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# Epigallocatechin Gallate Prevents Autoimmune Diabetes Induced by Multiple Low Doses of Streptozotocin in Mice

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Cytokines produced by immune cells infiltrating pancreatic islets have been incriminated as important mediators of β-cell destruction in insulin-dependent diabetes mellitus. In non insulin-dependent diabetes, cytokines are also associated with impaired β-cell function in high glucose condition. By the screening of various natural products blocking β-cell destruction, we have recently found that epigallocatechin gallate (EGCG) can prevent the *in vitro* destruction of RINm5F cell, an insulinoma cell line, that is induced by cytokines. In that study we suggested that EGCG could prevent cytokine-induced β-cell destruction by down-regulation of nitric oxide synthase (NOS) through inhibition of NF-κB activation. Here, to verify the *in vivo* antidiabetogenic effect of EGCG, we examined the possibility that EGCG could also prevent the experimental autoimmune diabetes induced by the treatment of multiple low doses of streptozotocin (MLD-STZ), which is recognized as an inducer of type I autoimmune diabetes. Administration of EGCG (100 mg/day/kg for 10 days) during the MLD-STZ induction of diabetes reduced the increase of blood glucose levels caused by MLD-STZ. *Ex vivo* analysis of β-islets showed that EGCG downregulates the MLD-STZ-induced expression of inducible NOS (iNOS). In addition, morphological examination showed that EGCG treatment ameliorated the decrease of islet mass induced by MLD-STZ. In combination these results suggest that EGCG could prevent the onset of MLD-STZ-induced diabetes by protecting pancreatic islets. Our results therefore revealed the possible therapeutic value of EGCG for the prevention of diabetes mellitus progression.

**Key words:** Epigallocatechin gallate, Autoimmune diabetes, Inducible nitric oxide synthase, Multiple low doses of streptozotocin

## INTRODUCTION

Epigallocatechin gallate (EGCG), a major ingredient of green tea, has healthy benefits including anticarcinogenic, antioxidant, antiangiogenic, and antiviral activity (Katiyar and Mukhtar, 1997; Nakayama *et al.*, 1993; Yang and Wang, 1993). It has been suggested that EGCG might also possess antidiabetic activity. In a recent report, injection of EGCG into lean and obese Zucker rats significantly lowered blood glucose and insulin levels, and green tea extract increased glucose metabolism in adipocytes (Broadhurst *et al.*, 2000; Kao *et al.*, 2000). Additionally, epicatechin, which is structurally similar to EGCG, is the active compound in the extract *Pterocarpus marsupium* Roxb bark, which is traditionally used in Indian folk medicine to treat diabetes (Ahmad *et al.*, 1989).

Insulin-dependent diabetes mellitus (IDDM) develops as a consequence of the selective destruction of insulin-producing β-cells due to a variety of factors including reactive oxygen species (ROS), toxins (microbial, chemical, dietary), and autoimmune responses (Rabinovitch and Suarez-Pinzon, 1998). Recent studies have provided evidence that β-cells are destroyed by autoimmune responses directed against certain β-cell constituents (autoantigens). It has been well shown that the cytotoxicity of pancreatic β-cells is mediated by cytokines secreted by the infiltrating immune cells in such conditions (Rabinovitch and Suarez-Pinzon, 1998; Tannous *et al.*, 2001). Tannous *et al.* (Tannous *et al.*, 2001) have demonstrated that cytokine-induced, pancreatic β-cell damage is primarily associated with the induction of inducible nitric oxide synthase (iNOS), leading to the generation of nitric oxide (NO) within the cell, which subsequently causes dysfunction of the

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pancreatic β-cell. Together, these results suggest that the cytokine-induced up-regulation of iNOS in pancreatic β-cells is involved in the generation of IDDM, and that therefore the blocking of iNOS-induction might be an important target of prevention or treatment of the disease. By the screening of various natural products blocking β-cell destruction, we have previously found that EGCG can prevent the in vitro destruction of RINm5F cell, an insulinoma cell line, that is induced by cytokines. In that study we suggested that EGCG could prevent cytokine-induced β-cell destruction by down-regulation of NOS through inhibition of NF-κB activation (Han, 2003).

The animal model of autoimmune diabetes has been well established by administrating multiple low doses of streptozotocin (MLD-STZ) in rodents (Mensah-Brown *et al.*, 2002; Stosic-Grujicic *et al.*, 2001). MLD-STZ-induced diabetes is a widely accepted experimental model exhibiting histo-immunological and clinical similarities to IDDM in humans (Like and Rossini, 1976). Accordingly, using the MLD-STZ-induced diabetes model in mice we demonstrated the *in vivo* antidiabetic effect of EGCG (Han, 2003).

