Dipeptidyl Peptidase IV Inhibition with MK0431 Improves Islet Graft Survival in Diabetic NOD Mice Partially via T-cell Modulation

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Submitted 12 August 2008 and accepted 4 December 2008.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org
ABSTRACT

Objective: The endopeptidase dipeptidyl peptidase-IV (DPP-IV) has been shown to N-terminally truncate the incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), thus abating their ability to potentiate glucose-stimulated insulin secretion (GSIS). Increasing the circulating levels of incretins through administration of DPP-IV inhibitors has therefore been introduced as a therapeutic approach for the treatment of Type 2 diabetes (T2DM). DPP-IV inhibitor treatment has also been shown to preserve islet mass in rodent models of T1DM. The current study was initiated in order to define the effects of the DPP-IV inhibitor Sitagliptin (MK0431) on transplanted islet survival in non-obese diabetic (NOD) mice, an auto-immune T1DM model.

Research Design and Methods: Effects of MK0431 on islet graft survival in diabetic NOD mice were determined with metabolic studies and microPET imaging, and its underlying molecular mechanisms were assessed.

Results: Treatment of NOD mice with MK0431, prior to and post islet transplantation, resulted in prolongation of islet graft survival whereas post-treatment alone showed only small beneficial effects compared to non-treated controls. Subsequent studies demonstrated that MK0431 pre-treatment resulted in decreased insulitis in diabetic NOD mice and reduced in vitro migration of isolated splenic CD4+ T lymphocytes. Furthermore, in vitro treatment of splenic CD4+ T cells with DPP-IV resulted in increased migration, as well as activation of protein kinase A (PKA) and Rac1.

Conclusions: Treatment with MK0431 therefore reduced the effect of autoimmunity on graft survival partially by decreasing the homing of CD4+ T cells into pancreatic β-cells, through a pathway involving cAMP/PKA/Rac1 activation.

Abbreviations: T1DM, Type 1 diabetes; T2DM, Type 2 diabetes; DPP-IV, dipeptidyl peptidase IV; sDPP-IV, soluble dipeptidyl peptidase IV GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; rAD-TK, recombinant adenovirus expressing HSV1-Sr39tk; rAD-β-gal, recombinant adenovirus expressing β-galactosidase; PET, positron emission tomography; NCD, normal chow diet; PRG, PET reporter gene; PRP, PET reporter probe; HSV1-Sr39TK, mutant form of herpes simplex virus 1 thymidine kinase; [18F]FHBG, 9-(4-[18F]-Fluoro-3-hydroxymethylbutyl)-guanine; MAPK, mitogen activated protein kinase; SAPK/JNK, stress-activated protein kinase/Jun-amino-terminal kinase; cAMP, cyclic 5’-adenosine monophosphate; PKA, Protein Kinase A; PKB, protein kinase B; TAC, time activity curve; MAP, maximum a posteriori; ROI, region of interest; m.o.i, multiplicity of infection; NOD mice, non-obese diabetic mice; STZ, streptozotocin; GSIS, glucose-stimulated insulin secretion; IPGTT, intraperitoneal glucose tolerance test; ANOVA, analysis of variance; SEM, standard errors of the mean.
The incretin hormones, glucose-dependent insulino tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), exert a number of actions that improve glucose homeostasis, including potentiation of glucose-stimulated insulin secretion (GSIS), promotion of β-cell proliferation and survival, and inhibition of glucagon secretion (1-7). Since GIP and GLP-1 are rapidly degraded by the endopeptidase dipeptidyl peptidase IV (DPP-IV; CD26), it has not been possible to directly take advantage of their beneficial actions for the treatment of Type 2 diabetes (T2DM, 8-9). Therefore, a number of strategies have been explored to circumvent this problem, including the development of small molecule (10-11) and DPP-IV resistant peptide (12-13) incretin receptor agonists, and DPP-IV inhibitors (14-15). Members of two classes of compounds have recently been approved by the FDA as type 2 diabetes (T2DM) therapeutics: the DPP-IV-resistant GLP-1 receptor agonist (incretin mimetic) exenatide (Byetta™) and the DPP-IV inhibitor sitagliptin (Januvia™).

Although DPP-IV inhibitors have been extensively studied in T2DM, there is little known about their potential for Type 1 diabetes (T1DM) therapy. Infusion of GLP-1 was shown to reduce glycemic excursions in T1DM patients, and this was attributed to reduced glucagon levels and delayed gastric emptying (16-17). In preclinical studies, the DPP-IV inhibitor isoleucine thiazolidide was shown to improve glucose tolerance in both streptozotocin (STZ)-induced (18-19) and BioBreeding (BB) (19) diabetic rats, associated with increased β-cell survival and, possibly, islet neogenesis (18). Additionally, we recently showed that the DPP-IV inhibitor MK0431 prolonged islet graft survival in STZ-induced diabetic mice (20). In the current study we show that MK0431 pre-treatment resulted in the prolongation of islet graft survival in an autoimmune T1DM model, the non-obese diabetic (NOD) mouse, through a mechanism that includes modulation of CD4+ T lymphocyte migration.

**EXPERIMENTAL PROCEDURES**

Mice— NOD/LtJ mice (NOD, H2b) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice (8-10 weeks old) were placed on either a normal chow diet (NCD, Purina Rodent Chow #5015) or diet containing Sitagliptin (MK0431) (21) (Purina Rodent Chow # 5015 plus 4 g MK0431 /Kg, Research Diets Inc., New Brunswick, NJ) ad libitum. From the mice that had been fed a normal chow diet (NCD), two groups of diabetic mice were studied. Immediately following the islet transplantation, Group I mice received the NCD for the remainder of the study (NCD Tx), whereas Group II mice were fed the MK0431-containing diet (Post MK0431 Tx). Group III mice were animals that had received the MK0431 diet for ~1 month prior to surgery and for the remainder of the study (Pre MK0431 Tx). In a second study, mice were fed either NCD or MK0431 for a period of 4 weeks. All animal experiments were conducted in accordance with the guidelines put forth by the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care.

