Inhibition of Human Insulin Gene Transcription by the Immunosuppressive Drugs Cyclosporin A and Tacrolimus in Primary, Mature Islets of Transgenic Mice

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ABSTRACT

Cyclosporin A and tacrolimus are clinically important immunosuppressive drugs. They share a diabetogenic action as one of their most serious adverse effects. The underlying mechanism is unknown. Previous studies have shown that tacrolimus can inhibit insulin gene transcription at high concentrations in tumor cell lines. To study insulin gene transcription in normal, mature pancreatic islet cells, we used a novel approach in the present study. Transgenic mice that carry a human insulin promoter-reporter gene were generated. The human insulin promoter directed transcription in pancreatic islets and conferred a normal, physiological glucose response to reporter gene expression in isolated islets. After stimulation with glucose, human insulin promoter-mediated gene expression was inhibited in normal, mature islet cells by both tacrolimus and cyclosporin A to a large extent (approximately 70%) and with high potency at concentrations that are known to inhibit calcineurin phosphatase activity (IC_{50} values of 1 and 35 nM, respectively). Furthermore, glucose stimulated calcineurin phosphatase activity in mouse pancreatic islets, further supporting the view that calcineurin phosphatase activity is an essential part of glucose signaling to the human insulin gene. The high potency of cyclosporin A and tacrolimus in normal islets suggests that inhibition of insulin gene transcription by cyclosporin A and tacrolimus is clinically important and is one mechanism of the diabetogenic effect of these immunosuppressive drugs.
inhibit insulin secretion immediately (Herold et al., 1993; Teuscher et al., 1994; Redmon et al., 1996), the primary effect of these drugs may be the inhibition of insulin gene transcription. Four reports have studied the effect of these immunosuppressants on insulin gene transcription. Whereas cyclosporin A and tacrolimus did not inhibit cAMP-induced rat insulin I gene transcription in the β-cell line HIT (Siemann et al., 1999), tacrolimus (1 to 10 μM) decreased rat insulin I gene transcription after stimulation by membrane depolarization, glucose, or cAMP in the β-cell line INS-1 (Lawrence et al., 2001, 2002). The transcriptional activity of the human insulin gene was found to be inhibited by tacrolimus (100 nM) in HIT cells incubated in a low (0.4 mM) or high (20 mM) glucose concentration (Redmon et al., 1996). However, in the reports that found an inhibition of insulin gene transcription, very high concentrations of tacrolimus (0.1 to 10 μM) were required to produce the effect, raising doubts about both the involvement of calcineurin and the clinical importance of the effect.

So far, only islet β-cell lines have been used to study the effect of cyclosporin A and tacrolimus on insulin gene transcription. However, β-cell lines differ in their properties from normal pancreatic islet β cells (Nielsen et al., 1985). Therefore, to study the effect of cyclosporin A and tacrolimus on insulin gene transcription in normal, mature β-cells, we used a novel approach in the present study. Transgenic mice were generated that carry a human insulin gene promoter-luciferase reporter gene. The insulin promoter conferred normal glucose responsiveness to reporter gene expression in isolated islets. Cyclosporin A and tacrolimus were found to inhibit glucose-induced human insulin gene transcription almost completely and with IC₅₀ values of 35 and 1 nM, respectively, which are similar to the known IC₅₀ values for the inhibition of calcineurin. These data demonstrate for the first time an inhibition by both cyclosporin A and tacrolimus of insulin gene transcription in normal mature β cells and at low concentrations, strongly supporting the view that the inhibition of insulin gene transcription is one of the mechanisms underlying the diabetogenic action of these immunosuppressive drugs.

Materials and Methods

Plasmid Construction. For the plasmid −339hInsLuc, the HindIII fragment of pNCAT1-Wt (Inagaki et al., 1992), kindly provided by Dr. H. Imura (Kyoto, Japan), was subcloned in the forward orientation into the HindIII site of pXP2 (Nordeen, 1988). Subcloning and plasmid isolation were performed by standard methods. The identity and the orientation of the construct was confirmed by sequencing, using the enzymatic method.

Generation and Analysis of Transgenic Mice. Transgenic mice were generated according to standard procedures. The 3.5-kilobase BamHI/RcaI fragment of −339hInsLuc, including the human insulin gene promoter from −339 to −112 and the firefly luciferase-coding gene, was gel-purified and microinjected into the male pronuclei of fertilized eggs (NMRI). Microinjected eggs were transferred to the oviducts of foster mothers (CD1). Genomic (tail) DNA from the offspring was digested with BglII, electrophoresed, and subjected to Southern blot analysis using a 1.6-kilobase XhoI luciferase fragment as the probe labeled with use of the Megaprime DNA labeling system (Amersham Biosciences Inc., Piscataway, NJ) and [α-32P]dATP. Genomic (tail) DNA from the offspring was analyzed by polymerase chain reaction with primers amplifying a 618-base pair fragment within the luciferase gene. Reporter gene expression was determined by measuring the reporter enzyme activity in tissue extracts as described previously (Beimesche et al., 1999). Protein was determined using a commercial kit (Bio-Rad Laboratories, Munich, Germany). All animal studies were conducted according to the National Institutes of Health’s Guidelines for Care and Use of Experimental Animals and were approved by the Committee on Animal Care and Use of the local institution and state.

