Results

Effect of thiazolidinediones on glycemic control of streptozotocin-induced diabetic rats

As shown in Fig. 1, the urine volume of the intensively insulin treated group as well as the AD-4833 and CS-045 groups decreased significantly (P<0.05), in contrast to high levels sustained in both the gum arabic group and the chlormethyl cellulose group. Urinary glucose excretion in the AD-4833 group decreased from 7.2±0.6 to 1.9±0.4 g/day, and in the CS-045 group from 4.2±1.2 to 0.9±0.8 g/day significantly (P<0.05), in concordance with the significant decrement in the intensive insulin treatment group from 6.2±1.8 to 0.1±0.1 g/day. Only minor changes in urinary glucose excretion from 5.9±2.2 to 4.5±1.3 g/day and from 5.5±1.5 to 5.8±1.2 g/day were observed in the gum arabic and chlormethyl cellulose groups, respectively. Almost the same changes in plasma glucose levels were apparent in these 5 groups during 7-day treatment. However, body weight gains in the AD-4833 and CS-045 groups were still lower than in the intensive insulin treatment group, as well as in the gum arabic and chlormethyl cellulose groups, as shown in the lowest row in Fig. 1.

Glucose clamp study

GIR during the 3 mU⋅kg⁻¹⋅min⁻¹ insulin infused clamp steady state in the intensively insulin treated and well controlled rats, as shown in Fig. 2, was 8.60±1.64 mg⋅kg⁻¹⋅min⁻¹, which was not different from the 9.40±1.16 mg⋅kg⁻¹⋅min⁻¹ for the normal control rats. GIR in both the AD-4833 and CS-045 groups were 9.00±2.23 and 7.44±2.01 mg⋅kg⁻¹⋅min⁻¹, respectively, also not different from the normal control. However, GIR in the gum arabic and chlormethyl cellulose group were 5.78±1.34 and 3.91±0.85 mg⋅kg⁻¹⋅min⁻¹, respectively, significantly lower than for the normal control and intensive insulin, AD-4833 and CS-045 groups, showing the presence of insulin resistance in the gum arabic and chlormethyl cellulose groups. Blood glucose levels during the clamp steady state of all groups were not different from each other, and insulin levels in all groups were 80–110 mU/l, also not different from each other.

The Gu for all groups during the basal steady state before insulin infusion shown in Fig. 3 was from 3 to 5 mg⋅kg⁻¹⋅min⁻¹, not very different from each other. The Gu for all groups during the insulin clamp steady state was also around 8
mg·kg⁻¹·min⁻¹, except for the significantly lower glucose uptake observed in the chlormethyl cellulose group (6.73±1.04 vs. 9.40±1.16 mg·kg⁻¹·min⁻¹ for the normal control).

However, in the basal state, the HGO for the intensive insulin group and the AD-4833 group was 5.13±1.18 and 5.15±0.72 mg·kg⁻¹·min⁻¹, respectively, significantly higher than 3.16±0.59 mg·kg⁻¹·min⁻¹ for the normal control, as shown in Fig. 4. The dissociation between nearly the same peripheral Glu and different HGO could be explained by considerable urinary glucose loss, shown as solid boxes in Fig. 4. The HGO for the other three groups was also significantly higher than for the control group. During the insulin infused glucose clamp steady state, HGO in the normal control as well as the intensive insulin, AD-4833, and CS-045 groups, were suppressed to almost zero. But, HGO in the gum arabic and chlormethyl cellulose groups was 1.59±0.78 (95 confidence range; 0.06–3.11) and 2.82±0.71 mg·kg⁻¹·min⁻¹, respectively, not suppressed completely. As shown more clearly in Fig. 5, % suppression of HGO during the clamp in the gum arabic and chlormethyl cellulose groups was 79.9±8.9 and 61.4±13.9%, respectively. These significant incomplete suppressions indicated the presence of hepatic insulin resistance in these two groups treated by minimum dose insulin without active agents. In contrast to these, 100% suppression was seen in the normal control and AD-4833 group; and there was 93.8±7.1 and 91.8±13.3% suppression in the intensive insulin treated and CS-045 groups, respectively.
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Fig. 2. Glucose clamp of normal control, intensive insulin, AD-4833, gum arabic, CS-045, and chlormethyl cellulose groups. Glucose infusion rates (GIR), calculated as the mean of every 2 min glucose infusion rate during the last 20 min of the clamp in intensive insulin, AD-4833, and CS-045 groups were 9.40±1.16 mg·kg⁻¹·min⁻¹, respectively, not very different from 8.60±1.64, 9.00±2.23, and 7.44±2.01 mg·kg⁻¹·min⁻¹ for the normal control. However, GIR in gum arabic and chlormethyl cellulose groups were 5.78±1.34 and 3.91±0.85 mg·kg⁻¹·min⁻¹, respectively, significantly lower than the normal control, indicating insulin resistance. Data are shown as the mean ± SEM.

Fig. 3. Glucose uptake during the basal steady state and in the insulin infused glucose clamp steady state of the normal control, intensive insulin, AD-4833, gum arabic, CS-045, and chlormethyl cellulose groups. Rates of glucose uptake (Gu) during the basal state of all groups were similar to each other. Gu during the clamp steady state in intensive insulin, AD-4833, gum arabic, and CS-045 group, was similar to the control. The Gu of chlormethyl cellulose group only was less than the normal control. Data are shown as the mean ± SD.