**DPP-IV activity assays**— To measure the plasma DPP-IV activity, a fluorometric assay was employed as previously described (20).

**Islet isolation and islet gene transfer**— Islets were isolated from non-diabetic male NOD/LtJ mice (8-12 week old) by collagenase digestion (22). Recombinant adenovirus expressing HSV1-Sr39TK was produced, expanded by infection of human embryonic kidney (HEK)-293 cells (23). Islets were exposed to rAD-TK as previously described (20, 24, 25).
Islet transplantation—A cohort of 70 female NOD/LtJ mice was divided into 2 randomly selected groups of 35 and, from the age of 8-10 weeks, one group of mice received a normal chow diet (NCD) while the second received a diet containing MK0431 (4 g/kg). When mice developed diabetes, defined as a blood glucose level of 15 mmol/l or greater for 3 consecutive days, they received an islet transplant. Islets (200 obtained from non-diabetic male NOD/LtJ mice, infected with 250 m.o.i. of rAD-TK) were transplanted under the kidney capsule as described earlier (20, 24, 25).

Plasma glucose determinations, intraperitoneal glucose tolerance tests (IPGTTs) and plasma hormone measurements—Non-fasting blood glucose levels were measured in mouse-tail blood using a SureStep Glucose analyzer (LifeScan) at the time points indicated in Figures 1 and 2. For the IPGTTs, mice were fasted for 4 h and blood glucose levels measured at 0, 15, 30, 60, 90 and 120 min following the glucose challenge (2 g/kg). Blood samples with glucose levels $\geq 27.8$ mmol/l were diluted with blood from non-diabetic mice and levels calculated. Plasma insulin, glucagon and active GLP-1 levels were determined using a Multiplex assay kit (Linco Research Inc.).

Synthesis of $[^{18}F]$FHBG—$[^{18}F]$FHBG was synthesized by a modification of the method of Ponde et al. (26) as previously described (24).

MicroPET—A PET reporter gene (PRG)/PET reporter probe (PRP) system was utilized, as previously described (20, 24, 25). HSV1-sr39tk was expressed in islets as described above. The probe, 9-(4-$[^{18}F]$-Fluoro-3-hydroxymethylbutyl)-guanine ($[^{18}F]$FHBG), was systemically administered and its retention in islets, following phosphorylation by rAD-TK, was quantified by microPET imaging. Transplanted mice were scanned using a FocusTM 120 (CTI Concorde) system that yield 63 slices, 1.2 mm apart with an in-plane resolution of 2 mm full-width at half-maximum (FWHM) (27). Image reconstruction and data analysis were performed as previously described (20, 24, 25).

Insulitis scoring and measurement of relative $\beta$-cell area—Paraffin-embedded pancreatic sections were stained with hematoxylin and eosin and islets were examined in a blinded manner under the microscope, to evaluate the degree of mononuclear cell infiltration. The degree of insulitis was scored as following five categories: 0 = intact islet; < 25 % of the islet infiltrated; 25 to 50 % of the islet infiltrated; 50 to 75 % of the islet infiltrated; and > 75 % of the islet infiltrated. The degree of insulitis in 20 to 25 islets per mouse was evaluated. $\beta$-cell area was measured on the sections of each pancreas stained for insulin. Morphometric evaluation of the total $\beta$-cell area was performed by computer-assisted image analysis (Northern Eclipse ver. 6) using light and immunofluorescence microscopy. The area of insulin-positive cells and the total area of the tissue section were evaluated for each section, and the percentage $\beta$-cell area was calculated by the ratio of the area occupied by insulin-positive cells to the total pancreatic area.

CD4$^+$ T lymphocytes isolation—For isolation of T lymphocytes, spleen cell suspensions were prepared from female NOD mice. CD4$^+$ T lymphocytes were enriched using the SpinSep® Mouse CD4$^+$ T Cell separation kit (StemCell Technologies Inc), and the purity of CD4$^+$ T cells confirmed by flow cytometry. Migration assay—CD4$^+$ T lymphocytes were plated on membrane inserts (8-$\mu$m pore size) in serum-free RPMI 1640 medium and cell migration was assayed using Transwell chambers (Corning), in the presence or absence of purified porcine kidney DPP-IV (32.1 units/mg; 100 mU/ml final concentration; kindly provided by Dr. Hans-Ulrich Demuth, Probiodrug GmbH, Halle (Saale), Germany) (28) $\pm$ DPP-IV inhibitor
(100 μM). After 1 h, cells on the upper surface were removed mechanically and cells that had migrated into the lower compartment were counted. The extent of migration was then expressed relative to the control sample. *Rac1 GTP binding assay*— Total cellular extracts were isolated from CD4+ T lymphocytes and Rac1 GTP binding assays performed using the fluorophore-based RhoGEF exchange assay kit (Cytoskeleton, Denver, CO), and data normalized to protein concentration.

*Cyclic adenosine monophosphate (cAMP) Measurements*— CD4+ T lymphocytes were incubated for 30 min with GIP, GLP-1 (100 nM) or purified porcine DPP-IV (100 μU/ml) in the presence of IBMX (0.5 mM). cAMP concentration in the cell extracts were determined using the Parameter™ cyclic AMP assay kit (R&D Systems).

*Protein Kinase A Activity Assay*—Activity was measured using a PKA kinase activity assay kit (Stressgen, Mississauga, Ontario) according to the manufacturer's protocol. The enzyme activity was normalized to protein concentration and data are shown as the relative activity to control.