## MATERIALS AND METHODS

### Animals

All experiments were carried out on male C57BL/KsJ mice aged 6 weeks, 18-20 g in weight, obtained from Jung-Ang animal lab (Korea). Mice were housed at 22°C with a 12 h light/dark cycle. They were kept under specific, pathogen-free conditions and received a rodent diet (Jung-Ang animal lab, Korea) and drinking water *ad libitum*.

### Autoimmune diabetes induction and EGCG treatment

STZ is widely used experimentally to induce diabetes in animals. A single, high dose (250 mg/Kg) of STZ with nicotinamide induces type II diabetes in rodents (Masiello *et al.*, 1998), as does an intraperitoneal injection of 90 mg/Kg of STZ on the second day after birth (Ryu *et al.*, 2003; Tsuji *et al.*, 1988). On the other hand, MLD-STZ induces T cell-mediated autoimmune diabetes in certain strains of mice such as C57BL/KsJ (Like and Rossini, 1976; Lukic *et al.*, 1998).

For the induction of diabetes with MLD-STZ (Sigma, USA) was dissolved in 0.01 M sodium citrate buffer solution and given intra-peritoneally for 5 consecutive days (40 mg/kg body weight). EGCG (100 mg/kg/day) was administered with STZ for 5 days and then EGCG alone was administered for a further 5 days. Blood glucose level was determined using an instant glucose detector (GLUCOTREND®, Boehringer Mannheim, Germany). The mice were considered to have developed diabetes when their blood glucose levels were elevated to higher than 12 mmol/L (control C57BL/KsJ mice had a normal blood glucose level of less than 6 mmol/L).

### Preparation of islets

Islets were isolated from male mice according to the collagenase method (An *et al.*, 2001). Briefly, the pancreas of ether-anesthetized mouse was distended by infusion of Hanks balanced salt solution (HBSS) (2 mM $CaCl_2$, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 20 mM Hepes [pH 7.3]) containing 1.5 mg/ml Type V collagenase (Sigma) via the common bile duct. After removing the destroyed acinar cells by washing with HBSS, intact islets were hand-picked with a Pasteur micropipette under a dissecting microscope.

### Quantitation of iNOS expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from islets by using TRIzol reagent following the manufacturers instructions (Invitrogen, CA, USA). One microgram of total RNA was transcribed into cDNA in a 20 μL final volume of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM each dNTP and 2.4 μM oligo-d(T) 16-primer, 1 unit RNase inhibitor, and 2.5 units M-MLV RNase H-reverse transcriptase) by incubation for 10 minutes at 21°C and 15 minutes at 42 °C. The reaction was stopped by incubation at 99°C for 5 minutes. For mouse iNOS RT-PCR, PCR aliquots of the synthesized cDNA were added to a 45 μL PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2 units Taq DNA polymerase, and 0.4 μM of each PCR primer. iNOS primers for a 111-bp fragment were designed for the forward primer, 5'-CAAGAGTTTGACCAGAGGACC-3', and the reverse primer, 5'-TGGAACCACTCGTACTTGGGA-3'. Amplification was initiated with 3 minutes of denaturation at 94°C, followed by 26 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. After the last cycle of amplification, the samples were further incubated for 5 minutes at 72°C. β-actin PCR was performed with 2.5 μL aliquots of synthesized cDNA using primers at a concentration of 0.15 μM. β-Actin primers for a 420-bp fragment were designed for the forward primer, 5'-ATGTACGTAGCCATCCAGGC-3', and the reverse primer, 5-AGGAAGGAAGGCTGGAAGAG-3'. Amplification was initiated with 3 minutes of denaturation at 94°C, followed by 24 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The obtained PCR products were analyzed on ethidium bromide-stained agarose (1.5%) gels.

### Morphological changes of islets

Mice were anesthetized with ether and the abdominal region was incised. Pancreases were then removed from

mice and fixed overnight in a 4% paraformaldehyde solution in PBS at 4°C. Fixed tissues were processed routinely for paraffin embedding, and 5-6 μm sections were used for hematoxylineosin staining as described previously (Kolb-Bachofen *et al.*, 1988).

### Assessment of cell viability

Cell viability of the isolated islets was assessed by the method described previously (Kumar *et al.*, 1994) with some modifications. Briefly, isolated islets in batches of 50 were incubated with EGCG at various concentrations of 0, 20, 50, 100 and 200 μg/mL in the presence of 50 U/mL IL-1β and 500 U/mL IFN-γ for 24 h, after which the medium was changed. Thereafter, 1 μg/mL 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 h. The viable cell number is directly proportional to the production of formazan which, following solubilization with DMSO, was measured spectrophotometrically at 570 nm.

