Original

Changes in Coagulative and Fibrinolytic Activities in Rats with Acute Diabetes Induced by Streptozotocin

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Abstract: Changes in coagulation and fibrinolysis were studied in Sprague-Dawley rats with acute diabetes induced by a single intravenous injection of 65 mg/kg or 45 mg/kg of streptozotocin (STZ). At 2, 24 and 48 hr after 65 mg/kg injection and 144 hr after 45 mg/kg injection, plasma glucose was drastically increased. Suppression of body weight gain and an increase in food and water intake were observed in the 45 mg/kg STZ-treated rats. Plasma lipids increased 24 and 48 hr after 65 mg/kg of STZ treatment, but only triglyceride increased 144 hr after 45 mg/kg injection. Partial thromboplastin time (PTT) was significantly shortened 48 hr after 65 mg/kg and 144 hr after 45 mg/kg. Antithrombin III activity (ATIII) increased significantly 144 hr after 45 mg/kg. Activity of factor XIII decreased 24 hr after 65 mg/kg injection. Neither 65 mg/kg nor 45 mg/kg induced changes in the fibrinogen level or fibrinolytic activity. A remarkable increase in plasma glucose was interrelated with shortening of PTT and increased ATIII activity in STZ-treated rats. These results demonstrated enhancement of intrinsic coagulation in rats with acute diabetes induced by STZ.

Key words: streptozotocin, diabetes, coagulation, fibrinolysis, rat

Introduction

Diabetes mellitus is a chronic disorder characterized by a rise of the glucose level in blood. The risk of atherosclerotic complications is markedly increased in diabetes mellitus because of their vascular complications¹⁾. Several studies in the last decade showed that adult diabetic patients frequently have atherosclerotic diseases such as myocardial infarction and stroke, and that they have increased platelet aggregation, particularly those with retinopathy and advanced vascular changes^{2,3)}. Recently, the elevation of plasma concentrations of thromboxane B₂, Factor VIII, von Willebrand factor-related antigen, and ristocetin cofactor indicating a hypercoagulable state was demonstrated in diabetes mellitus⁴⁾. However, there are few reports which discuss the changes in coagulative and fibrinolytic activity in diabetic animals as experimental models. To know the relation between the blood coagulative system and diabetic condition is important for revealing the mechanisms of atherosclerotic development. To examine the coagulative and fibrinolytic activities in diabetes mellitus, we used streptozotocin (STZ)-treated rats. Because different doses of STZ, which is derived from *Streptomyces achromogenes*⁵⁾, selectively destroy the pancreatic B-cell, it is possible to produce

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graded states of diabetes in rats⁶⁾. We have already reported increased coagulative activity and indirect enhancement of fibrinolytic activity in hyperlipidemic rats⁷⁾. The purpose of the present study was to investigate the changes in the coagulation and fibrinolysis and plasma lipid contents in STZ-treated rats, and discuss the relation between STZ-induced acute diabetes and atherosclerosis.

Materials and Methods

Experimental animals

Male Sprague-Dawley rats, 6 weeks old at the start of the experiment, were fed a stock diet (MF, Oriental Yeast Co., Ltd., Japan) and tap water *ad lib.* under standard laboratory conditions ($21 \pm 2^\circ\text{C}$, $55 \pm 15\%$ humidity). In preliminary experiments to determine the dosage of STZ, 65 mg/kg of STZ induced an increase in the mortality rate 72 hr after injection. To determine the effect of the sustained high plasma levels of glucose, rats treated with 45 mg/kg of STZ were also examined. Each animal was housed individually for at least 5 days prior to the experiment.