*Western blot analysis*— Total cellular extracts were separated on a 15 % sodium dodecyl sulfate (SDS)/polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad). Probing of the membranes was performed with antibodies to phospho-p38 MAP Kinase (Threonine-180/Tyrosine-182), p38 MAP Kinase, phospho-p42/44 MAP Kinase (Threonine-202/Tyrosine-204), p42/44 MAP Kinase, phospho-SAPK/JNK (Threonine-183/Tyrosine-185), phospho-PKB (Serine-473), phospho-PKB (Threonine-308), PKB and β-actin (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using horseradish peroxidase-conjugated IgG secondary antibodies.

*Statistical analysis*— Data are expressed as means ± Standard Errors of the Mean (SEM). Data were analyzed using the linear regression analysis program PRISM (GraphPad, San Diego, CA) and area under the curves (AUCs) were calculated using the algorithm provided in the Prism software package. Significance was tested using analysis of variance (ANOVA) with Newman-Keuls hoc test (P < 0.05) as indicated in figure legends.

**RESULTS**

*MK0431 treatment improves islet graft survival in diabetic NOD mice.* Islet transplanted, diabetic NOD mice were studied to determine the effect of MK0431 treatment on graft survival. Three groups of mice were studied, as described in Materials and Methods: Group I received NCD throughout the study period (NCD Tx), Group II were fed MK0431 only following transplant (Post MK0431 Tx) and Group III received MK0431 prior to and post-transplant (Figure 1A). Importantly, of mice that were treated with MK0431 prior to receiving the transplant, only 22.8 % (8/35) became diabetic, compared to 40 % (14/35) of the mice receiving NCD.

For the transplants, islets were infected with a rAD-TK, recombinant adenovirus expressing HSV1-sr39tk, the gene for a mutant form of herpes simplex virus 1 thymidine kinase, to enable PET imaging (29-30), and inserted under the kidney capsule of diabetic female NOD mice. The effect of recombinant adenoviral infection on NOD islet function was addressed with *in vitro* studies. Insulin secretory capacity was preserved in 250 m.o.i. rAD-TK-treated islets, when compared with untreated or control rAD-β-gal-virus treated islets (Supplementary Figure 1A). Furthermore, although small decreases in mean cell viability were observed with both rAD-TK and rAD-β-gal-treated islets when compared to untreated
islets, these reductions did not reach statistical significance (Supplementary Figure 1B), indicating that 250 m.o.i. was an appropriate dose for further studies. The DPP-IV activity in plasma from non-diabetic NOD mice was 67.2 ± 4.3 mU/ml, whereas levels measured at the time of islet transplantation were 100.7 ± 7.1 mU/ml in the NCD diabetic group, 93.8 ± 16.5 mU/ml in the Post MK0431 Tx group and 52.1 ± 11.8 mU/ml in the Pre MK0431 Tx group (n = 5 ~ 7 per group). Plasma DPP-IV activity in the NCD Tx group progressively increased to levels that were ~300 - 400 % greater than at the initiation of the study. Both Pre and Post MK0431 Tx groups showed significantly reduced DPP-IV activity compared to the NCD group (Figure 1B). Plasma levels of active (N-terminally intact) GLP-1 were significantly increased in both Pre and Post MK0431 Tx groups, demonstrating protection of circulating incretin from DPP-IV mediated degradation (Figure 1C). For the first 0.5 week following transplantation, Pre MK0431 Tx group tended to be slightly heavier (Figure 1D), eating less food (Figure 1E), and drinking less water (Figure 1F) than the Post MK0431 Tx group, although it didn’t reach to the statistically significant point. The NCD and Post MK0431 Tx groups then lost body weight (Figure 1D), despite their greater food and water intake (Figure 1E and 1F), whereas the Pre MK0431 Tx group maintained body weight and food and water intake remained stable (Figures 1D, 1E and 1F). By 0.5 weeks following transplantation, non-fasting blood glucose levels were normalized in the Pre MK0431 Tx group, whereas levels in NCD and Post MK0431 Tx groups progressively increased from 1 week on (Figure 1G). A similar trend was evident for fasting blood glucose (Figure 1H). During the course of the study, ~80 % of the NCD Tx group and 40 % of the Post MK0431 Tx group died, probably as a result of the severe hyperglycemia, whereas all of the Pre MK0431 Tx group survived (Figure 1I).

Intraperitoneal glucose tolerance tests (IPGTTs) were performed following transplantation (n = 5~7 animals/group). As expected, with the sub-optimal transplanted islet mass, IPGTTs showed a rapid deterioration of glucose handling in NCD and Post MK0431 Tx groups. By contrast, the Pre MK0431 Tx group preserved their capacity to regulate blood glucose levels normally until the end of the study (Figure 2A). Both basal and glucose-stimulated plasma insulin levels in the NCD and Post MK0431 Tx groups were below 6.2 pM (the detection limit of the assay), whereas the Pre MK0431 Tx group showed stable insulin secretory responses to glucose stimulation (Figure 2B). While plasma glucagon levels increased substantially in the NCD Tx group over the 3 weeks following transplantation, glucagon levels in the Post MK0431 Tx group remained low, and were further decreased in the Pre MK0431 Tx group (Figure 2C). The effect of MK0431 on islet graft survival was further assessed by microPET imaging. The PET signal from transplanted islets in the NCD and Post MK0431 Tx groups decreased dramatically within 1 week, whereas mice in the Pre MK0431 Tx group showed sustained PET signals for up to 4 weeks following transplantation (Figure 2D). These results indicated that MK0431 pre-treatment resulted in prolongation of islet graft survival.