## RESULTS AND DISCUSSION

Cytokines such as IL-1β, interferon γ and TNF α, destroy pancreatic β-cells and are associated with the pathogenesis of type 1 diabetes mellitus (Rabinovitch and Suarez-Pinzon, 1998). We have previously demonstrated that EGCG could inhibit this cytokine-induced RINm5F cell destruction (Han, 2003). In this study we further tested whether EGCG could also prevent the destruction of isolated β-islets induced by IL-1β and interferon γ. We used MTT assay to analyze the cell viabilities of isolated islet cells treated with IL-1β and IFN-γ. As shown in Table I, these cytokines were able to cause isolated islet cell death, with a cell-death rate of approximately 45% following treatment with IL-1β (50 U/mL) in the presence of IFN-γ (500 U/mL). However, treatment of islet cells with EGCG inhibited IL-1β and IFN-γ induced cell death in a dose-dependent manner. At a dose of 200 μg/mL EGCG, the islet cell death caused by IL-1β and IFN-γ was nearly completely blocked. These data showed that EGCG could protect the cytokine-induced cell death of isolated islet cells as well as of RINm5F cells. From these findings, we hypothesized that EGCG might have an antidiabetogenic effect in type 1 diabetes, which is known to be induced by cytokine mediated β-cell damage. Accordingly, in order to evaluate the ability of EGCG to interfere with type 1 autoimmune diabetogenic process, we used the model of autoimmune diabetes induced in mice by administration of five low doses of STZ and examined the *in vivo* effects of EGCG on the disease development. MLD-STZ-treated mice received EGCG intraperitoneally (100 mg/kg/day) for 10 days, starting from the first treatment of STZ. The effects of EGCG administration on the disease manifestations defined by hyperglycemia are shown in Fig. 1. The control MLD-SZ-treated group of mice developed hyperglycemia (16 mM serum glucose at 10 day after induction), whereas the treatment groups with EGCG had significantly reduced MLD-STZ-induced hyperglycemia (Fig. 1).

We have previously claimed that EGCG could inhibit cytokine-induced RINm5F cell damage by down regulation of iNOS expression through inhibition of NF-κB activation (Han, 2003). To present an *in vivo* relevance for the action mechanism of EGCG, we tested whether EGCG inhibits the expression of iNOS or not in an experimental autoimmune diabetic model. RNA was isolated from islet cells after 10 days of disease induction and RT-PCR with iNOS

**Table 1.** Protective effect of EGCG on IL-1β/IFN-γ-induced cytotoxicity in isolated β-islets

| Treatment | Cell viability (% of control) |
|---|---|
| Control | 100 |
| IL-1β (50 U/mL) + IFNγ (500 U/mL) | 55 ± 7 |
| IL-1β (50 U/mL) + IFNγ (500 U/mL) + 20 μg/mL EGCG | 55 ± 8 |
| IL-1β (50 U/mL) + IFNγ (500 U/mL) + 50 μg/mL EGCG | 68 ± 7 |
| IL-1β (50 U/mL) + IFNγ (500 U/mL) + 100 μg/mL EGCG | 78 ± 7 |
| IL-1β (50 U/mL) + IFNγ (500 U/mL) + 200 μg/mL EGCG | 89 ± 6 |

Isolated β-cells were cultured with IL-1β and IFN-γ in the presence or absence of EGCG for 24 h. The cell viability percentage after these treatments was determined by MTT colorimetric assay and calculated as a ratio of $A_{570}$ of treated- and control cells. Each value is the mean ± SEM of four independent experiments.

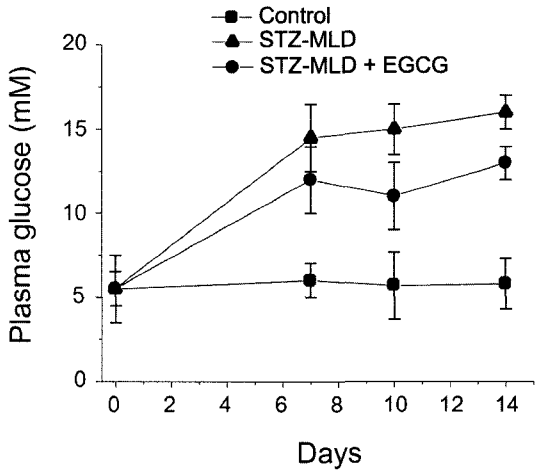


**Fig. 1.** The effect of EGCG on plasma glucose levels in MLD-STZ treated mice. C57BL mice were treated intraperitoneally with STZ at a dose of 40 mg/kg per day (low-dose) for five consecutive days. EGCG was given intraperitoneally at a dose of 100 mg/kg per day for 10 days from STZ treatment. At 0, 7, 10 and 14 days from STZ treatment, plasma was obtained. The assay of plasma glucose concentration was performed as described in Materials and Methods. Each value is the mean ± SEM of three independent experiments.

