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IN VIVO AND *IN VITRO* EFFECTS OF CYCLOSPORIN A ON GLUCOSE TRANSPORT BY SOLEUS MUSCLES OF MICE

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Abstract—The effect of cyclosporin A (CyA) on 2-deoxyglucose (2DG) uptake by soleus muscles of ICR mice was studied *in vivo* and *in vitro*. The basal and insulin-stimulated uptakes of 2DG by the muscles as well as the plasma insulin level were significantly decreased by the *in vivo* treatment of mice with 20 mg/kg/day of CyA for 6 weeks ($P < 0.01$ and $P < 0.05$), whereas the insulin binding was increased inversely in the muscles from 20 mg/kg/day CyA-treated mice. The insulin-stimulated uptake of 2DG by the muscles was significantly decreased by the *in vitro* treatment of the muscles with 1, 10 and 100 μg/mL of CyA ($P < 0.05$ and $P < 0.01$, respectively), while the basal uptake of 2DG was not changed by the *in vitro* treatment of the muscles with CyA. The insulin binding to the muscles was not altered by the *in vitro* treatment of the muscles with CyA. These findings suggest that CyA affects not only the insulin secretion from the pancreatic islets but also the postbinding mechanisms of insulin action on the glucose transport by the muscles, which may account for a part of the diabetogenic effect of CyA.

Cyclosporin A (CyA†) is a potent immunosuppressive agent and is used clinically not only for organ transplantations but also for the treatment of autoimmune diseases including insulin-dependent diabetes mellitus [1–3]. The diabetogenic effect of CyA has been shown in human subjects and experimental animals treated with CyA [4–10] and this was explained in part by the reduced insulin secretion due to damages of pancreatic islets as demonstrated *in vivo* and *in vitro* [5–9, 11, 12]. On the other hand, the impaired glucose tolerance has been shown in CyA-treated patients and CyA-treated animals with increased C-peptide or insulin levels in blood [4, 10]. This observation in humans and animals has raised a possibility that CyA may have a direct effect on the mechanism(s) of the insulin action on the glucose transport in the muscles. In this study, the 2-deoxyglucose (2DG) uptake and insulin binding by soleus muscles from the mice treated with CyA *in vivo* and by soleus muscles treated directly with CyA *in vitro* have been investigated to elucidate the mechanisms of diabetogenic effect of CyA.

MATERIALS AND METHODS

Animals. Male ICR Mice were obtained from Japan Clea Co. Ltd (Tokyo, Japan) and were kept in an animal room with an ambient temperature of 25 ± 1° and illumination from 7 a.m. to 7 p.m. They were given free access to water and lab. chow MF (Oriental Yeast Co. Ltd, Tokyo, Japan). They were given olive oil alone, or 5, 10 and 20 mg/kg body weight of CyA (Sandoz Ltd, Basel, Switzerland) dissolved in olive oil orally, with a stomach tube every day for 6 weeks from the age of 4 weeks. At the age of 9 weeks, the mice underwent the glucose tolerance test (OGTT) (after a 16 hr fast) in which blood was collected from the orbital sinus before and after the 2 g/kg glucose load (0, 60 and 120 min). At the age of 10 weeks, they were killed by heart puncture (in the fed state at 9.00–10.00 a.m.) under anesthesia with 100 mg/kg body weight of sodium pentobarbital. Their plasma, immediately separated from the collected heparinized blood, was kept frozen at −80° until the insulin determination, and their soleus muscles were immediately subjected to experiments on the *in vivo* effect of CyA. The soleus muscles of normal male ICR mice at the age of 12 weeks were subjected to experiments on the *in vitro* effect of CyA.

Measurement of 2DG uptake. 2DG uptake by soleus muscles was measured as previously described [13]. The procedure was, briefly, as follows. For experiments on the *in vivo* effect of CyA, the soleus muscles, separated from the mice of each group, were randomly used for the 2DG uptake assay. The muscles were preincubated in 4 mL of Krebs–Ringer–bicarbonate (KRB)–Hepes buffer (120 mM NaCl, 4.75 mM KCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM $NaHCO_3$ and 30 mM Hepes, pH 7.4) containing 20 mg/mL of bovine serum albumin (BSA) fraction V and 2 mM pyruvate at 25° for 30 min to remove endogenous insulin and then they were further incubated with or without insulin in 4 mL of KRB–Hepes buffer (pH 7.4) containing 20 mg/mL of BSA (pH 7.4) at 25° for 180 min. The muscles were then finally incubated with or without insulin in 4 mL of KRB–Hepes buffer (pH 7.4) containing 20 mg/mL of BSA, 1 mM 2-deoxy-D-[^{3}H]glucose (1 μCi/tube) and 1 mM L-[^{14}C]glucose (0.5 μCi/tube) (New England Nuclear Inc., Boston, MA, U.S.A.) at 25° for 20 min

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† Abbreviations: CyA, cyclosporin A; 2DG, 2-deoxyglucose; OGTT, glucose tolerance test; KRB, Krebs–Ringer–bicarbonate; IRI, immunoreactive insulin; Hepes, BSA, bovine serum albumin; STZ, streptozotocin.