Modulation of the immune system in MK0431-treated mice. In view of the reduced incidence of diabetes in the Pre MK0431 Tx group, as well as the prolonged islet graft survival, we considered it likely that the action of MK0431 in NOD mice involved modulation of the immune system during the 1-month pre-treatment period. To determine whether MK0431 administration could impact on immune cell infiltration of transplanted islets, the development of insulitis was examined in female NOD mice
receiving NCD or MK0431 diets for 1 month starting at 8-10 weeks of age (Figure 3A). By 12-14 weeks of age, the incidence of diabetes was substantially decreased in the MK0431 group compared with the NCD group; 17.6% (3/17) of the mice developing diabetes in the MK0431 group and 35% (6/17) in the NCD group. Animals with glucose <15 mmol/L were grouped as ‘Normal’ and those with glucose ≥15 mmol/L as ‘Diabetic’. There were no significant differences in blood glucose levels between the diabetic mice that had been fed NCD 21.1 ± 2.5 (n = 6) and those receiving MK0431 diet 20.7 ± 2.8 (n = 3) (Figure 3B). However, histochemical analysis revealed that islets in sections from the diabetic MK0431 group exhibited a more intact structure, whereas the majority of islets in the diabetic NCD group showed severe insulitis: approximately 90% and 70% of the islets in NCD Diabetic and MK0431 Diabetic mice, respectively, were infiltrated by more than 25% (Figure 3C). Treatment with MK0431 resulted in significantly increased islet β-cell area in both the mice with normal plasma glucose and the diabetic mice: Normal mice β-cell area- NCD: 0.37 ± 0.03%; MK0432: 0.61 ± 0.05%; Diabetic mice β-cell area- 0.01 ± 0.003%; MK0431: 0.12 ± 0.02% (Figure 3D).

Mechanisms underlying the DPP-IV mediated reduction in lymphocytic infiltration

Potential mechanisms by which DPP-IV inhibitor treatment protected islets from severe lymphocytic infiltration were next assessed. CD4+ T cells have been considered to be essential for the development of diabetes through recognition of β-cell antigens in the context of the class II MHC I-A<sup>ª</sup>. The identification of an islet-specific CD4<sup>+</sup> T cell clone for the acceleration of diabetes in young NOD mice (31) led us to consider the potential involvement of DPP-IV mediated CD4<sup>+</sup> T cells migration. The in vitro migration of splenic CD4<sup>+</sup> T cells isolated from the diabetic NCD group was significantly increased, when compared to the normal NCD group, and MK0431 treatment partially restored the levels towards normal (Figure 3E). The extent of CD4<sup>+</sup> T cell migration correlated well with plasma DPP-IV activity and blood glucose levels (R² = 0.85, P < 0.0001 for plasma DPP-IV activity and R² = 0.79, P < 0.0001 for fasting blood glucose levels, Figures 3F and 3G). These results suggested that the prolonged islet graft survival observed in the Pre MK0431 Tx group was at least partially due to immunomodulation.

The effect of MK0431 treatment on signaling modules potentially involved in the regulation of CD4<sup>+</sup> T cell migration was next studied. Rac1 is a member of the Rho-GTPase family, and has been shown to play an important role in cytoskeletal reorganization, membrane trafficking, cell growth and development (32-33). In view of this role, we examined the Rac1 GTP binding activity in splenic T cells from NCD and MK0431-treated NOD mice. As shown in Figure 4A, Rac1 GTP binding activity in CD4<sup>+</sup> T cells of the diabetic NCD group was substantially reduced in the diabetic MK0431 treated group. On the other hand, there were no significant changes in the level of phosphorylation of a number of other signaling molecules, including p38 mitogen activated protein kinase (MAPK) (Thr180/Tyr182), stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) (Thr183/Tyr185), p42/44 MAPK (Thr202/Tyr204), protein kinase B PKB (Ser473) or PKB (Thr308) (Figure 4B-4F). Further in vitro studies were next performed to determine whether elevated circulating incretins or DPP-IV itself were likely responsible for the increase in T cell migration and Rac1 activity. CD4<sup>+</sup> T cells were isolated from the spleen of non-diabetic female NOD mice and treated with DPP-IV, GIP or GLP-1 in the presence or absence of DPP-IV inhibitor. As shown in Figure 5A,
treatment of CD4⁺ T cells with DPP-IV resulted in ∼1.6-fold increase in T cell migration that was abolished by DPP-IV inhibitor treatment. Neither GIP nor GLP-1 exhibited significant effects on T cell migration. In preliminary studies, similar effects of DPP-IV on migration of human T cell lymphoma Jurkat cells were observed. Treatment resulted in ∼3.8-fold increases in migration that were abolished by DPP-IV inhibitor treatment (Supplementary Figure 2).

Treatment with DPP-IV also increased Rac1 GTP binding activity, whereas both GIP and GLP-1 had no effect (Figure 5B). There were also no significant effects of DPP-IV, GIP or GLP-1, in the presence or absence of DPP-IV inhibitor, on phosphorylation of other signaling proteins examined (Figures 5C-5G), strongly suggesting that DPP-IV regulates the migration of CD4⁺ T cells directly, via a pathway involving Rac1.

