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Effect of metformin on advanced glycation endproduct formation and peripheral nerve function in streptozotocin-induced diabetic rats

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Abstract

The effects of metformin treatment on advanced glycation endproduct formation and peripheral nerve function in streptozotocin-induced diabetic rats were examined. Streptozotocin-induced diabetic rats were treated with low dose metformin (50–65 mg kg^{-1} daily) or high dose metformin (500–650 mg kg^{-1} daily) for 10 weeks. While the metformin-untreated diabetic group showed a significant increase of advanced glycation endproducts (6.1-fold in the lens, 1.6-fold in the sciatic nerve, 2.3-fold in the renal cortex, and 1.9-fold in plasma; all $P < 0.01$) compared with the healthy control group, both metformin-treated groups had significantly less advanced glycation endproduct deposition. The % decrease in the diabetes-induced increase in advanced glycation endproduct formation by low and high dose metformin treatment was 25% and 72% in the lens (both $P < 0.01$), 31% and 42% in the sciatic nerve (both $P < 0.05$), and 16% and 33% in the renal cortex ($P < 0.05$ and $P < 0.01$), respectively. However, the plasma advanced glycation endproduct level showed no significant difference from that in the untreated diabetic group, in spite of slight decrease in plasma glucose and glycated hemoglobin levels in the metformin-treated groups. The diabetes-induced sciatic nerve conduction velocity deficits were improved by 46% and 42% by low and high dose metformin treatment, respectively (both $P < 0.01$). These data suggest that metformin may have a direct antiglycative action, which in turn contributes to amelioration of peripheral nerve function. Thus, metformin treatment may be effective in the prevention of diabetic complications through not only lowering plasma glucose, but also directly inhibiting advanced glycation endproduct formation.

Keywords: Metformin; Antiglycative action; Advanced glycation endproduct; Peripheral nerve conduction velocity; Streptozotocin-induced diabetes; (Rat)

1. Introduction

Metformin hydrochloride is a biguanide that is an amino group-rich compound like aminoguanidine (Bailey et al., 1996). Aminoguanidine is a potent inhibitor of the formation of advanced glycation endproducts which contribute to the etiology of diabetic complications (Brownlee et al., 1983; Brownlee, 1992). The amino groups of aminoguanidine can react with dicarbonyl metabolites, such as 3-deoxyglucosone, and block the progression of the late steps of the Maillard reaction (Brownlee et al., 1986; Igaki et al., 1990; Edelstein and Brownlee, 1992a). Thus, aminoguanidine may be a beneficial agent for the prevention of diabetic complications (Edelstein and Brownlee, 1992b; Yagihashi et al., 1992). Although the conformational structure of metformin differs from that of aminoguanidine in terms of the sites and directions of the amino groups based on computer analysis (personal communication from Sumitomo Pharmaceutical, Osaka, Japan), a very recent report has shown that metformin reduces the levels of a dicarbonyl compound, methyglyoxal, in type 2 diabetic subjects (Beisswenger et al., 1999). Furthermore, we have already observed that metformin inhibits in vitro advanced glycation endproduct formation induced by glucose in bovine serum albumin, and its potency is similar to that of aminoguanidine (Tanaka et al., 1997). However, the in vivo inhibiting action of metformin on advanced glycation endproduct formation and its preventive effect on diabetic complications have not been examined. Accordingly, we evaluated the effect of metformin on advanced glycation endproduct deposition in the lens, sciatic nerve, renal cortex, and plasma, and on sciatic motor nerve conduction

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velocity in streptozotocin-induced diabetic rats, which were used as an insulin-deficient overt hyperglycemic model to minimize the influence of the hypoglycemic effect of metformin on advanced glycation endproduct formation.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats aged 6 weeks and weighing 160–180 g were randomized into control and diabetic groups. Diabetes was induced by intravenous injection of streptozotocin (Sigma, St. Louis, MO, USA; 60 mg kg^{-1}) in 50 mM citrate buffer (pH 4.5). Control animals were injected with buffer only.

