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ORIGINAL PAPER

Experimental diabetes attenuates calcium mobilization and proliferative response in splenic lymphocytes from mice

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Abstract The present study was conducted to investigate the effects of the diabetic condition on cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, and the proliferation of splenic lymphocytes from mice. Diabetes was induced in mice by intraperitoneal injection of alloxan. $[Ca^{2+}]_i$ and the proliferation ex vivo of splenic lymphocytes isolated from mice were examined using fura-2 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, respectively. Diabetes caused a significant increase in resting $[Ca^{2+}]_i$ and significantly reduced the ability of concanavalin A (Con A; a T-lymphocyte-selective mitogen) to increase $[Ca^{2+}]_i$, but not that of lipopolysaccharide (LPS; a B-lymphocyte-selective mitogen). In addition, diabetes significantly reduced Con A-stimulated but not LPS-stimulated lymphocyte proliferation. Verapamil (an L-type Ca^{2+} channel blocker) inhibited Con A-induced increases in $[Ca^{2+}]_i$ and proliferation in lymphocytes from control and diabetic mice to a similar extent, respectively. These results suggest that diabetes attenuates Con A-stimulated T-lymphocyte proliferation by decreasing $[Ca^{2+}]_i$ via reduction of Ca^{2+} entry through L-type Ca^{2+} channels.

Keywords Diabetes · Cytosolic free Ca^{2+} · Lymphocyte proliferation · Concanavalin A · Lipopolysaccharide · Mouse spleen

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Introduction

Impaired function of immune system cells is a common feature of human diabetes, and patients with diabetes are generally more susceptible to infections and resulting complications [1, 2]. Experiments on cultured human immunological cells have shown profound differences between the normal and diseased states. In this regard, the ability of peripheral blood mononuclear cells from diabetic patients to produce cytokines is reduced [3, 4], and the proliferative responses of lymphocytes to primary protein antigens or mitogens are significantly reduced [5–8]. Several studies on laboratory animals have revealed that some immune defects observed in diabetes result from impairment of lymphoid cell function and viability [9–13]. On the other hand, production of cytokines in peripheral blood mononuclear cells is dose- and time-dependently suppressed by elevation of glucose concentrations [14], and high glucose levels also decrease the proliferative responses of lymphocytes [12] and peripheral blood mononuclear cells [14] to primary protein antigen or mitogen. However, the mechanisms responsible for impaired lymphocyte proliferation in diabetic patients and animals remain largely unclear.

The spleen is primarily involved in dealing with blood-borne pathogens, such as encapsulated bacteria [15]. The spleen cell population is heterogeneous, consisting mostly of lymphocytes, i.e. T- and B-lymphocytes [16], with the former and latter playing an important role in cellular and humoral immunity, respectively.

Calcium (Ca^{2+}) plays an essential role in lymphocyte activation and maturation. Cross-linking of antigen or Fc receptors on T- and B-lymphocytes rapidly increases the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [17, 18], which activates many signaling factors, including the nuclear

factor of activated T-lymphocytes, nuclear factor-κB, and c-Jun NH_2-terminal kinase-1 [19, 20]. These factors, in turn, regulate the expression of several inducible genes that mediate diverse genetic programs, including effector immune function and cell proliferation, differentiation and death [21].

It has been reported that resting $[Ca^{2+}]_i$ is significantly higher in lymphocytes from human type 2 (non-insulin-dependent) diabetic subjects than in lymphocytes from normal subjects [5, 22]. However, there was no apparent difference between the amplitude of an increase in $[Ca^{2+}]_i$ in mitogen-stimulated lymphocytes from human type 1 (insulin-dependent) diabetic and normal subjects [8] or non-obese diabetic (a model of spontaneous type 1 diabetes) and normal mice [23]. Since type 1 diabetes and the condition in non-obese diabetic mice are a chronic autoimmune disease caused by T-lymphocytes-mediated immune destruction of pancreatic β-cells [24], lymphocyte activation occurs in spontaneous type 1 diabetic patients and non-obese diabetic mice. To our knowledge, the effect of the diabetic condition on mitogen-stimulated Ca^{2+} mobilization in lymphocytes has not yet been investigated, nor has the relationship between $[Ca^{2+}]_i$ and the immune response in lymphocytes under the diabetic condition. Therefore, in the present study, we investigated the effects of the diabetic condition on mitogen-stimulated $[Ca^{2+}]_i$ increase and proliferation of splenic lymphocytes from mice using an alloxan-induced diabetes model.