Table 1. Glucose tolerance and plasma IRI levels in mice treated with or without CyA *in vivo*

CyA (mg/kg/day)	Body weight (g)	Muscle weight	ΣBS (mg/dL)	Plasma IRI (ng/mL)
0 (17)	38.5 ± 0.5	16.1 ± 0.4	395 ± 11	3.54 ± 0.32
5 (20)	40.0 ± 0.4	16.5 ± 0.3	480 ± 20	2.60 ± 0.23*
10 (21)	40.1 ± 0.5	16.5 ± 0.3	506 ± 32†	2.23 ± 0.22†
20 (18)	41.3 ± 0.4	16.8 ± 0.3	631 ± 57†	2.48 ± 0.31*

Data are expressed as mean ± SEM. Numbers in parentheses indicate the number of animals.

ΣBS is the sum of the blood glucose values at OGTT (0, 60 and 120 min) performed after 16 hr fasting at the age of 9 weeks. The fasting blood glucose levels were not different significantly among the four groups. The bloods for plasma IRI determination were collected in the fed state at the age of 10 weeks.

* $P < 0.05$, † $P < 0.01$ vs controls by analysis of variance with Duncan's multiple range tests.

Table 2. 2DG uptake by soleus muscles from mice treated with or without CyA *in vivo*

CyA treatment (mg/kg/day)	2DG uptake		
	Basal	Maximal (pmol/mg muscle/min)	Net
0	402 ± 20 (10)	651 ± 28 (13)	249 ± 30
5	399 ± 33 (10)	649 ± 21 (13)	250 ± 26
10	353 ± 14 (12)	513 ± 34 (13)†	160 ± 26*
20	282 ± 23 (10)†	445 ± 19 (10)†	163 ± 21*

Data are expressed as mean ± SEM. Numbers in parentheses indicate the number of muscles examined.

Statistical evaluation was performed by analysis of variance with Duncan's multiple range tests in the case of basal and maximal 2DG uptake and by two-tailed unpaired *t*-test in the case of net 2DG uptake. Maximal insulin-stimulated 2DG uptake was obtained at the insulin concentration of 100 ng/mL. Net insulin-stimulated 2DG uptake was calculated as the difference between maximal insulin-stimulated uptake and basal uptake.

* $P < 0.05$, † $P < 0.01$ (vs controls).

to determine the initial rate of 2DG uptake. After incubation, the muscles were washed with chilled saline and dissolved in 0.2 mL of 30% KOH. The muscle solution was neutralized with acetic acid and dissolved in ACS II (Amersham, Buckinghamshire, U.K.), and the radioactivities of ^{3}H and ^{14}C were counted with a liquid scintillation counter Tri-Carb 300 C (Packard Instrument Co. Inc., Downers Grove, IL, U.S.A.). For the experiment on the *in vitro* effect of CyA, the soleus muscles, separated from normal ICR mice, were randomly used for the 2DG uptake assay. The muscles were preincubated with CyA dissolved in ethanol and polyoxycastor oil or with ethanol and polyoxycaster oil alone in 4 mL of KRB–Hepes buffer (pH 7.4) containing 20 mg/mL of BSA and 2 mM pyruvate at 37° for 150 min to remove endogenous insulin and to let CyA exert the effect before the incubation with insulin. The following procedures were the same as those for the experiments on the *in vivo* effect of CyA except for adding CyA or solvent alone to the reaction mixture during the entire incubation.

The radioactivity of L-[^{14}C]glucose trapped by the muscles was designated to be the non-specific uptake by the simple diffusion and extracellular trapping. All uptake values were corrected for this factor and were shown in picomoles of 2DG taken up specifically per mg muscle per min. The specific uptake of 2DG by the muscles was linear for 40 min either in the presence or absence of insulin and CyA in the incubation medium. The ATP content was 0.53 ± 0.06 nmol/mg in the muscles treated with 100 μg/mL of CyA *in vitro* (N = 5) and this was not different from the value of 0.51 ± 0.05 nmol/mg in the control muscles (N = 5). The maximal insulin-stimulated uptake of 2DG by the muscles was obtained at the insulin concentration of 100 ng/mL.