Experimental procedures

Diabetes was induced by a single intravenous injection of streptozotocin (STZ: 2-deoxy-2-(methylnitrosoamino)-carbonyl-amino-D-glucopyranose), purchased from Sigma Chemical Co. (St. Lois, MO, U.S.A.). Each animal was fasted for 18 hr after prefeeding for 5 days, and then received STZ (65 mg/kg or 45 mg/kg in 0.05 M citrate buffer, pH 4.5) via tail vein injection. Control rats were injected intravenously with a corresponding volume of citrate buffer. In acute diabetes induced by 65 mg/kg dose, we sacrificed rats for examination at 2, 7, 24 and 48 hr after injection. Treated rats were fasted until sacrifice and control rats were fasted for corresponding periods. In acute diabetes induced by 45 mg/kg STZ, the animals were sacrificed 144 hr after treatment. In rats treated with 45 mg/kg STZ and allowed free access to food and water, changes in daily body weight and intake of food and water were examined for 5 days.

Blood collection and preparation

Blood specimens were taken from the inferior vena cava with a plastic syringe and a silicon-coated needle under pentobarbital anesthesia (40 mg/kg, i.p.). Whole blood from rats was mixed 3.2% sodium citrate solution in a volume ratio of nine to one, and then centrifuged at 3000 r.p.m. for 15 min at 4°C . The supernatant was used for specimens as citrated plasma.

Blood examination

Ethylenediaminetetraacetic acid disodium salt-treated blood was analyzed for platelet, white blood cell (WBC) and red blood cell (RBC) counts and the values of hematocrit (Ht) and hemoglobin (Hb) using automated equipment (Coulter Counter, S-plus size distribution, Coulter Electronics, Inc., Japan).

Plasma glucose and plasma lipid levels

Plasma glucose levels were determined using commercially available diagnostic kits (Glucose Test Wako: Wako Pure Chemical Ind., Ltd., Japan). Total plasma lipid was determined by the method of Bragdon⁸⁾. Total cholesterol was determined by colorimetry (Cholesterol-B Test). Phospholipid and triglyceride contents were determined enzymatically (Phospholipid-B Test and Triglyceride-G Test, respectively). Nonesterified fatty acid (NEFA) was determined by the method of Duncombe⁹⁾. These assays were performed using commercial kits (Wako Pure Chemical Ind., Ltd., Japan).

Assay of coagulative and fibrinolytic activities

1. Prothrombin time (PT) and partial thromboplastin time (PTT). PT was measured using Simplastin (Warnar-Lambert Co., Ltd.) in a modified one-stage method. Simplastin contains a tissue thromboplastin reagent (rabbit brain and lung), CaCl_2 and NaCl. PTT was measured using Platelin (Warnar-Lambert Co., Ltd.) by the method of Langdell et al.⁽¹⁰⁾. Platelin contains a plasma thromboplastin reagent (rabbit brain phospholipid) and NaCl. Measurement of PT and PTT was conducted using Clot-Digitim TE-20 (Erma Optical Works, Ltd., Japan).

2. Fibrinogen level. Fibrinogen level was measured by the method of Tomikawa et al.⁽¹¹⁾. Citrated plasma was mixed with CaCl_2 and tranexamic acid (Sigma Chemical Co. St. Lois, MO, U.S.A.) and the mixture was incubated at 37°C. After removal of the non-clottable proteins from the diluted plasma clot by centrifugation, the protein content of the fibrin precipitate was determined by the method of Lowry et al.⁽¹²⁾.

3. Factor XIII. We followed the conventional method using a commercial kit (F. XIII, Iatron Laboratories, Inc., Japan). The activities of factor XIII were determined on the basis of the intensity of fluorescence from the dansylcadaverine complex by a fluorescence spectrofluorometer (Hitachi 650-10M Fluorescence spectrophotometer, Japan).

4. Antithrombin III (ATIII). ATIII activity was determined by synthetic chromogenic substrates (Tosyl-Gly-Pro-Arg-pNa: Chromolate ATIII, Iatron Laboratories, Inc., Japan).