Materials and methods

Chemicals

Alloxan monohydrate, concanavalin A (Con A) and Tris (hydroxymethyl) aminomethane were purchased from Wako Pure Chemicals (Osaka, Japan). Fura-2-acetoxymethyl ester (fura-2-AM), HEPES and EGTA were supplied by Dojindo Laboratories (Kumamoto, Japan) and lipopolysaccharide (LPS), verapamil hydrochloride and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) by Sigma (St. Louis, MO, USA). DMEM was supplied by Invitrogen (Carlsbad, CA, USA) and fetal bovine serum by Cambrex Bio Science (Walkersville, MD, USA). Other chemicals were of reagent grade or the highest quality available.

The standard incubation medium for the measurement of $[Ca^{2+}]_i$ contained the following (in mM): 125 NaCl, 5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 5 $NaHCO_3$, 6 glucose, 1 $CaCl_2$ and 25 HEPES, with the pH adjusted to 7.4. For the Ca^{2+}-free medium, Ca^{2+} was omitted and 0.2 mM EGTA added. DMEM containing 12 mM HEPES, 100 U/ml penicillin G potassium, and 100 μg/ml streptomycin sulfate was used for cell culture.

Animals

Male *ddY* strain mice (3 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Animals were housed under standard light (lights on from 0600 to 1800 hours) and temperature (22 ± 2°C) conditions. Food and water were provided ad libitum. Mice were euthanized by cervical dislocation at 1–2 days or 5 weeks after injection of alloxan or the vehicle. All procedures pertaining to the care and use of experimental animals were approved by the Animal Research Committee of Obihiro University, and conducted in accordance with the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Induction of experimental diabetes

Mice (4 weeks old) were rendered diabetic by a single intraperitoneal injection of alloxan (150 mg/kg) dissolved in a sterile 0.9% NaCl solution following a 22- to 23-h fast. Age-matched control mice were injected with sterile 0.9% NaCl solution alone. Both the alloxan-injected and control animals were kept on the same diet. Some of the former were used for experiments at 1–2 days after injection of alloxan (alloxan–control group). The remaining alloxan- and the 0.9% NaCl-injected animals were used for experiments at 5 weeks after injection of the respective solutions. Glucose concentrations in tail vein blood samples were measured using a blood glucose monitoring system (Accu-Chek Compact Plus; Roche Diagnostics, Fermoy, Ireland). Mice were considered diabetic if their blood glucose level exceeded 400 mg/dl 4 days after injection of alloxan and remained >500 mg/dl for 4 weeks (diabetic group). The blood glucose levels of the control mice were in the range 137–269 mg/dl (control group).

Cell suspensions

Spleens were isolated from the control and alloxan-injected mice and passed through a 1-mm metal mesh. The spleen cells were suspended in the standard medium or DMEM and then filtered through a 10-μm nylon mesh. Next, the cells were treated with pH 7.65 buffer (17 mM Tris–HCl and 0.83% ammonium chloride) to lyse erythrocytes, after which they were washed three times in the standard medium or DMEM. For $[Ca^{2+}]_i$ measurement or further cell culture, spleen cells were resuspended in standard medium or DMEM supplemented with 10% heat-inactivated fetal calf serum, respectively. Cell viability was >90% as

determined by the trypan blue exclusion test, which is consistent with that for human lymphocytes as reported by Falciola et al. [25].

$[Ca^{2+}]_i$ determination

Intracellular $[Ca^{2+}]$ levels were measured by monitoring the intensity of fura-2 fluorescence as described previously [26]. Briefly, a spleen cell suspension (10^7 cells/ml) was incubated for 30 min at 37°C with 5 μM fura-2-AM dissolved in DMSO. The cells were washed three times and then resuspended in 5 ml of standard medium. Next, aliquots (0.5 ml) of the cell suspension were placed in a cuvette and pre-incubated for 10 min before addition of mitogen. Measurement of fluorescence was initiated 4 min before addition of mitogen and continued for 8 min thereafter. Samples in the cuvette were maintained at 37°C and mixed by means of a magnetic stirrer. Fura-2 fluorescence was measured with a model CAF-100 spectrofluorometer (Jasco, Tokyo, Japan) using the ratio mode. Excitation wavelengths were 340 and 380 nm, and the emission wavelength was 500 nm. For each cell suspension, fluorescence due to extracellular fura-2 was determined through the addition of 50 μM Mn^{2+}, and this was subtracted from the maximal fluorescence in order to calculate the free Ca^{2+} concentration of subsequent samples of the same suspension [27]. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. [28].