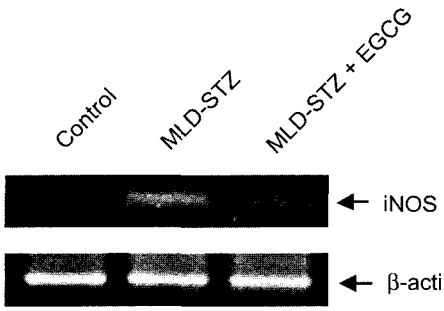


**Fig. 2.** The effect of EGCG on iNOS mRNA levels in islets of MLD-STZ treated mice. C57BL mice were treated intraperitoneally with STZ at a dose of 40 mg/kg per day (low-dose) for five consecutive days. EGCG was given intraperitoneally at a dose of 100 mg/kg per day for 10 days from STZ treatment. At 10 days from STZ treatment, islets were isolated as described in Materials and Methods. INOS mRNA expression was quantified by RT-PCR as described in Materials and Methods. The data are representatives of three experiments.

and β-actin specific primer was performed. The treatment with MLD-STZ strongly induced the expression of iNOS mRNA, but this expression was markedly suppressed by the treatment with EGCG (Fig. 2). These results indicated that EGCG could also down-regulate the *in vivo* iNOS expression induced by MLD-STZ and prevent the damage of islets induced by MLD-STZ. In addition, histological analysis of the MLD-STZ treated mice showed markedly decreased islet mass (Fig. 3). In contrast, islets in the EGCG treated group were partially intact. Hyperglycemia elicited with MLD-STZ might be induced by islet cell damage due to NO toxicity, as evidenced by the fact that EGCG can down-regulate iNOS expression and protect islet cell damage in MLD-STZ treated mice. Taken together, these data indicated that EGCG prevented MLD-STZ-induced diabetes by protecting pancreatic islets through inhibition of iNOS expression.

Here we showed that EGCG could prevent the cytokine-induced destruction of β-cells and furthermore prevent

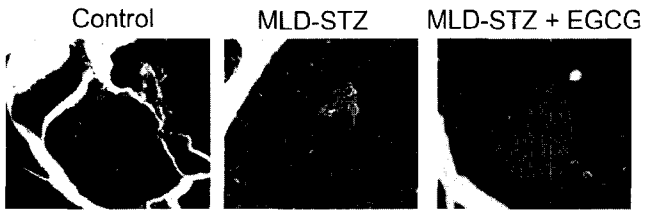


**Fig. 3.** Hematoxylin-Eosin staining of pancreatic islets showing recovery of islet mass by EGCG treatment in MLD-STZ treated mice. C57BL mice were treated intraperitoneally with STZ at a dose of 40 mg/kg per day (low-dose) for five consecutive days. EGCG was given intraperitoneally at a dose of 100 mg/kg per day for 10 days from STZ treatment. At 10 days from STZ treatment, pancreases were removed. Hematoxylin-eosin staining of the pancreases was performed as described in Materials and Methods. The data are representatives of three experiments.

MLD-STZ-induced diabetes. EGCG suppressed expression of iNOS induced by MLD-STZ. It has been known that MLD-STZ-induceddiabetes is caused by local and systemic proinflammatory cytokines and mimics type 1 autoimmune diabetes (Stosic-Grujicic *et al.*, 2001). The protective effect of EGCG on MLD-STZ-induced diabetes might be due to the blockage of the cytokine-oxygen radical-NFκB-iNOS pathway by EGCG. We also cannot exclude the possibility that inhibition of iNOS expression by EGCG might be mediated by the inhibition of cytokine production. Irrespectively, the protective effect of EGCG on β-cell destruction indicates that EGCG can be used to prevent the onset of type 1 diabetes.

In type 2 diabetes, chronic hyperglycemia is suggested to be detrimental to pancreatic β-cells, causing impaired insulin secretion. A recent report suggested that the pathogenesis of glucotoxicity in type 2 diabetes could be associated with an inflammatory process and with the IL-1β/NF-κB pathway (Maedler *et al.*, 2002). From our results, EGCG might also be helpful to inhibit the progression of type 2 diabetes occurring through the β-cell failure induced by high glucose levels.

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