Isolation and Culture of Islets. Pancreatic islets were isolated as described previously (Lacy and Kostianovsky, 1967). Briefly, the pancreata of mice were mechanically dispersed in Krebs-Ringer buffer supplemented with 5 mM glucose and digested for 3 to 5 min at 37°C with collagenase P (Roche Diagnostics, Mannheim, Germany). Tissue was shaken, and the islets were hand-picked. Isolated islets were preincubated in a humidified atmosphere of 95% air/5% CO₂ for 12 h in RPMI 1640 medium containing 5 mM glucose and supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 μg/ml. Cyclosporin A and tacrolimus (concentrations as indicated) were added 7 h before harvest, and forskolin (10 μM) or glucose (concentrations as indicated) was added 6 h before harvest. The islets were collected, washed once with phosphate-buffered saline buffer, and resuspended in potassium phosphate buffer, pH 7.8, followed by three freeze-thaw cycles. Luciferase activity (Schwaninger et al., 1993) and protein content (Protein Assay; Bio-Rad Laboratories) were determined in the supernatant.

In Vivo Study. Reporter mice were kept at 22°C on a 12-h/12-h light/dark cycle with free access to water and standard laboratory diet. Transgenic mice received once daily at 6 PM for 7 days an intraperitoneal injection of the vehicle or 10 mg/kg of body weight of cyclosporin A dissolved in sunflower seed oil. Mice were killed on the eighth day between 6 AM and 7 AM, and the pancreata were collected. Reporter gene expression was determined by measuring reporter enzyme activity in tissue extracts as described previously (Beimesche et al., 1999). Reporter gene expression in a given cyclosporin A-treated animal is expressed as the percentage of reporter gene expression in a transgenic mouse from the same litter that was treated with vehicle (control).

[3H]Uridine Incorporation. Approximately 35 isolated mouse islets per group were incubated in RPMI 1640 medium containing 5 mM glucose and supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 μg/ml. After 1 h of preincubation, culture medium was replaced by fresh medium. Cyclosporin A (200 nM), tacrolimus (20 nM), or actinomycin D (5 mg/ml) was added 7 h before harvest. Glucose (15 mM; final concentration of 20 mM) or 20 μCi of [5,6-3H]uridine (specific activity, 41.0 Ci/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) were added 6 h before harvest. [3H]Uridine incorporation was then measured as described previously (Eun et al., 1987). Briefly, islets were collected and washed twice in phosphate-buffered saline and resuspended in 45 μl of ice-cold Tris buffer (10 mM), pH 7.4, containing 1 mM EDTA. Islets were lysed by three freeze-thaw cycles. After the addition of 10 μl of Nonidet P-40 (5%), the islets were incubated for 15 min on ice with intermittent agitation. Islets were centrifuged (15,000g for 3 min), and 50 μl of the supernatant was transferred into a new sterile tube containing 30 μl of 20× buffer (3M NaCl/0.3 M citrate, pH 7.4) and 20 μl of 37% (w/w) formaldehyde, followed by an incubation at 67°C for 15 min. Aliquots (35 μl) were spotted on GF/C filters (Whatman, Maidstone, UK), precipitated with 10% trichloroacetic acid, washed twice with 5% trichloroacetic acid and twice with 80% ethanol, and counted in 5 ml of organic counting scintillant (QuickSafe A safety scintillator; Zinsser Analytic, Frankfurt, Germany) on an LS 5000 TD counter (Beckman Coulter, Inc., Fullerton, CA).

Calcineurin Phosphatase Assay. Isolated mouse islets (approximately 45 per group) were preincubated in RPMI 1640 medium containing 5 mM glucose and supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 μg/ml for 4 h. Cyclosporin A (5 μM) or tacrolimus (100 nM) was added 90 min before harvest, and glucose (15 mM; final concentration of 20 mM)
was added 30 min before harvest. Cells were washed twice in Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) and resuspended in lysis buffer (green cellular calcineurin assay kit plus, BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), followed by three freeze-thaw cycles and centrifugation (20,000 g for 45 min at 4°C). The supernatant was then placed on a mini Quick Spin Oligo Column (Roche Diagnostics) to remove free phosphates. Calcineurin phosphatase activity was then measured nonradioactively with RII phosphopeptide substrate and Malachite green using a commercial kit and following the manufacturer’s instructions (Biomol green cellular calcineurin assay kit plus), except that the lysis buffer and assay buffer were supplemented with 5 mM ascorbic acid (Mitsushashi et al., 2000).