Proliferation assay

Proliferation was determined in 24-well plates containing 1-ml aliquots of spleen cells (4×10^6 cells per well) in DMEM supplemented with 10% heat-inactivated fetal calf serum. Cells were adjusted to 0.2, 0.6, or 2 μg/ml of Con A or 10, 20, or 40 μg/ml of LPS by adding 0.5 ml of the respective test solution. Control cultures received 0.5 ml of culture medium without Con A or LPS. The spleen cells were then cultured for 72 h at 37°C in an atmosphere containing 5% CO_2. Mitogenic activity was measured by mitochondrial conversion of MTT detected at 570 nm as described by Mosmann [29] using a model Ubest-35 spectrophotometer (Jasco).

Statistical analysis

All data were expressed as the group mean ± SEM of the indicated numbers of mice. Statistically significant differences were assessed using the Student's *t* test for unpaired samples or, when three or more groups were compared, two-way ANOVA for repeated measures as appropriate using the Tukey's post hoc test. Differences between means were considered significant at $P < 0.05$.

Table 1 Body weight and blood glucose level in control and diabetic mice

Group	Body weight gain (% of initial body weights)	Blood glucose concentration (mg/dl)
Control	178.5 ± 4.6 ($n = 7$)	175.9 ± 6.1 ($n = 7$)
Diabetic	147.3 ± 3.9** ($n = 7$)	586.3 ± 8.2** ($n = 7$)

Body weight and blood glucose level were measured 5 weeks after injection of alloxan. Values express group mean ± SEM

n Number of mice per group

** $P < 0.0001$ versus control group

Table 2 Resting $[Ca^{2+}]_i$ in control and diabetic mice

Group	Resting $[Ca^{2+}]_i$ (nM)
Control	94.2 ± 3.7 ($n = 7$)
Diabetic	115.1 ± 4.6* ($n = 7$)

Fura-2-loaded splenic lymphocytes were incubated without addition of mitogen and the fluorescence of extracellular fura-2 was quenched by Mn^{2+}. Values express group mean ± SEM (7 independent experiments performed in duplicate)

n Number of mice per group

* $P < 0.01$ versus control group

Results

Body weight and blood glucose level

The body weight gain of the diabetic mice was significantly lower than that of the control mice during the course of the experiment ($F_{1,69} = 102.67$, $P < 0.0001$). Five weeks after injection of alloxan, the body weight gain in the diabetic animals was significantly decreased (Table 1; $P < 0.0001$). Blood glucose levels were significantly higher in the diabetic mice than in the control mice 5 weeks after injection of alloxan (Table 1; $P < 0.0001$).

Resting $[Ca^{2+}]_i$

We measured resting $[Ca^{2+}]_i$ in the mouse splenic lymphocytes under basal conditions and after inducing diabetes with alloxan, which produced a significant increase in resting $[Ca^{2+}]_i$ (Table 2; $t_{1,12} = 3.817$, $P < 0.01$). The resting level in the control was consistent with that reported by Grinstein and Dixon [30].

Mitogen-stimulated $[Ca^{2+}]_i$ elevation

Next, we examined changes in $[Ca^{2+}]_i$ induced by a T- or B-lymphocyte-selective mitogen in splenic lymphocytes from the control and diabetic mice. Treatment with either mitogen caused a gradual increase in $[Ca^{2+}]_i$ that plateaued