5. Plasminogen (PLG) and α_2 -plasmin inhibitor. PLG assay was conducted by the chromogenic method using S-2251 as a substrate (Kabi Diagnostica, Daiichi Chemical Pharmacy Co., Ltd. Japan). We used urokinase (Mochida Pharmaceutical Co., Ltd. Japan) as the PLG activator because the PLG of the rat and mouse is activated by urokinase but not by streptokinase⁽¹³⁾. Activity of α_2 -PI was determined by a method using the synthetic chromogenic substrate, S-2251, the same substrate used for PLG assay.

Statistical analysis

Results were expressed as mean $\pm$ S.D. of 7 to 9 rats per group. Statistical significance was evaluated using Student's *t*-test.

Results

Body weight, and food and water intake

Figure 1 shows daily body weight and intake of food and water after STZ injection (45 mg/kg) in rats. There was no increase in body weight gain in STZ-treated rats, although that of control rats increased naturally. The amount of food consumption increased significantly on the 4th and 5th days, whereas water intake increased drastically day by day and the amount of water consumption on the 5th day was about 8 times that of control rats that received citrate buffer injection alone.

Plasma glucose and blood examination

Figure 2 shows changes in plasma glucose levels after STZ injection in rats. After injection of 65 mg/kg STZ, rats could not eat anything due to STZ toxicity, although they were allowed free access to food and water. In this acute diabetes, plasma glucose levels were increased at 2, 24 and 48 hr, but were decreased significantly at 7 hr. However, in control rats, there were no apparent changes in plasma glucose levels due to the fasting. At 144 hr after STZ injection of 45 mg/kg in rats which were allowed free access to food and water after injection, plasma glucose levels increased to about 2.2 times that of the

Fig. 1. Changes in body weight and intake of food and water after intravenous injection of 45 mg/kg streptozotocin (STZ) in rats. Each animal was fasted for 18 hr after prefeeding for 5 day, and then received STZ (×-× and □ : 45 mg/kg in 0.05 M citrate buffer, pH 4.5; arrow). Control rats (●-● and ■) received a corresponding volume of 0.05 M citrate buffer. Body weights and food intake amounts were shown in g and water intake amounts were shown in ml. Each point or column is mean±S.D. of 6 or 7 rats. *, **, *** significant difference from the citrate buffer control value with $p<0.05$, $p<0.01$, $p<0.001$, respectively.

Fig. 2. Changes in plasma glucose levels after streptozotocin (STZ) injection in rats. Plasma glucose levels were determined using commercial kits. Each animal was fasted for 18 hr before intravenous STZ injection (□ : 65 mg/kg or 45 mg/kg in 0.05 M citrate buffer, pH 4.5). Control rats (■, C_n; n=1-4) received a corresponding volume of 0.05 M citrate buffer and fasted for the same periods as STZ-treated rats. C₁: total fasting time is 18 hr. C₂: total fasting time is 42 (18+24) hr. C₃: total fasting time is 66 (18+48) hr. C₄: fasting time is 18 hr. Each point or column is mean±S.D. of 6 or 7 rats. **, *** significant difference from the C₁ value with $p<0.01$, $p<0.001$, respectively.

Table 1. Counts of various blood cells and hemoglobin (Hb) and hematocrit (Ht) values after streptozotocin injection (65 or 45 mg/kg) in rats.

	Control	STZ 65 mg/kg, 48 hr	STZ 45 mg/kg, 144 hr
PLA ($\times 10^5/\mu\text{l}$)	10.00 $\pm$ 1.39	11.90 $\pm$ 1.83*	8.75 $\pm$ 1.71#
WBC ($\times 10^3/\mu\text{l}$)	6.38 $\pm$ 1.14	4.35 $\pm$ 0.95**	4.08 $\pm$ 1.11**
RBC ($\times 10^6/\mu\text{l}$)	7.25 $\pm$ 0.58	7.38 $\pm$ 0.36	7.83 $\pm$ 0.24
Hb (g/dl)	14.80 $\pm$ 0.77	15.30 $\pm$ 0.75	15.60 $\pm$ 0.64
Ht (%)	42.20 $\pm$ 3.80	43.60 $\pm$ 2.74	47.50 $\pm$ 1.29*#