Both PKA activation of Rac1 (34) and Rac1 activation of PKA (35) have been described in different cell types. Involvement of the cAMP/PKA pathway in DPP-IV action on CD4⁺ T lymphocytes was therefore studied. Treatment of splenic CD4⁺ T lymphocytes with DPP-IV (100 mU/ml) for 30 mins resulted in ∼2.7-fold increase in cAMP (cyclic 5’-adenosine monophosphate) concentration compared to control, and DPP-IV inhibitor abolished the effect (Figure 6A). By contrast, treatment with GIP or GLP-1 (100 nM) had no significant effects on cAMP levels (Figure 6A). MK0431 decreased DPP-IV mediated PKA activation in a concentration dependent manner (Figure 6B). The low concentrations that resulted in inhibition support a specific action of MK0431 on DPP-IV. Additionally, MK0431 did not inhibit the PKA stimulatory effect of 6-Bnz-cAMP, a PKA-selective, PDE resistant cAMP analog (Figure 6C). This suggests that the activation is upstream of cAMP production, probably at an extracellular level. As expected, when PKA was inhibited by either H-89 or Rp-cAMPS, DPP-IV was no longer capable of stimulating PKA, and DPP-IV inhibition exhibited no further effect (data not shown). Together, these results strongly support a mechanism for DPP-IV mediated PKA activation proximal to, or at the level of, adenylate cyclase. Forskolin, a direct activator of adenyl cyclase, was found to mimic the effect of DPP-IV on CD4⁺ T cell migration, and responses to both DPP-IV and forskolin were greatly reduced by administration of the selective PKA inhibitor H-89 (Figure 6D). Similarly, DPP-IV and forskolin both increased Rac1 GTP binding, whereas H-89 abolished their effects (Figure 6E). Together, these results strongly suggest that DPP-IV activates a pathway involving the production of cAMP and activation of PKA and Rac1, resulting in increased CD4⁺ T cell migration.

**DISCUSSION**

Two major developments have been responsible for dramatically improving the long-term success of islet transplantation and allowing islet-alone grafts: the development of automated methods for human islet isolation (36) and improved immunosuppression (37), culminating in what is known as the ‘Edmonton Protocol’ (37, 38). However, despite major successes, with current procedures at least two donor pancreases are generally needed to achieve insulin independence, due to the loss of islet viability both during isolation and following transplantation. Additionally, only 67 % (39) and 10 % (40) of transplant recipients have been shown to be insulin-independent at the end of 1 year and 5 years, respectively. The causes of post-transplant loss are multiple and apoptosis is thought to play a critical role. As the incretin hormones GIP and GLP-1 have been reported to stimulate β-cell proliferation and survival (1-3, 5-7), increasing the concentrations of bioactive incretin hormones through DPP-IV inhibitor treatment could offer therapeutic advantages in T1DM
patients receiving islet transplants. In the present study, MK0431 pre-treatment was shown to exert beneficial effects on glycemia in mice receiving islet transplants, as indicated by metabolic studies and microPET imaging (Figures 1 and 2). In contrast to the preserved GSIS of the Pre MK0431 Tx group (Figure 2A), glucose tolerance rapidly deteriorated in both the NCD and Post MK0431 Tx groups and they showed greatly attenuated PET signals (Figure 2D). In a subsequent study it was shown that, despite improved islet structural integrity in the diabetic MK0431 group when compared to the diabetic NCD group (Figure 3C), there were no significant differences in non-fasting blood glucose levels between the groups (Figure 3B). Additionally, no significant differences in non-fasting blood glucose levels were observed even in 2-month MK0431 pre-treated mice (data not shown). However, since MK0431 treatment resulted in the preservation of pancreatic β-cells in both mice with normal glucose tolerance and the diabetic group (Figure 3D), we cannot exclude a significant contribution from the residual islets to the overall beneficial effects of MK0431 pre-treatment on glycemia in the mice receiving islet transplants.

There is considerable evidence supporting a critical role for DPP-IV in immune regulation, including delivery of a co-stimulatory signal for T cell activation (41). Acute rejection in experimental models of cardiac allograft transplantation was shown to be associated with increased serum DPP-IV activity, and inhibition resulted in abrogated acute and accelerated rejection (42-43). Additionally, inhibition of intragraft DPP-IV significantly reduced ischemia/reperfusion-associated pulmonary injury, allowing for successful lung transplantation (44). In the present study, islet graft survival was only prolonged significantly in the Pre MK0431 group. Despite similar active GLP-1 levels in the Pre and Post MK0431 Tx groups (Figure 1C), the Post MK0431 Tx group showed only minor improvements in glucose homeostasis, with mean fasting glucose levels slightly lower than the NCD Tx group over the first 2 weeks (Figure 1H), and a small enhancement in glucose tolerance at 4th week (Figure 2A). Secondly, NCD and Post MK0431 Tx groups showed significantly attenuated PET signals from the 1st week, compared to Pre MK0431 Tx group (Figure 2D). These results strongly suggested that MK0431 modulated the immune function of DPP-IV during the 1-month pre-treatment period, thus contributing to islet graft survival in the NOD mice. The pathogenesis of T1DM involves activation of autoimmune T cells followed by their homing in on the pancreatic islets, resulting in the destruction of β-cells. It was previously reported that soluble DPP-IV (sCD26) had an enhancing effect on transendothelial T cell migration, mediated through its intrinsic DPP-IV enzyme activity (45). In the present study, migration of splenic CD4+ T cells prepared from the diabetic NCD group was significantly increased, when compared to the non-diabetic NCD group, and MK0431 treatment partially restored the levels towards normal (Figure 3E).

Active, GTP-bound, Rac1 plays an important role in the control of cell migration by regulating actin-rich lamellipodial protrusions that are critical for the generation of driving force of cell movement (46). Recently, it was shown that basal activation of Rac1 via MHC class II molecule stimulation is essential for CD4+ T cell motility and self-ligand deprivation is associated with reduced levels of active Rac1 (47). In the present study, enhanced Rac1 GTP binding activity in CD4+ T cells of diabetic NCD group was found to be decreased in the diabetic MK0431 treated group (Figure 4A). Furthermore, DPP-IV directly influenced the migration of CD4+ T cells and Rac1 GTP binding activity (Figure 5). It is therefore conceivable that increased
plasma DPP-IV activity in diabetic NCD mice, led to increased CD4+ T cell migration by regulating Rac1 GTP binding activity, whereas MK0431 attenuated auto-immune diabetes partially through decreasing CD4+ T cell migration. Strong evidence for the involvement of increased cAMP production and PKA activation in DPP-IV-mediated CD4+ T cell migration was obtained (Figure 6A, 6B, 6C and 6D), although it is unclear as to the protein activation sequence. In the current studies, DPP-IV and forskolin activation of Rac1 (Figure 6E) were all ablated by treatment with the PKA inhibitor, H89, suggesting that Rac1 is downstream of PKA. Although the effect of cAMP/PKA on cell migration has been reported to be positive and negative depending on the cell type, the spatial-temporal distribution and activation of cAMP/PKA during the regulation of cell migration are likely to be a critical component of its action (48).