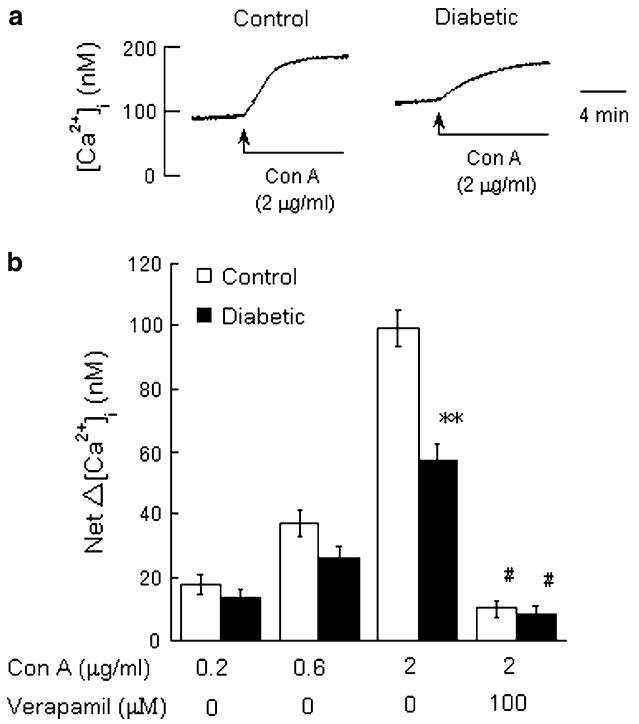

Fig. 1 **a** Time course of changes in $[Ca^{2+}]_i$ in response to Con A (2 μg/ml) in splenic lymphocytes from control and alloxan-induced diabetic mice. **b** Effects of Con A and Con A plus verapamil on $[Ca^{2+}]_i$ in splenic lymphocytes from control ($n = 7$ per group) and alloxan-induced diabetic ($n = 7$ per group) mice. Fura-2-loaded lymphocytes were incubated for 8 min with or without Con A. Verapamil was added 10 min before exposure to Con A. The net $\Delta[Ca^{2+}]_i$ is the difference between the increase in $[Ca^{2+}]_i$ at 8 min after addition of Con A or Con A plus verapamil, and the vehicle, respectively. Data express group mean ± SEM (7 independent experiments performed in duplicate). Statistically significant differences are indicated as: $^{**}P < 0.0001$ versus control group, $^{\#}P < 0.0001$ versus corresponding Con A (2 μg/ml) group

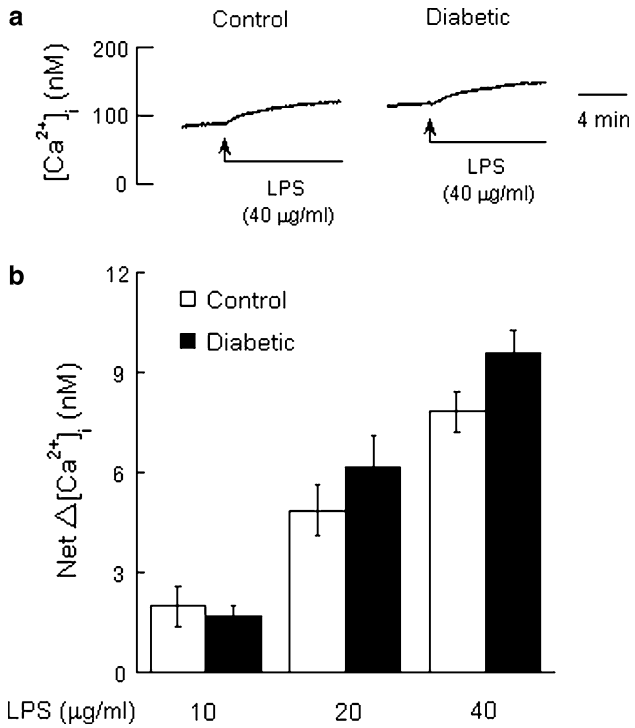

Fig. 2 **a** Time course of changes in $[Ca^{2+}]_i$ in response to LPS (40 μg/ml) in splenic lymphocytes from control and alloxan-induced diabetic mice. **b** Effects of LPS on $[Ca^{2+}]_i$ in splenic lymphocytes from control ($n = 7$ per group) and alloxan-induced diabetic ($n = 7$ per group) mice. Fura-2-loaded lymphocytes were incubated for 8 min with or without LPS. The net $\Delta[Ca^{2+}]_i$ is the difference between the increase in $[Ca^{2+}]_i$ at 8 min after addition of LPS and the vehicle. Data express group mean ± SEM (7 independent experiments performed in duplicate)