PLA, platelet; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit. At 48 hr after 65 mg/kg and at 144 hr after 45 mg/kg of STZ, blood specimens were collected after 18 hr fasting. Control rats were sacrificed 48 hr and 144 hr after 0.05 M citrate buffer treatment after fasting times of 18 hr. Each value is mean $\pm$ S.D. of 6–8 specimens. **, * significant difference from the citrate buffer control value with $p < 0.01$, $p < 0.05$, respectively, # significant difference from the value for 65 mg/kg STZ treated rats with $p < 0.05$ (Student's *t*-test).

Fig. 3. Changes in PT and PTT in streptozotocin (STZ)-induced diabetic rats. PT and PTT were measured by using Simplastin and Platelin within 4 hr after blood collection and each column shows sec. Each rat was fasted for 18 hr after prefeeding for 5 days, and then received citrate buffer (C: ■) or STZ (□: 65 or 45 mg/kg in 0.05 M citrate buffer) and the fasting time was from 18 to 66 hr. Each column is mean $\pm$ S.D. of 6 or 7 rats. *, ** significant difference from the citrate buffer control value with $p < 0.05$, $p < 0.01$, respectively.

control rats. At 48 hr and 144 hr after STZ injection of 65 mg/kg and 45 mg/kg, respectively, plasma glucose levels exceeded 250 mg/dl. Table 1 shows the various values of blood parameters after STZ injection in rats. Platelet count increased 48 hr after the treatment of 65 mg/kg STZ and tended to decrease 144 hr after 45 mg/kg injection of STZ. White blood cells decreased significantly after STZ treatment. Hematocrit values increased with a tendency for red blood cells to increase at 144 hr after 45 mg/kg injection of STZ.

PT and PTT

Figure 3 shows changes in PT and PTT in diabetic rats induced by STZ. In citrate control rats, we found no significant changes in PT or PTT due to the different fasting times. PT tended to be elongated 7 hr after 65 mg/kg of STZ injection, whereas PTT was significantly shortened 48 hr after 65 mg/kg treatment and 144 hr after 45 mg/kg.

Fig. 4. Changes in the activity of coagulation and fibrinolytic factors in rats with streptozotocin-induced diabetes.

Fibrinogen (Fib: ■—□) levels are shown in mg/dl, and the activity of factor XIII (XIII: ●—○) is shown as intensity of dansylcadaverine complex fluorescence determined by a fluorescence spectrofluorometer. Plasminogen (PLG: ◆—◇) content and activities of antithrombin III (ATIII: ▲—△) and α_2 -plasmin inhibitor (α_2 PI: ×—×) are shown as ratios relative to normal human plasma. Each filled mark shows the control value. Each value is mean $\pm$ S.D. of 6–8 specimens. *, ** significant difference from the citrate buffer control value with $p < 0.05$, $p < 0.01$, respectively. For details, see Fig. 3.

Fibrinogen, factor XIII, ATIII, PLG and α_2 -PI

Figure 4 shows changes in the activity of coagulative and fibrinolytic factors in STZ-induced diabetes. In control rats, there were no significant effects of the different fasting times on fibrinogen level, factor XIII, or ATIII activity. No significant change in fibrinogen level was observed. The activity of factor XIII decreased 24 hr after 65 mg/kg STZ injection. At 144 hr after 45 mg/kg STZ, an apparent increase in ATIII activity was observed. We found no significant change induced by STZ in PLG content or α_2 -PI activity in acute diabetic rats.