Fig. 4. Hepatic glucose output during the basal the steady state and in the insulin infused glucose clamp steady state of the normal control, intensive insulin, AD-4833, gum arabic, CS-045, and chlormethyl cellulose groups. The rates of hepatic glucose output (HGO), the sum of glucose uptake plus urinary glucose loss indicated as a solid box, of all streptozotocin-induced diabetic rats during basal steady state were higher than the control. During the insulin infused glucose clamp steady state, HGO in the normal control, intensive insulin, AD-4833, and CS-045 groups were suppressed to almost zero, but not in the gum arabic or chlormethyl cellulose group. Data are shown as the mean ± SD.
Discussion

Thiazolidinediones, new oral antidiabetic agents, have been expected to normalize insulin resistance, one of the most important abnormalities in non-insulin dependent diabetes mellitus \cite{11,12}. Indeed, it has been reported that pioglitazone (AD-4833) lowers the blood glucose levels of NIDDM model animals within 3 days \cite{1}. Kobayashi et al. reported that the insulin receptor kinase activity of muscle in insulin resistant Wistar fatty rats recovered through the administration of pioglitazone \cite{13}. Treatment with other thiazolidinediones, ciglitazone \cite{14}, CS-045 \cite{2} and englitazone \cite{15} of insulin resistant animals was similarly effective.

However, the concept that poorly controlled insulin-dependent diabetes mellitus is characterized by insulin resistance, which worsens hyperglycemia, is now widely accepted \cite{16,17}. So the question whether or not thiazolidinediones is also effective on insulin resistance in insulin deficient diabetes mellitus should be raised. It has been reported that pioglitazone has no effect on IDDM model animals, such as streptozotocin-induced diabetic rats \cite{1}. If so, there are two types of insulin resistance. The type of insulin resistance seen in IDDM would not be correctable by thiazolidinediones, and the other type of insulin resistance seen in NIDDM would be thiazolidinediones-sensitive.

This investigation clearly showed that even in an IDDM model animal thiazolidinediones had a glucose-lowering effect, when the agents were given concomitantly with the minimal dose of insulin to prevent ketosis but not enough to prevent hyperglycemia. Recently, using the same streptozotocin-induced diabetic rats, Hofmann et al. reported that pioglitazone treatment along with minimal insulin replacement, the procedure we used, restored the reduction in GLUT4 mRNA expression in peripheral tissue \cite{18}. Although the difference between the insulin resistance present in IDDM and NIDDM is still unknown, it is clear that thiazolidinediones can improve the insulin resistance of IDDM, when the agent is given with exogenous insulin.

Our present study indicates that the insulin resistance is restored by thiazolidinediones mainly due to the improvement of hepatic insulin resistance, different from Hofmann's study \cite{18}. With regard to the mechanisms of thiazolidinediones, the question which is the more important or primary action of the agents, to correct impaired peripheral glucose uptake or to suppress overproduction of glucose from the liver, remains unanswered. As mentioned above, Kobayashi et al. clearly showed the peripheral effect of thiazolidinediones on NIDDM model animals \cite{13}. However, Sugiyama et al. reported the presence of hepatic insulin resistance in Wistar fatty rats, a model of NIDDM \cite{19}, in which hyperglycemia was corrected with thiazolidinediones \cite{1}. Hofmann et al. recently reported that two-fold elevated activity and mRNA expression of hepatic phosphoenolpyruvate carboxykinase returned to normal levels following pioglitazone treatment in obese KKAY insulin-resistant mice \cite{20}. Finally, a strong correlation between a reduction in HGO and improvement in the fasting blood glucose levels of patients with NIDDM induced by CS-045 was observed \cite{3}. These findings are in accord with the results of our present study of IDDM model rats, in which the site of action of thiazolidinediones is suggested to be primarily in the liver. Although it is still not clear which is the primarily organ responsible for insulin resistance, peripheral tissue or the liver, in vivo studies including ours.

![Graph: Percent suppression of the hepatic glucose output during the clamp steady state of the normal control, intensive insulin, AD-4833, gum arabic, CS-045 and chlormethyl cellulose groups. One hundred percent suppression of hepatic glucose output (HGO) during clamp \((1-\text{HGO}_{\text{clamp}}/\text{HGO}_{\text{basal}}) \times 100\) was seen in the normal control and AD-4833 groups; and 93.8±7.1 and 91.8±13.3 % suppression was seen in the intensive insulin and CS-045 groups, respectively. In contrast to these, significant incomplete suppression was observed in the gum arabic and chlormethyl cellulose groups. Data are shown as the mean ± SD.]

\textbf{Fig. 5.} Percent suppression of the hepatic glucose output during the clamp steady state of the normal control, intensive insulin, AD-4833, gum arabic, CS-045 and chlormethyl cellulose groups.
have shown the importance of the liver in understanding the mechanism of thiazolidinediones. In conclusion, it is suggested that thiazolidinediones improve hepatic insulin resistance both in IDDM and NIDDM in the presence of insulin.

Acknowledgment

The authors thank Dr. H. Ikeda of Takeda Chemical Industries and Dr. H. Horikoshi of Sankyo Company for their support of this investigation.

References