within 8 min (Figs. 1a and 2a). Con A (0.2–2 μg/ml), the T-lymphocyte-selective mitogen, caused a concentration-dependent increase in $[Ca^{2+}]_i$ in the splenic lymphocytes ($F_{2,41} = 127.51$, $P < 0.0001$; Fig. 1b). In the diabetic mice, the increase in $[Ca^{2+}]_i$ was reduced significantly ($F_{1,41} = 26.81$, $P < 0.0001$) and at 2 μg/ml of Con A it was reduced by 42% ($P < 0.0001$) (Fig. 1b). The pretreatment of splenic lymphocytes with verapamil (an L-type Ca^{2+} channel blocker, 100 μM; [31]) inhibited Con A-induced increases in $[Ca^{2+}]_i$ in the control and diabetic mice to a similar extent (control $t_{1,12} = 16.375$, $P < 0.0001$; diabetic $t_{1,12} = 12.618$, $P < 0.0001$) (Fig. 1b). For the control mice, this inhibitory effect was in agreement with the findings for human T-lymphocytes and mouse splenic lymphocytes reported by Nakabayashi et al. [32] and Satoh et al. [33]. The removal of external Ca^{2+} abolished Con A-induced increases in $[Ca^{2+}]_i$ for both control and diabetic mice (data not shown).

LPS (10–40 μg/ml), the B-lymphocyte-selective mitogen, also caused a concentration-dependent increase in $[Ca^{2+}]_i$ in lymphocytes from the control and diabetic mice ($F_{2,41} = 53.14$, $P < 0.0001$; Fig. 2b), but diabetes failed to inhibit the LPS-induced increase in $[Ca^{2+}]_i$ (Fig. 2a, b).

Mitogen-stimulated proliferation

We also examined the proliferative responses to a T-lymphocyte- or B-lymphocyte-selective mitogen in splenic lymphocytes derived from the control and diabetic mice. Con A (0.2–2 μg/ml) produced a concentration-dependent increase in the proliferation of lymphocytes from both types of mice, with a sharp rise in the concentration–response curve from 0.6 to 2 μg/ml ($F_{2,41} = 153.74$, $P < 0.0001$; Fig. 3). In diabetic mice, the increase in lymphocyte proliferation was reduced significantly ($F_{1,41} = 29.75$, $P < 0.0001$) and at 2 μg/ml of Con A it was reduced by 46% ($P < 0.0001$) (Fig. 3). The pretreatment of splenic lymphocytes with verapamil (100 μM) completely suppressed Con A-stimulated lymphocyte proliferation in both the control and diabetic mice (Fig. 3).

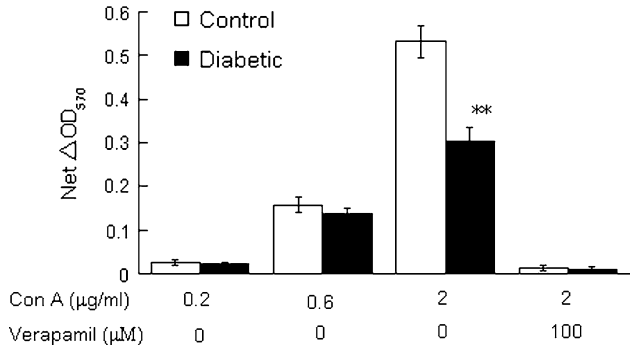

Fig. 3 Proliferative effects of Con A and Con A plus verapamil on splenic lymphocytes from control ($n = 7$ per group) and alloxan-induced diabetic ($n = 7$ per group) mice. Lymphocytes were stimulated for 72 h with Con A or Con A plus verapamil, and proliferation was measured by MTT assay. Verapamil was added 10 min before exposure to Con A. The net ΔOD_{570} is the difference between the increase in OD_{570} (optical density at 570 nm) at 72 h after addition of Con A or Con A plus verapamil, and the vehicle, respectively. Data express group mean ± SEM (7 independent experiments performed in duplicate). Statistically significant difference is indicated as: $^{**}P < 0.0001$ versus control group