Although the results reported in the present study suggest that direct inhibitor effects on DPP-IV, and not increased levels of active GIP and GLP-1, are mainly responsible for the improvements in graft retention, we have not completely ruled out a contribution from the incretins, or other factors. Additionally, the reduced incidence of diabetes in the MK0431-treated group may well involve the incretins. The long-acting GLP-1 receptor agonist, exendin-4 has been shown to synergize with anti-CD3 monoclonal antibody treatment in reversing the diabetes in NOD mice by enhancing the recovery of β-cells (49). Furthermore, continuous administration of GLP-1 to prediabetic NOD mice reduced diabetic incidence by regulating β-cell proliferation and apoptosis (50). More recently, the GLP-1 receptor was shown to be expressed in lymphoid tissue and exendin-4 treatment increased the number of CD4+ and CD8+ T cells in the lymph nodes and reduced the number of CD4+CD25+Foxp3+ regulatory T cells in the thymus, suggesting direct effects of GLP-1 on the immune system (51). However, exendin-4 treatment was not associated with significant changes in the number of CD4+ and CD8+ T cells, or B cells in the spleen (51). In the present study, neither GIP nor GLP-1 treatment produced significant effects on splenic CD4+ T cell migration in vitro (Figure 5), whereas DPP-IV was stimulatory. The reason for the diverse effects of GLP-1/exendin-4 on the different subset of T cells in vivo is not understood, but the potential for DPP-IV to modify additional subsets of lymphocytes, including those in lymph nodes and the thymus, requires further investigation. The beneficial actions of DPP-IV inhibitors and DPP-IV resistant GLP-1 receptor agonists in T2DM have mainly been attributed to increased incretin-receptor mediated responses. Although it has been recognized that DPP-IV also plays a significant role in modulating immune function, no beneficial sequelae of inhibiting such actions in diabetes have been described. The ability of the DPP-IV inhibitor MK0431 to prolong islet graft survival by reducing immunocyte migration and islet infiltration, suggests that the underlying cAMP/PKA/Rac1 could be an additional drug target.

ACKNOWLEDGEMENTS
These studies were generously supported by funding to CHSMc from Merck Frosst, Canada. We would like to thank Dr. Thomas J Ruth, Salma Jivan and TRIUMF for support and the preparation of [18F]FHBG, Siobhan McCormick for excellent technical assistance, S. Gambhir (Stanford University) for the HSV1-sr39TK construct and Dr. H-U. Demuth (Probiodrug, Germany) for the GIP, GLP-1 and DPP-IV.
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Figure 1. The effects of MK0431 in diabetic NOD mice following islet transplantation. A. Schematic diagram of group design. Islets isolated from non-diabetic NOD mice were infected with 250 m.o.i. of rAD-TK, 200 of which were transplanted under the right kidney capsule of diabetic female NOD mice. Islet transplanted diabetic NOD mice were fed: Group I- Normal chow diet throughout (NCD Tx); Group II-MK0431 diet post-transplant (Post MK0431 Tx); Group III: MK0431 diet ~1 month before and post-transplant (Pre MK0431 Tx). B-C. The effects of MK0431 on plasma DPP-IV activity (B), and plasma active GLP-1 levels (C) in diabetic NOD mice following islet transplantation. D-I. The effects of MK0431 on food consumption (D), body weight (E), water intake (F), non-fasting (G) and fasting (H) glycemic control and survival rates (I) in diabetic NOD mice following islet transplantation.

Number of animals: \( n = 5 \sim 7 \) per group: NCD Tx group (open circle), Post MK0431 Tx group (asterisk) and Pre MK0431 Tx group (filled circle). All data represent the mean ± S.E.M. and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents \( p<0.05 \) vs NCD Tx.
Figure 1

A. Schematic timeline showing Islet Tx:

B. Time course of DPP-IV activity (% baseline) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

C. Time course showing active plasma Glu1 Levels (nmol/L) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

D. Time course of Body Weight (g) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

E. Time course of Food Consumption (g/day) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

F. Time course of Water Intake (ml/day) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

G. Time course of Blood Glucose Levels (mmol/L) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.

H. Time course of Fasting Blood Glucose Levels (mmol/L) for 1st, 2nd, 3rd, and 4th time points.

I. Survival Rate (%) for NCD-Tx, Post MK0431-Tx, and Pre MK0431-Tx.
Figure 2. Time-course monitoring of glucose responses, plasma chemistry, and islet graft survival following islet transplantation. On the indicated days following transplantation, A. IPGTTs, B. Plasma insulin and C. Glucagon levels were determined in the mice described in the legend to Figure 1A. Blood glucose levels measured at 0, 15, 30, 60, 90 and 120 min following the glucose challenge (2 g/kg): NCD Tx group (open symbols), Post MK0431 Tx group (asterisk) and Pre MK0431 Tx group (filled symbols). D. Time-course monitoring of MicroPET signals detected from recipient diabetic mice following islet transplantation. Mice described in the legend to Figure 1A were injected with [18F]FHBG on the indicated days following transplantation and scanned. Regions of interests (ROIs) were placed on the kidney area of the microPET image and two peak values of % ID/g from the 2 time activity curves (TACs) for each region were used for determination of the signals. All data represent the mean ± S.E.M. and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents p<0.05 vs NCD Tx and ## represents p<0.05 vs 0 mins, Pre MK0431 Tx.
Figure 2

A.