Measurement of insulin binding. Insulin binding to the soleus muscles was measured as previously described [14]. In the experiments on the *in vivo* effect of CyA, the muscles were preincubated in 4 mL of Tris–Hepes buffer (pH 7.4) containing 10 mg/mL of BSA and 2 mM pyruvate for 30 min at 25° to remove endogenous insulin and were further incubated in 2 mL of Tris–Hepes buffer (pH 8.0) containing 10 mg/mL of BSA and 0.2 ng/mL of A14 labeled ^{125}I-insulin (New England Nuclear Inc.) in the presence or absence of 200 μg/mL of unlabeled

insulin at 15° for 4.5 hr. The muscles were then washed with chilled saline and the radioactivity of ^{125}I was measured with a gamma counter ARC-600 (Aloka Co. Ltd, Tokyo, Japan). The radioactivity in the presence of unlabeled insulin was designated to be the non-specific binding. The specific insulin binding was calculated by subtracting the non-specific binding from the total binding measured as the radioactivity in the absence of unlabeled insulin. The soleus muscle of one side was used for the measurement of total insulin binding and that of the other side was used for the measurement of non-specific insulin binding. In the experiments on the *in vitro* effect of CyA, the muscles were preincubated with or without CyA in 4 mL of Tris–Hepes buffer (pH 7.4) containing 10 mg/mL of BSA and 2 mM pyruvate at 37° for 150 min to make the same *in vitro* conditions as in the 2DG uptake experiment before the incubation with ^{125}I-insulin, and the following insulin binding was measured at 15° for 4.5 hr as above.

Other procedures. The plasma immunoreactive insulin (IRI) concentration was measured in the same assay by the radioimmunoassay technique using rat insulin as a standard (Dainabot Radioisotope Lab., Tokyo, Japan) [15]. The blood glucose concentration was measured by the glucose oxidase method [16]. All results were expressed as the mean ± SEM. The two-tailed unpaired *t*-test and Duncan's multiple range test after analysis of variance were employed for the statistical analyses as appropriate.

RESULTS

In vivo *effect of CyA*

ΣBS, the sum of blood glucose concentrations for the OGTT was significantly ($P < 0.01$) increased in mice treated with 10 and 20 mg/kg of CyA (Table 1), while the fasting blood glucose levels were not different significantly among the four groups (data not shown). The plasma IRI concentration was significantly decreased in mice treated with 5, 10 and 20 mg/kg of CyA ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively) compared with those in control mice (Table 1). The basal 2DG uptake by the muscles was significantly ($P < 0.01$) decreased by 30% in mice treated with 20 mg/kg of CyA (Table 2). Maximal and net insulin-stimulated 2DG uptake by the muscles were also significantly decreased in mice treated with 10 and 20 mg/kg of CyA ($P < 0.01$ and $P < 0.05$, respectively) (Table 2). Insulin binding to the muscles was significantly increased in mice treated with 10 and 20 mg/kg body weight of CyA ($P < 0.05$) (Table 3).

In vitro *effect of CyA*

2DG uptake by the muscles preincubated with CyA *in vitro* was measured under the various insulin concentrations of 0, 2, 5, 10, 50 and 100 ng/mL. The basal 2DG uptake by the muscles was not changed by the preincubation with 1, 10 and 100 μg/mL of CyA. The submaximal insulin-stimulated 2DG uptake was significantly decreased in the muscles treated with 100 μg/mL of CyA ($P < 0.01$). The maximal insulin-stimulated 2DG uptake was significantly decreased by 11, 12 and 25% in the muscles treated with 1, 10 and 100 μg/mL of CyA *in vitro* ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively) (Fig. 1) and the net insulin-stimulated 2DG uptake was

Table 3. Insulin binding to soleus muscles with or without CyA treatment *in vivo* and *in vitro*

CyA treatment	Bound ^{125}I-insulin (%/mg muscle)
In vivo (mg/kg/day)	
0 (9)	0.104 ± 0.007
5 (10)	0.102 ± 0.010
10 (10)	0.139 ± 0.007*
20 (10)	0.138 ± 0.007*
In vitro (μg/mL)	
0 (8)	0.085 ± 0.009
1 (9)	0.081 ± 0.005
10 (9)	0.083 ± 0.004
100 (9)	0.080 ± 0.004

Data are expressed as mean ± SEM. Numbers in parentheses indicate the number of animals examined.
* $P < 0.05$ vs controls by analysis of variance with Duncan's multiple range tests.