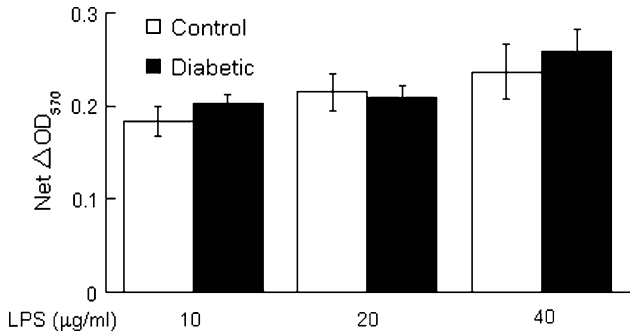

Fig. 4 Proliferative effect of LPS on splenic lymphocytes from control ($n = 7$ per group) and alloxan-induced diabetic ($n = 7$ per group) mice. Lymphocytes were stimulated for 72 h with various concentrations of LPS, and proliferation was measured by MTT assay. The net ΔOD_{570} is the difference between the increase in OD_{570} (optical density at 570 nm) at 72 h after addition of LPS and the vehicle. Data express group mean ± SEM (7 independent experiments performed in duplicate)

The inhibitory effect of verapamil in the control mice was in agreement with the findings for human T-lymphocytes and mouse splenic lymphocytes reported by Birx et al. [34], Nakabayashi et al. [32] and Satoh et al. [33]. At the concentration used in the present study, it had no effect on cell viability as assessed by the trypan blue exclusion test (data not shown).

LPS (10–40 μg/ml) brought about a large increase in the proliferation of lymphocytes from the control and diabetic mice (Fig. 4). The increases in lymphocyte proliferation due to 10 μg/ml of LPS were 80–90% of those induced by 20 and 40 μg/ml (Fig. 4). Diabetes in the mice did not affect the increase in lymphocyte proliferation. The concentration–response curves for Con A- and LPS-stimulated proliferation in the control mouse lymphocytes were similar to those reported by Lysle et al. [35], Mosmann [29], Sakowicz-Burkiewicz et al. [11] and Silberman et al. [36]. The concentrations of Con A and LPS needed to induce a maximal response were 2 and 40 μg/ml, respectively.

Discussion

The purpose of this study was to investigate the effects of the diabetic condition on mitogen-stimulated $[Ca^{2+}]_i$ and proliferation of splenic lymphocytes from mice. We chose the alloxan-induced diabetes mouse model because it had previously proved useful for evaluating immunological changes associated with hyperglycemia and diabetes, with no impairment of lymphoid cell function and viability [37]. Our experiments showed that blood glucose levels in alloxan-induced diabetic mice were significantly elevated, and associated with known diabetic symptoms such as polyuria and weight loss. We did not observe any difference in Ca^{2+} signaling or proliferative response between the control and alloxan–control groups, and splenic lymphocytes from the alloxan–control group did not show the same changes as were obtained in the diabetic group (data not shown). Therefore, we assumed that the alterations observed were not due to toxic effects of alloxan but were a consequence of the diabetic state. Type 1 diabetes, such as that in insulin-dependent diabetic patients, diabetic Bio Breeding rats and non-obese diabetic mice, is a chronic autoimmune disease caused by T-lymphocyte-mediated immune destruction of pancreatic β-cells [24]. As aberrant T-lymphocyte activation and proliferation occur in patients and animals with autoimmune diabetes [38], the use of lymphocytes from genetically diabetic patients and diabetic animals was not considered appropriate for the purpose of this study.

We demonstrated for the first time that alloxan-induced diabetes significantly increases the resting $[Ca^{2+}]_i$ in splenic lymphocytes from mice. Three transport systems, i.e. plasma membrane Ca^{2+}-ATPase (PMCA), endoplasmic reticulum Ca^{2+}-ATPase, and Na^+/Ca^{2+} exchanger, primarily act in concert to regulate Ca^{2+} homeostasis in lymphocytes [39, 40], and Balasubramanyam et al. [22] concluded that cellular Ca^{2+} accumulation in lymphocytes from human type 2 diabetic subjects results from reduction in PMCA activity and modulation of Na^+/Ca^{2+} exchanger. We assume that the diabetic condition modified one, two or all three transport systems, causing an increase in resting $[Ca^{2+}]_i$.

Con A and LPS increase $[Ca^{2+}]_i$ in T- and B-lymphocytes, respectively [41–43]. We demonstrated for the first

time that alloxan-induced diabetes reduces a Con A-induced but not a LPS-induced increase in $[Ca^{2+}]_i$ in mouse splenic lymphocytes, which indicates that diabetes reduces the increase in $[Ca^{2+}]_i$ in T-lymphocytes in particular.