1st week AUC

Blood Glucose Levels (mmol/l)

Time (mins)

NCD Post Pre

2nd week AUC

Blood Glucose Levels (mmol/l)

Time (mins)

NCD Post Pre

3rd week AUC

Blood Glucose Levels (mmol/l)

Time (mins)

NCD Post Pre

4th week AUC

Blood Glucose Levels (mmol/l)

Time (mins)

NCD Post Pre

B.

Plasma Insulin Levels (pU)

Time (weeks after Tx)

1st 2nd 3rd 4th

Post ShK0431 Tx

C.

Picosomal Glucose Levels (mg/l)

Time (weeks after Tx)

1st 2nd 3rd 4th

D.

[18F]-FDG (%) SUV

Time (Weeks after Tx)

NCD Post ShK0431 Tx

Pre ShK0431 Tx
Figure 3. MK0431 regulates the migration of CD4⁺ T cells. A. Schematic diagram of group design. Female NOD mice (8-10 weeks old) were placed on normal chow diet (NCD group) or MK0431 diet (MK0431 group) for 1 month. B. Non-fasting blood glucose levels. C. Representative pancreatic sections from 12- to 14-week-old littermate female NOD mice stained with hematoxylin and eosin were shown. The extent of insulitis was assessed as described in Experimental Procedures. D. Relative β-cell area. β-cell area was expressed as the percentage of sectional pancreas area as described in Experimental Procedures. E. CD4⁺ T cells were isolated from the spleen of NCD and MK0431 group. The migration of CD4⁺ T cells was determined using Transwell chamber as described in Experimental Procedures. Correlation between migration of splenic CD4⁺ T cells and plasma DPP-IV activity (F) and blood glucose levels (G). Data were analyzed using the linear regression analysis program PRISM. All data represent the mean ± S.E.M. and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents p<0.05 vs Normal NCD group, ## represents p<0.05 vs Diabetic NCD group, N.S. represents not significant.
Figure 3

A.

Weeks: 0 1st 2nd 3rd 4th
NCD: .................................................. MK0431: .............................................

B.

Blood Glucose Levels (mmol/l)

N.S.

Normal Diabetic Normal Diabetic
NCD MK0431 Diet

C.

Percentage of cells

Normal Diabetic Normal Diabetic
NCD MK0431 Diet

D.

Relative β-cell Area (%)

Normal Diabetic Normal Diabetic
NCD MK0431 Diet

E.

CD4+ T Cell Migration (fold difference vs Control)

Normal Diabetic Normal Diabetic
NCD MK0431 Diet

F.

CD4+ T Cell Migration (fold difference vs Control)

R² = 0.85
P < 0.0001

0 50 100 150
DPP-IV activity (mU/ml)

G.

CD4+ T Cell Migration (fold difference vs Control)

R² = 0.79
P < 0.0001

0 5 10 15 20 25 30
Blood Glucose Levels (mmol/l)
Figure 4. Signaling modules potentially involved in the effect of MK0431 on splenic CD4⁺ T cells. A. Rac1 GTP binding activity. Total cellular extracts were isolated from CD4⁺ T cells described in Figure 3 legend, and Rac1 GTP binding activity was determined as described in Experimental Procedures and the data are expressed as fold difference vs Normal NCD group. Western blot analyses were performed with antibodies against phosphorylated p38MAPK (Thr180/Tyr182) (B), SAPK/JNK (Thr183/Tyr185) (C), p42/44 MAP Kinase (Thr202/Tyr204) (D), PKB (Ser473) (E), PKB (Thr308) (F) and actin. Western blots are representative of n = 3, and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents p<0.05 vs Normal NCD group, ## represents p<0.05 vs Diabetic NCD group.
**Figure 4**

**A.**
GTP bound to Rac1 (fold difference vs. Control)

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image1) | ![Graph](image2) | ![Graph](image3) | ![Graph](image4) |

**B.**

p-p38 MAP Kinase (Thr180/Tyr182) →

p38 MAP Kinase →

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image5) | ![Graph](image6) | ![Graph](image7) | ![Graph](image8) |

**C.**

p-SAPK/JNK (Thr183/Tyr185) →

β-actin →

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image9) | ![Graph](image10) | ![Graph](image11) | ![Graph](image12) |

**D.**

p-p42/44 MAP Kinase (Thr202/Tyr204) →

p42/44 MAP Kinase →

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image13) | ![Graph](image14) | ![Graph](image15) | ![Graph](image16) |

**E.**

p-PKB (Ser473) →

PKB →

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image17) | ![Graph](image18) | ![Graph](image19) | ![Graph](image20) |

**F.**

p-PKB (Thr308) →

PKB →

|        | Normal | Diabetic | NCD | MK0431 Diet |
|--------|--------|----------|-----|-------------|
|        | ![Graph](image21) | ![Graph](image22) | ![Graph](image23) | ![Graph](image24) |
Figure 5. The effect of sDPP-IV and incretins on splenic CD4\(^+\) T cells. CD4\(^+\) T cells were isolated from the spleen of non-diabetic female NOD mice placed on NCD. T cells were stimulated with purified porcine DPP-IV (100 mU/ml), GIP or GLP-1 (100 nM) in the presence or absence of DPP-IV inhibitor (100 \(\mu\)M) for 1 hr. **A. Splenic CD4\(^+\) T cells migration.** The migration of splenic CD4\(^+\) T cells were determined using Transwell chamber (Corning) as described in Experimental Procedures. **B. Rac1 GTP binding activity.** Total cellular extracts were isolated and Rac1 GTP binding activity was determined. **C-G.** Total cellular extracts were isolated and Western blot analyses performed with antibodies against phosphorylated p38MAPK (Thr180/Tyr182) (C), SAPK/JNK (Thr183/Tyr185) (D), p42/44 MAP Kinase (Thr202/Tyr204) (E), PKB (Ser473) (F), PKB (Thr308) (G) and actin. Western blots are representative of \(n = 3\), and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents \(p < 0.05\) vs Control.
Figure 5

A. 