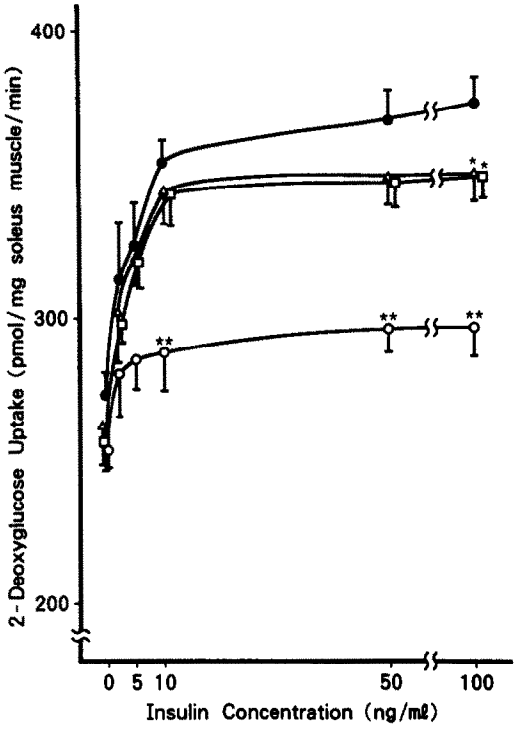

Fig. 1. 2DG uptake by soleus muscles. Soleus muscles separated from normal ICR mice were randomly used for this experiment. Soleus muscles were preincubated *in vitro* with 0 (●), 1 (△), 10 (□) and 100 μg/mL of CyA (○) at 37° for 150 min and the initial rate of 2DG uptake was measured under the various concentrations of insulin as described in Materials and Methods. Values are the mean ± SEM, N = 11. * $P < 0.05$, ** $P < 0.01$ vs controls by analysis of variance with Duncan's multiple range tests.

also decreased by 25, 21 and 51%, respectively ($P < 0.01$). Insulin binding to the muscles was not significantly changed by the treatment of the muscles with CyA *in vitro* (Table 3).

DISCUSSION

The question addressed by the present study was by what mechanisms CyA deteriorates the insulin action and induces the glucose intolerance in animals. In the *in vivo* study, CyA was shown to reduce the insulin secretion significantly with a reciprocal increase in insulin binding to its receptors as presented in Tables 1 and 3. This is in line with many previous studies reporting the decrease of insulin levels in the blood and pancreas or morphological changes of pancreatic islets in CyA-treated animals [5–9]. These findings suggest that reduced insulin secretion resulting from the damage of pancreatic islets by CyA may lead to glucose intolerance in animals. On the other hand, basal and insulin-stimulated 2DG uptake by the muscles were shown to be decreased in CyA-treated mice manifesting the glucose intolerance. This observation is compatible with the previous findings of ours and others that basal and insulin-stimulated 2DG uptake by the muscles were decreased in streptozotocin (STZ)-induced diabetic rats and mice, and in spontaneously diabetic non-obese mice [13, 17]. Furthermore, glucose transporters were shown to be reduced in the adipocytes of STZ-induced diabetic rats [18]. It was further reported that glucose transport by muscles was shown to be regulated by the glucose concentration in the medium *in vitro* [19]. It is, therefore, suggested that a decrease in 2DG uptake by the muscles in CyA-treated mice might result from a decrease in glucose transporters due to hypoinsulinemia and/or the following hyperglycemia *in vivo*.

The plasma insulin concentration was decreased only by 25% in CyA-treated mice manifesting a remarkable glucose intolerance. The plasma insulin concentration and pancreatic insulin content were reported to be decreased by 50% or more in STZ-induced diabetic rats with the same degree of glucose intolerance as in the present study [20]. Furthermore, it was reported that glucose tolerance was impaired in CyA-treated patients even at the time when the serum C-peptide level was increased [4]. It was also reported that the glucose tolerance was impaired in CyA-treated rats with increased insulin levels in the early period of the CyA treatment [10] and, on the contrary, the glucose tolerance was improved at the time when the serum insulin level was still low after discontinuing the CyA treatment [6, 10]. These discrepancies between insulin secretion and glucose tolerance in CyA-treated patients and animals suggest that CyA may cause the glucose intolerance not only by reducing the insulin secretion but also by inducing the insulin resistance of muscles which are responsible for the glucose tolerance *in vivo*.

In the *in vitro* study, the insulin-stimulated 2DG uptake was significantly reduced in CyA-treated muscles while insulin binding to the muscles was not changed. These findings *in vitro* suggest that CyA may affect the postbinding mechanisms of insulin action on the glucose transport by the muscles. Insulin action on glucose transport is believed to promote the translocation of glucose transporters from intracellular compartments to the plasma membranes, which is an energy-dependent process requiring ATP [21]. There may be many steps in this insulin action, from insulin binding on receptors to the expression of glucose transporters on plasma membranes, autophosphorylation of receptors, activation of tyrosine kinase, the transduction of signals, and some other unknown steps. It is unknown at the moment on which steps of insulin action CyA works. At least, a possibility that CyA causes a depletion of ATP by its cytotoxicity is denied by the fact that the ATP content was not changed by the *in vitro* treatment with CyA. But, strictly speaking, the problem of ATP compartmentalization has still remained because we only measured whole cell ATP. Further studies are needed to elucidate the mechanisms by which CyA affects insulin action on the glucose transport of the muscles and this might provide a clue to understanding insulin action on glucose transport by muscles.

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