Plasma lipids

Figure 5 shows changes in TC, FC, TG, PL and NEFA contents in rat plasma. At 24 and 48 hr after 65 mg/kg STZ injection, TC and FC contents increased significantly. TG content was increased at 2, 7, 24 and 48 hr after 65 mg/kg STZ and 144 hr after 45 mg/kg STZ injection. NEFA content increased apparently after 65 mg/kg STZ injection.

Relation between plasma glucose and PT, PTT and ATIII

Table 2 shows interrelations between the plasma glucose level and coagulative activity in STZ-induced diabetes. In control rats, no significant relation was found between the plasma glucose and coagulative activity. However, there was an interrelated shortening of PTT and an increase in ATIII activity with a remarkable increase in the plasma glucose level in STZ-induced diabetic rats.

Fig. 5. Changes in plasma lipid levels in streptozotocin-treated rats. Plasma lipid concentrations are shown in mg/dl, and each value is mean $\pm$ S.D. of 6–8 specimens. *, **, *** significant difference from the control value with $p<0.05$, $p<0.001$, respectively. TC (●—○): total cholesterol, FC (×—×): free cholesterol, TG (▲—△): triglyceride, PL (■—□): phospholipid, NEFA (◆—◇): non-esterified fatty acid. For details, see Fig. 3.

Table 2. Relation between plasma glucose and coagulation activity in streptozotocin (STZ)-induced diabetic rats.

	Control	STZ
GL & PTT	$y=0.012x+28.98$ $r=-0.059$ n.s. (n=18)	$y=-0.032x+36.96$ $r=-0.507$ $p<0.05$ (n=23)
GL & PT	$y=-27.8x+441.79$ $r=-0.618$ n.s. (n=15)	$y=-43.8x+721.37$ $r=-0.519$ $p<0.01$ (n=22)
GL & ATIII	$y=-0.160x+149.63$ $r=-0.257$ n.s. (n=23)	$y=0.066x+129.95$ $r=0.403$ $p<0.05$ (n=29)

GL, plasma glucose; r, correlation coefficient.

Discussion

Diabetic patients have a higher incidence of micro- and macrovascular abnormalities associated with functional aberrations of the coagulation system¹⁴⁾, platelet aggregation^{15–17)} and prostaglandin metabolism^{16,17)}. In the present paper, we demonstrated the shortening of PTT in STZ-induced diabetic rats. Considering that PTT is a test for factors included in the intrinsic system except platelet factor III, these acute diabetic rats showed enhancement of

intrinsic coagulation. In addition, the PTT shortening was related significantly to the increase in plasma glucose in this experimental diabetic model.

STZ has been shown to be a selective B-cell toxin⁶¹. In the present study, treatment of rats with 65 mg/kg of STZ induced triphasic plasma glucose change, that is, an increase in plasma glucose after 2 hr, a decrease after 7 hr, and an increase after 24 and 48 hr. These changes were, respectively, induced by the suppression of insulin release, by the temporary increase in insulin release owing to the destruction of B-cells, and by the decrease in insulin release⁶¹. Additionally, a single injection of 45 mg/kg of STZ to rats drastically increased food and water consumption without increasing body weight gain. These are the most typical symptoms of diabetes.

From the epidemiologic research, diabetes is associated with higher levels of the atherogenic risk factors, including hyperfibrinogemia, hypertension, hypertriglyceridemia and obesity¹⁸). Fibrinogen concentration was significantly higher in diabetics than in non-diabetics, and a small but significant correlation was found between fibrinogen concentrations and plasma cholesterol concentrations¹⁹). In our experiments, no increase in fibrinogen levels was observed, but we did observe that plasma from STZ-treated rats tended to form fibrin clots easily during the determination of fibrinogen content. We speculate that fibrinogen in the plasma had been consumed to form clots in acute diabetic rats before determination of fibrinogen levels. Simultaneously, indirect suppression of the hypercoagulation was suggested by the increase in ATIII activity and decrease in factor XIII activity. We found a decrease in factor XIII activity 24 hr after injection of 65 mg/kg of STZ. This tendency may be a result of physiological reaction to counterbalance the enhancement and the suppression of coagulation. Our results indicated that the STZ treatment of rats induced enhancement of intrinsic coagulation demonstrated by the shortening of PTT. Significant relations between high plasma glucose levels and PTT shortening were also observed. The enhancement of intrinsic coagulation is considered to accompany activation of the kinin-kallikrein system, so it increases vascular permeability²⁰). From our results with the acute diabetic model, a high plasma level of glucose can be considered to trigger thrombosis. In addition, the subchronic diabetic rats also showed PTT shortening, which meant the enhancement of intrinsic coagulation (M. Okazaki *et al.*, unpublished data). These changes in acute and subchronic diabetes will contribute to the development of atherosclerosis.