CDA + T Cell Migration (fold difference vs. Control) 

| Condition                      | GIP (100 nM) | GLP-1 (100 nM) | DPP-IV (100 mU/ml) | DPP-IV inhibitor (100 μM) |
|--------------------------------|--------------|----------------|-------------------|--------------------------|
|                                | —            | —              | —                 | —                        |

B. 

GIP bound to Start (fold difference vs. Control) 

| Condition                      | GIP (100 nM) | GLP-1 (100 nM) | DPP-IV (100 mU/ml) | DPP-IV inhibitor (100 μM) |
|--------------------------------|--------------|----------------|-------------------|--------------------------|
|                                | —            | —              | —                 | —                        |

C. 

p-95 MAP Kinase (Thr183/Tyr185) 

| Condition                      | Control | DPP-IV | Control | DPP-IV |
|--------------------------------|---------|---------|---------|---------|
|                                |         |         |         |         |

D. 

p-ERK1/2 (Thr202/Tyr204) 

| Condition                      | Control | GIP | Control | GLP-1 | GIP |
|--------------------------------|---------|-----|---------|-------|-----|
|                                |         |     |         |       |     |

E. 

p-p38 MAP Kinase (Thr180/Tyr182) 

| Condition                      | Control | DPP-IV | Control | DPP-IV |
|--------------------------------|---------|---------|---------|---------|
|                                |         |         |         |         |

F. 

p-p38 (Ser307) 

| Condition                      | Control | GIP | Control | GLP-1 | GIP |
|--------------------------------|---------|-----|---------|-------|-----|
|                                |         |     |         |       |     |

G. 

p-Pod2 (Thr308) 

| Condition                      | Control | DPP-IV | Control | DPP-IV |
|--------------------------------|---------|---------|---------|---------|
|                                |         |         |         |         |
Figure 6. The effect of sDPP-IV on cAMP accumulation and the involvement of cAMP/PKA/Rac1 pathway in DPP-IV mediated CD4$^+$ T cells migration. 

A. DPP-IV mediated cAMP accumulation. Splenic CD4$^+$ T lymphocytes were stimulated for 30 min with GIP, GLP-1 (100 nM) or purified porcine DPP-IV (100 mU/ml) in the presence of IBMX (0.5 mM) and cAMP concentrations determined in the cell extracts.

B. Effect of MK0431 on DPP-IV mediated PKA activation. Splenic CD4$^+$ T lymphocytes were treated with purified porcine DPP-IV (100 mU/ml) in the presence of indicated concentration of MK0431, and PKA activity determined.

C. Effect of MK0431 on 6-Bnz-cAMP mediated PKA activation. Splenic CD4$^+$ T lymphocytes were treated with 6-Bnz-cAMP (100 μM) in the presence of indicated concentration of MK0431, and PKA activity determined.

D. Effect of PKA inhibition on DPP-IV mediated T-cell migration. CD4$^+$ T lymphocytes were stimulated for 1 h with DPP-IV (100 mU/ml) or forskolin (10 μM) in the presence or absence of H-89 (10 μM) and cell migration determined.

E. Effect of PKA inhibition on DPP-IV mediated Rac1 activation. CD4$^+$ T lymphocytes were treated as described above and Rac1 GTP binding activity determined. All data represent the mean ± S.E.M. and significance was tested using ANOVA with a Newman-Keuls post hoc test, where ** represents p<0.05 vs Control, ## represents p<0.05 vs DPP-IV.
Figure 6

A.

CAMP Concentration (pmol/ml)

| Condition                  | CAMP Concentration |
|----------------------------|--------------------|
| GIP (100 nM)                | − − − + + − − − |
| GLP-1 (100 nM)              | − − − − − + + |
| DPP-IV (100 mU/ml)          | − + + − − − |
| DPP-IV inhibitor (100 µM)   | − − + − + + |

B.

Relative PKA activity (fold difference vs Control)

| Condition                  | PKA Activity |
|----------------------------|--------------|
| DPP-IV (100 mU/ml)         | − + + + + + |
| MK0431 (µM)                | − − 0.01 0.1 1 10 100 |

C.

Relative PKA activity (fold difference vs Control)

| Condition                  | PKA Activity |
|----------------------------|--------------|
| 6-Brn-cAMP (100 µM)        | − + + + + + |
| MK0431 (µM)                | − − 0.01 0.1 1 10 100 |

D.

CD4+ T Cell Migration (fold difference vs Control)

| Condition | CD4+ T Cell Migration |
|-----------|-----------------------|
| Control   | 1.0 ± 0.2             |
| DPP-IV    | 1.5 ± 0.3             |
| FSK       | 2.0 ± 0.4             |
| H-89      | 0.5 ± 0.1             |
| DPP-IV    | 1.0 ± 0.2             |
| FSK + H-89| 1.5 ± 0.3             |

E.

GTP bound to Rac1 (fold difference vs Control)

| Condition | GTP bound to Rac1 |
|-----------|-------------------|
| Control   | 1.0 ± 0.2         |
| DPP-IV    | 2.0 ± 0.4         |
| FSK       | 3.0 ± 0.5         |
| H-89      | 0.5 ± 0.1         |
| DPP-IV    | 1.0 ± 0.2         |
| FSK + H-89| 1.5 ± 0.3         |