2.2. Experimental design

To determine the effects of metformin, diabetic rats were divided into three groups 2 weeks after streptozotocin injection: (i) an untreated group, (ii) a low dose metformin group, and a (iii) high dose metformin group. The low dose metformin and high dose metformin groups were given 0.05% or 0.5% metformin-containing laboratory chow (provided by Sumitomo Pharmaceutical), respectively, and water ad libitum for 10 weeks. The control and untreated groups were maintained on metformin-free laboratory chow and water ad libitum for 10 weeks. All rats were housed in individual cages to measure food intake. Right side sciatic motor nerve conduction velocity was measured at 10 weeks after the start of metformin treatment, and a few days later, the rats were killed under thiopental (40 mg kg^{-1}) anesthesia.

2.3. Blood analysis and tissue sample preparation

Blood samples were obtained at the time of death, and tissue samples for advanced glycation endproduct measurement were prepared by the methods reported previously (Nakayama et al., 1993). Briefly, the bilateral lenses were decapsulated and homogenized in 1.0 ml of 0.1 N NaOH, followed by centrifugation at $8000 \times g$ for 15 min at 4°C. The bilateral renal cortexes and left sciatic nerve were minced and washed three times in cold phosphate-buffered saline (PBS; pH 7.4), and then defatted by shaking in chloroform and methanol (2:1). To avoid an influence of measurement of motor nerve conduction velocity on advanced glycation endproduct content, we sampled the nerve on the left side only. The solvents were removed by suction, and the pellets were washed sequentially three times with methanol and three times with deionized water. Then, the pellets were homogenized in 1.0 ml of 0.1 N NaOH, followed by centrifugation at $8000 \times g$ for 15 min at 4°C. After the protein concentration was determined as described previously (Lowry et al., 1951), the supernatants were stored at −80°C until advanced glycation endproduct analysis. Plasma glucose, glycated hemoglobin, and plasma insulin levels were measured by the glucose dehydrogenase method, an HbA1c kit using an anti-glycated hemoglobin antibody and latex beads (SRL, Tokyo, Japan), and a rat insulin radioimmunoassay (RIA) kit (Incstar, MN, USA), respectively.

2.4. ELISA for advanced glycation endproducts

The advanced glycation endproduct content was measured by a competitive enzyme-linked immunosorbent assay (ELISA) using an anti-advanced glycation endproduct-keyhole limpet hemocyanin antibody. This antibody cross-reacts with N^{e}-(carboxymethyl)lysine and pentosidine, major advanced glycation endproducts in vivo, but does not react with Amadori products (Ono et al., 1998). Briefly, 50 l of sample, 50 l of 0.6% sodium dodecyl sulfate in 10 mM Tris–HCl saline (pH 7.4), and 5 l of 2 M $NaBH_4$ in 50 mM NaOH were mixed and heated at 100°C for 10 min. The mixture was then cooled in ice water and diluted by more than 5 volumes of PBS. Then 100 l of sample or advanced glycation endproduct-bovine serum albumin standards was added to wells coated with advanced glycation endproduct-bovine serum albumin, followed by 100 l of diluted anti-AGE antiserum. The plate were then incubated at 4°C overnight. After the wells were washed with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20), horseradish peroxidase-labelled anti-rabbit antibody was added to each well and incubated at 25°C for 4 h. After the wells were washed with PBS containing 0.05% Tween 20, 100 l of 3,3′,5,5′-tetramethybenzidine solution was added to each well and incubated for 40 min. The optical density was measured at 450 nm with a micro-plate ELISA reader after the addition of 50 l of 2 N H_2SO_4. Results are expressed as arbitrary advanced glycation endproduct units adjusted to the protein content of the sample (g), and 1 mU of advanced glycation endproduct was defined as 4 μg of standard advanced glycation endproduct-bovine serum albumin. The lower limit of the dynamic range for the ELISA is 1 mU ml^{-1} with coefficient variance (CV) of about 5.0%.