Proust et al. [41] and Tsien et al. [43] reported that an increase in $[Ca^{2+}]_i$ in mouse T-lymphocytes induced by Con A was abolished by removal of extracellular Ca^{2+}. We obtained the same result (data not shown). This shows that an increase in $[Ca^{2+}]_i$ induced by Con A needs Ca^{2+} entry from the extracellular medium. It has been demonstrated that T-lymphocytes express an L-type Ca^{2+} channel [44]. As verapamil inhibited Con A-induced increases in $[Ca^{2+}]_i$ in the control and alloxan-induced diabetic mice to a similar extent in the present study, this suggests that diabetes attenuates a Con A-induced increase in $[Ca^{2+}]_i$ by reducing Ca^{2+} entry through L-type Ca^{2+} channels.

Lysle et al. [35] and Silberman et al. [36] observed that Con A and LPS stimulate the proliferation of mouse splenic lymphocytes, more specifically the proliferation of T- and B-lymphocytes, respectively. In the present study, we demonstrated that alloxan-induced diabetes reduces Con A-stimulated but not LPS-stimulated lymphocyte proliferation in mice, indicating that diabetes reduces T-lymphocyte proliferation. Jang et al. [45] have already shown that streptozotocin-induced diabetes reduces Con A-stimulated T-lymphocyte but not LPS-stimulated B-lymphocyte proliferation in mice. These findings indicate that T-lymphocyte is more vulnerable to high blood-glucose than B-lymphocyte. However, Rubinstein et al. [46] observed that streptozotocin-induced diabetes reduces LPS-stimulated B-lymphocyte proliferation in mice. This disagreement with our finding could be due to various differences between the studies, such as mouse strain, sex, age, diabetic state and period of hyperglycemia.

Con A and LPS mobilize Ca^{2+} by activating T- and B-lymphocyte receptors, respectively [41–43], and an increase in $[Ca^{2+}]_i$ constitutes an essential triggering signal for T- and B-lymphocyte proliferation [47, 48]. In the present study, there was a positive correlation between $[Ca^{2+}]_i$ and T-lymphocyte proliferation in the alloxan-induced diabetic state, and verapamil completely suppressed the proliferative response to Con A in lymphocytes from both the control and diabetic mice. These results suggest that diabetes attenuates Con A-stimulated T-lymphocyte proliferation by decreasing $[Ca^{2+}]_i$ via reduction of Ca^{2+} entry through L-type Ca^{2+} channels.

The lymphocyte proliferative response to primary protein antigen or mitogen has often been used as an in vitro test of cell-mediated immune function, and hyperglycemia in human type 1 [6, 8] and type 2 [5, 7] diabetes and experimental diabetes in rats [11–13] has been seen to be associated with a decrease in lymphocyte proliferation in response to primary protein antigen or mitogen. We also observed a decreased mitogen-induced proliferative response in the splenic lymphocytes from alloxan-induced diabetic mice. Since the spleen is primarily involved in dealing with blood-borne pathogens, such as encapsulated bacteria [15], a diabetes-induced decrease in splenic lymphocyte function could impair protection against blood-borne pathogens, and might be partially responsible for the increased occurrence of infections in diabetic patients and animals.

In conclusion, the present study demonstrates that experimental diabetes in mice attenuates Con A-stimulated T-lymphocyte proliferation by decreasing $[Ca^{2+}]_i$ via reduction of Ca^{2+} entry through L-type Ca^{2+} channels. The pathogenesis of complications of diabetes is likely to be a multifactorial process involving: (1) the polyol pathway and increased polyol pathway flux; (2) increased formation of advanced glycation end products; (3) hyperglycemia-induced activation of protein kinase C isoforms; and (4) increased hexosamine pathway flux and consequent over-modification of proteins by *N*-acetylglucosamine [49]. A consistent differentiating feature common to all cell types that are damaged by hyperglycemia is increased production of reactive oxygen species [49]. These factors could play a role in mediating the effects of the diabetic condition on lymphocytes. Additional studies are now needed to further clarify how diabetes attenuates Ca^{2+} mobilization and T-lymphocyte proliferation.

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