We observed no significant changes in fibrinolytic activity in rats with STZ-induced diabetes. Almer and Nilsson showed that the fibrinolytic defense system is impaired in diabetes, and they suggested that fibrin deposits that had not been removed contributed to the development of vascular lesions²¹). A more recent report also revealed that fibrinogen produced concentration-dependent inhibition of plasmin activity²²) in an acute diabetic model in which the rats retained their fibrinolytic activity. It is interesting to examine the long-term effects of high plasma glucose level on fibrinolysis using a chronic diabetic model.

Examination of the blood of STZ-treated rats revealed an increase in the platelet count 48 hr after 65 mg/kg of STZ and a tendency to decrease 144 hr after 45 mg/kg of STZ. This suggests that treatment with a high dose of STZ can induce thrombosis. However, the prolonged high blood glucose level had the opposite tendency. One reason for this could be that platelets might be consumed in the process of coagulation. White blood cells were decreased in both diabetic rat models, which may be direct effects of STZ, because cells incubated *in vitro* with STZ contained numerous chromosomal abnormalities indicative of DNA

strand breakage²³⁾. On the other hand, non-enzymatic glucosylation of protein was increased due to the prolonged high blood glucose. Suzuki et al.²⁴⁾ reported that the polymerization and cross linking of fibrin monomer were accelerated in the glucosylated fibrinogen. In addition, fibrinolysis by plasmin was delayed in fibrin formed from the glucosylated fibrinogen. That is, the glucosylation of plasma fibrinogen in diabetic patients may induce some different effects on blood coagulation and fibrinolysis.

Diabetes mellitus is often associated with lipid abnormalities. Recent experiments showed that serum cholesterol and triglyceride concentrations were significantly elevated at 1 and 6 months after induction of diabetes by a single injection of 65 mg/kg STZ²⁵⁾. In the present paper in rats with acute diabetes, we also demonstrated significant increases in total cholesterol, free cholesterol, triglyceride and non-esterified fatty acid 24 and 48 hr after the injection of 65 mg/kg STZ. Since STZ induced decreased bile flow, bile acids output and BSP biliary excretion²⁶⁾, one of explanation for the abnormal lipid metabolism might be the transient toxic effects of STZ on hepatocytes. In our previous paper, an increased fibrinogen level accompanied by increased plasma lipids was evident in triton WR-1339-induced hyperlipidemic rats⁷⁾. In the present acute diabetic model, we could not demonstrate significance in the relation between the plasma lipids and fibrinogen level, and no relation between plasma fibrinogen and glucose levels was found. However, there was a positive relation between the shortening of PTT and the increase in plasma lipids in the STZ-induced diabetic model (data not shown). From these data, it could not be determined whether the increase in glucose level or lipid level enhanced the activity of the intrinsic coagulation in the acute diabetic rats. But changes in the coagulation system might be secondary results of changes in plasma glucose.

In conclusion, STZ-induced acute diabetes showed an increase in plasma lipids and enhancement of intrinsic coagulation. Both were considered to induce trigger and/or promotion of atherosclerosis. These data further suggest that alteration of coagulative activity is important in the pathogenesis of atherosclerosis.

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