2.5. Measurement of sciatic motor nerve conduction velocity

Motor nerve conduction velocity was recorded in the right sciatic nerve with a modification of the method described previously (Sharma and Thomas, 1974). The rats were anesthetized by inhalation of ethyl ether, and the skin temperature was kept at 37°C with a body temperature control system (TK-4, Nihon Bioresearch, Gihu, Japan). After stimulation electrodes were inserted percutaneously in the sciatic notch and just above the internal malleus, they were stimulated supramaximally with 0.2-ms^2 wave pulses, and muscle action potentials were picked up from

the plantar muscle and recorded by a nerve conduction velocity measurement system (MEB-7102, Nihon Koden, Tokyo, Japan). Motor nerve conduction velocity was calculated by dividing the distance between the points of stimulation by the difference in the latencies from these two points.

2.6. Statistical analysis

All results are expressed as means ± S.E.M., and the equality of variances was evaluated with Barttlet's test. For results with a significant equality in variances, statistical significances were determined by one-way ANOVA and Scheffe's multiple comparison test for a post hoc test. For the results with no significant equality in variances, statistical significance were evaluated by Kruskal–Wallis' test and Scheffe's type multiple comparison test for non-parametric tests.

3. Results

3.1. Characteristics and blood analysis

As shown in Table 1, all diabetic groups with or without metformin treatment has a much lower body weight, a remarkably lower plasma insulin level, and much higher plasma glucose and glycated hemoglobin levels than the control group. Both plasma glucose and glycated hemoglobin levels in the low dose metformin and high dose metformin group were slightly but significantly lower than those in the untreated group. From measurement of the consumed and spilled chow and daily body weight data in the low dose metformin and high dose metformin groups, the actual intake of metformin was calculated to be about 50–65 and 500–650 mg kg^{-1} day^{-1}, respectively. Since metformin is clinically used at a maximal dose of 2550 mg daily, the metformin dose in the low dose metformin group was equivalent to the clinical dose used in the treatment of diabetic patients.

3.2. Tissue and plasma advanced glycation endproducts

Data on the tissue advanced glycation endproduct content are displayed in Fig. 1. While the untreated group showed markedly higher tissue levels of advanced glycation endproducts (6.1-fold in the lens, 1.6-fold in the sciatic nerve, 2.3-fold in the renal cortex, and 1.9-fold in the plasma; all $P < 0.01$) compared with the control group, metformin treatment decreased advanced glycation endproduct levels in lens and renal cortex in a dose dependent manner and in sciatic nerve in a non-dose dependent manner. The % decrease of the diabetes-induced increase in advanced glycation endproduct levels in the low dose metformin and the high dose metformin groups was 25% and 72% in the lens (both $P < 0.01$), 31% and 42% in the

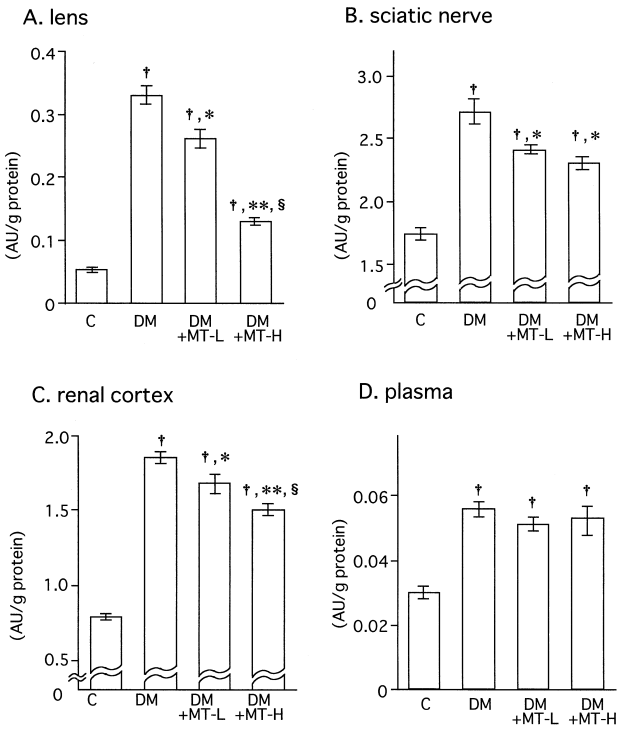

Fig. 1. Advanced glycation endproduct content in the lens, sciatic nerve, renal cortex and plasma measured by competitive ELISA in control and streptozotocin-induced diabetic rats. C: control group, DM: non-metformin-treated diabetic group, DM + MT-L: low dose metformin group, DM + MT-H: high dose metformin group. Data are expressed as the means ± S.E.M. AU: arbitrary units. Data for lens and plasma were analyzed by one-way ANOVA, followed by Scheffe's test, and data for sciatic nerve and renal cortex were analyzed by Kruskal–Wallis' test, followed by Scheffe's type multiple comparison test; $^{+}P < 0.01$ vs. control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. non-metformin-treated diabetic group, $^{\S}P < 0.05$ vs. low dose metformin group.

Table 1
Metabolic data from control and streptozotocin-induced diabetics rats (18 weeks). Data are expressed as means ± S.E.M.[1,2] The metformin treatment protocol was described in Research Design and Methods. The actual daily intake of metformin was calculated as 50–65 mg kg^{-1} in the low dose group and 500–650 mg kg^{-1} in the high dose group. All data were analyzed by one-way ANOVA, followed by Scheffe's test

	Control	Diabetic		
Metformin treatment	untreated	untreated	low dose[1]	high dose[2]
Number of rats	12	8	10	10
Body weight (g)	499 ± 14	265 ± 15[a]	279 ± 13[a]	279 ± 12[a]
Plasma glucose (mmol l^{-1})	6.4 ± 0.3	30.7 ± 1.4[a]	25.5 ± 1.1[a,b]	25.3 ± 1.3[a,b]
Glycated hemoglobin (%)	2.6 ± 0.1	10.3 ± 0.3[a]	9.2 ± 0.2[a,c]	8.3 ± 0.5[a,c]
Plasma insulin (μU ml^{-1})	12 ± 2	0.6 ± 0.1[a]	0.7 ± 0.2[a]	0.9 ± 0.2[a]

[a] $P < 0.01$ vs. control group, [b] $P < 0.05$, [c] $P < 0.01$ vs. metformin-untreated diabetic group.

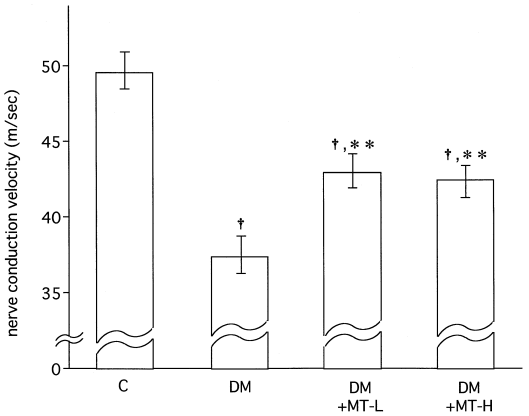

Fig. 2. Sciatic motor nerve conduction velocity in control and streptozotocin-induced diabetic rats. C: control group, DM: non-metformin treated diabetic group, DM + MT-L: low dose metformin group, DM + MT-H: high dose metformin group. Data are expressed as the means ± S.E.M. All data were analyzed by one-way ANOVA, followed by Scheffe's tets; $^{+}P < 0.01$ vs. control group, $^{**}P < 0.01$ vs. non-metformin-treated diabetic group.

sciatic nerve (both $P < 0.05$), and 16% and 33% in the renal cortex ($P < 0.05$ and $P < 0.01$), respectively. However, the plasma advanced glycation endproduct level showed no significant difference between the untreated group and the metformin-treated groups.

3.3. Sciatic motor nerve conduction velocity

Data on the sciatic motor nerve conduction velocity are expressed in Fig. 2. While the untreated group showed significantly slowed motor nerve conduction velocity compared with the control group (37.4 ± 0.7 vs. 49.6 ± 1.4 m s^{-1}, $P < 0.01$), such diabetes-induced motor nerve conduction velocity deficits were significantly improved by 46% in the low dose metformin (43.0 ± 1.0 m s^{-1}) and by 42% in the high dose metformin group (42.5 ± 1.1 m s^{-1}; both $P < 0.01$), respectively.

4. Discussion

The present study demonstrated that 10 weeks of metformin treatment at both a low dose (50–65 mg kg^{-1}) and a high dose (500–650 mg kg^{-1}) inhibited advanced glycation endproduct formation in the lens (% decrease in the diabetes-induced increase in advanced glycation endproduct formation was 25% and 72%), sciatic nerve (31% and 42%), and renal cortex (16% and 33%), but not in the plasma of streptozotocin-induced diabetic rats with a slight but significant decrease in plasma glucose and glycated hemoglobin levels. In vivo treatment of streptozotocin-diabetic rats with aminoguanidine decreased the diabetes-induced increase in advanced glycation endproduct formation by 46% in sciatic nerve (25 mg kg^{-1} for 16 weeks; Yagihashi et al., 1992), by 40% in lens (25 mg kg^{-1} for 13 weeks; Mastumoto et al., 1997), and by 44% in renal cortex (50 mg kg^{-1} for 16 weeks; Miyauchi et al., 1996). Although it is difficult to compare the inhibitory effect of metformin on in vivo advanced glycation endproduct formation with that of aminoguanidine because of the different treatment times and doses used, the low dose of metformin (50–65 mg kg^{-1}) may have a slightly lower potency than that of aminoguanidine (25–50 mg kg^{-1}).

We previously reported that 1 mM metformin could inhibit the in vitro glycation of bovine serum albumin induced by 1.7 M glucose (metformin:glucose molar ratio, 1:1700) (Tanaka et al., 1997). However, since the advanced glycation endproduct-bovine serum albumin compounds induced by such a high concentration of glucose may not be formed under physiological conditions, the inhibitory effect of metformin on in vivo advanced glycation endproduct formation remained unclear. A previous study showed that the maximum plasma metformin level was obtained at 0.5 h after oral administration (50 mg kg^{-1} containing [^{14}C]-metformin) in streptozotocin diabetic rats (35.4 ± 5.8 μmol l^{-1} in the inferior vena cava or 61.5 ± 8.0 μmol l^{-1} in the hepatic vein), while the tissue metformin concentrations in the gastrointestinal tract, salivary gland, muscle, liver, heart, and kidney remained much higher than the plasma level up to 8 h (Wilcock and Bailey, 1994). Although we did not measure tissue and plasma metformin concentrations in the present study, sufficient accumulation of metformin to directly inhibit advanced glycation endproduct formation may have occurred in the lens, sciatic nerve, and renal cortex of streptozotocin-induced diabetic rats after 10 weeks of treatment. Furthermore, Beisswenger et al. (1999) very recently reported that metformin treatment of type 2 diabetic subjects significantly reduced the plasma level of methylglyoxal, a potent dicarbonyl metabolites of advanced glycation endproduct formation, compared with the level of HbA1c matched non-metformin treated type 2 diabetic subjects. These reports suggest that metformin treatment may have a direct antiglycative action even at clinical doses.

While the plasma levels of glucose and glycated hemoglobin significantly decreased in the metformin-treated groups, plasma levels of advanced glycation endproducts were not different between the untreated group and metformin-treated groups. Plasma levels of advanced glycation endproducts were much lower than those of tissues in the present study, which may mean that plasma advanced glycation endproducts are constantly eliminated whereas tissue advanced glycation endproducts accumulate over time, and thus, a slight but significant decrease in plasma glucose and glycated hemoglobin levels may be effective for inhibition of advanced glycation endproduct formation. As for reliability of plasma advanced glycation endproduct measurement, the lower limit of the ELISA system is 1 mU ml^{-1} with CV of about 5.0%, and the

levels of plasma advanced glycation endproducts varied from 1.4 to 3.0 mU ml^{-1}, or from 0.024 to 0.064 U g $protein^{-1}$ after adjustment for the plasma protein concentration. Therefore, plasma advanced glycation endproducts were detected reliably in the present study. Taken together, the inhibiting effect of metformin on tissue advanced glycation endproduct formation seen in the present study may be associated with both a direct antiglycative action and an indirect antihyperglycemic action. Since all antihyperglycemic agents can indirectly inhibit in vivo advanced glycation endproduct formation by lowering plasma glucose, it is difficult to design a study that evaluates the direct antiglycative action of metformin. Although we used streptozotocin-induced diabetic rats as an insulin-deficient overt hyperglycemic model to minimize any influence of an improvement of glycemic control by metformin, the metformin-treated groups showed a slight but significant decrease in plasma glucose and glycated hemoglobin levels. Therefore, comparison study of inhibitory effect of metformin on advanced glycation endproduct formation with other diabetes treatment such as insulin or other oral agents by the level of glycated hemoglobin-matched design may indicate direct antiglycative action of metformin in the future.

The 10-week metformin treatment improved the diabetes-induced sciatic motor nerve conduction velocity deficit in the present study. Previous reports showed that normalization or near normalization of plasma glucose levels by chronic insulin treatment could partially ameliorate diabetes-induced slowed motor nerve conduction velocity in streptozotocin-induced diabetic rats (Yasuda et al., 1988; Van dam et al., 1996) and type 1 diabetic patients (Pietri et al., 1980; Service et al., 1985). Thus, although the role of the slight decrease in plasma glucose and glycated hemoglobin levels on the amelioration of sciatic motor nerve conduction velocity was unclear, the antihyperglycemic effect of metformin was not negligible for the improvement of nerve function in the present study. As for advanced glycation endproduct-associated peripheral nerve dysfunction in diabetic rats, previous reports showed an ameliorating effect of aminoguanidine treatment on sciatic motor nerve conduction velocity and nerve blood flow in streptozotocin-induced diabetic rats (Kihara et al., 1991; Yagihashi et al., 1992). Although aminoguanidine has been shown to act via both an inhibition of advanced glycation endproduct formation (Brownlee et al., 1986; Igaki et al., 1990; Edelstein and Brownlee, 1992a) and an inhibition of aldose reductase activity (Kumari et al., 1991), it was reported that aminoguanidine-treated diabetic rats showed no change in sciatic nerve sorbitol, fructose, and myo-inositol contents (Cameron et al., 1992), or in sciatic nerve ATPase activity (Miyauchi et al., 1996) compared with untreated diabetic rats. Thus, the beneficial effect of aminoguanidine on nerve function may be explained by inhibition of advanced glycation endproduct formation and improvement of nerve blood flow, and, inversely, advanced glycation endproducts may contribute partially to the etiology of diabetic neuropathy. Interestingly, recent reports showed the effect of metformin on tyrosine kinase activity in rat smooth muscle cells (Dominguez et al., 1996) and hemodynamic rheological responses in diabetic patients (Marfella et al., 1996). These reports suggest that metformin may influence nerve function by modifying cell signaling and nerve blood flow. Although we examined neither focal nitric oxide production, nor tyrosine kinase activity in nerve tissue, the ameliorating effect of metformin treatment on nerve function may be associated not only with its antiglycative action and antihyperglycemic action, but also with other mechanisms. Furthermore, it remains unclear why there were no dose-response effects of metformin on both sciatic nerve advanced glycation endproduct level and motor nerve conduction velocity. Thus, further studies are required to elucidate the exact mechanism of metformin on peripheral nerve function.

In conclusion, the present study has demonstrated for the first time that a clinical dose of metformin inhibits tissue advanced glycation endproduct formation, which in turn may contribute to the prevention of diabetic complications. The UK Prospective Diabetes Study (UKPDS) Group has recently reported that metformin treatment of obese type 2 diabetic subjects appears to decrease the risk of diabetes-related endpoints of complications, and they also discussed the possibility of an aminoguanidine-like action of metformin (UK Prospective Diabetes Study Group, 1998). Therefore, a further study of long-term metformin treatment is needed to verify whether its direct inhibition of advanced glycation endproduct formation has clinical significance for the prevention of diabetic complications.

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