Materials. Luciferin, Tween 80, sunflower seed oil, and forskolin were purchased from Sigma Chemical Co. (Taufkirchen, Germany). Tacrolimus was a gift from Fujisawa Pharmaceutical (Osaka, Japan), and cyclosporin A was a gift from Novartis (Basel, Switzerland). Forskolin was dissolved in dimethyl sulfoxide, and tacrolimus was dissolved in ethanol. For in vitro studies, a stock solution of cyclosporin A was prepared in ethanol with 20% Tween 80 and further diluted in RPMI 1640 medium. Controls received the solvent only.

Results

Transgenic Reporter Mice. For the generation of transgenic reporter mice, the promoter of the human insulin gene from −339 to +112 was fused to the firefly luciferase gene. The human insulin promoter-luciferase reporter gene (Fig. 1) was microinjected as a linear fragment into the pronuclei of 1-cell embryos. Three independently derived transgenic mouse lines expressed the luciferase reporter gene in the pancreas (data not shown). As shown in Fig. 2, luciferase activity per mg of protein in the islets of two different mice was 540- and 330-fold higher than in the total pancreas, indicating that the human insulin gene promoter directs transcription specifically in pancreatic islets. The treatment of isolated islets with the adenylate cyclase activator forskolin (10 μM), glucose (20 mM), or forskolin plus glucose enhanced reporter gene expression 5.3-, 11.4-, and 31-fold, respectively (Fig. 3). Cyclic AMP is a major intracellular second messenger of insulin gene transcription (Fehmann and Habsener, 1992; Docherty and Clark, 1994; Ohneda et al., 2000), whereas glucose is a major extracellular regulator of insulin gene transcription (Docherty and Clark, 1994; Ohneda et al., 2000). A synergistic stimulation of rat insulin I promoter-reporter gene transcription by cAMP and glucose has been reported previously in transfected fetal rat islet cell cultures (German et al., 1990). Increasing concentrations of glucose stimulated reporter gene expression in a concentration-dependent manner (Fig. 4). Human insulin promoter-reporter gene expression was stimulated by glucose at concentrations of 5 to 20 mM (Fig. 4), indicating a normal, physiological responsiveness. The addition of 30 mM glucose did not further enhance human insulin promoter-reporter gene expression. When taken together, all these results suggest that these transgenic mice are useful in the study of the regulation of human insulin promoter-reporter gene expression in primary, mature pancreatic islets.

Effect of Cyclosporin A and Tacrolimus on Human Insulin Promoter-Reporter Gene Expression in Primary Mature Islets. To study whether the immunosuppres-
sive drugs cyclosporin A and tacrolimus inhibit human insulin gene transcription, isolated pancreatic islets from human insulin promoter-reporter transgenic mice were treated with these drugs. As shown in Fig. 5, cyclosporin A (5 \mu M) and tacrolimus (100 nM) inhibited forskolin-induced reporter gene expression by approximately 70%. Cyclosporin A (5 \mu M) as well as tacrolimus (100 nM) also inhibited glucose-induced reporter gene expression by 60 to 80% (Fig. 5). Tacrolimus and cyclosporin A inhibited glucose-stimulated human insulin gene transcription in a concentration-dependent manner, with IC_{50} values of approximately 1 and 35 nM, respectively (Fig. 6). These values are consistent with the reported IC_{50} values for the inhibition of calcineurin phosphatase activity in pancreatic islet cells (Schwaninger et al., 1993, 1995; Ho et al., 1996).

High concentrations of cyclosporin A are known to inhibit RNA synthesis nonspecifically in rat islet cells (Eun et al., 1987). To investigate whether the inhibitory effect of both immunosuppressive drugs on glucose-stimulated human insulin gene transcription may be determined by a general toxic effect on RNA synthesis, isolated mouse pancreatic islets were incubated with [\textsuperscript{3}H]uridine to label RNA. Whereas an inhibitor of transcription, actinomycin D (5 mg/ml), decreased [\textsuperscript{3}H]uridine incorporation to 1.9 \pm 0.4\% of that of controls, cyclosporin A and tacrolimus at concentrations that produce a maximum inhibition of human insulin promoter activity (200 and 20 nM, respectively) had no effect on general RNA synthesis ([\textsuperscript{3}H]uridine incorporation of 102 \pm 2 and 113 \pm 7\% of controls, respectively; \(n = 4\)).

Rapamycin is an analog of tacrolimus. Rapamycin binds to the cytosolic receptor of tacrolimus with similar affinity, but in contrast to the tacrolimus/immunophilin complex, the rapamycin/immunophilin complex does not inhibit calcineurin (Sigal and Dumont, 1992). Using a concentration (100 nM) at which tacrolimus produces a maximum effect (Fig. 6), rapamycin had no effect on human insulin promoter-directed gene expression after stimulation by 20 mM glucose (luciferase activity of 135 \pm 16\% of controls; \(n = 4\)). This is in contrast to results at the rat insulin I gene promoter (Leibiger et al., 1998; Lawrence et al., 2001). Taking together

**Fig. 4.** Stimulation of reporter gene expression in isolated islets of transgenic mice by increasing concentrations of glucose. Final glucose concentrations are shown. Luciferase activity is expressed relative to the value in each experiment of the activity in the control induced by glucose (30 mM). The values are presented as means \(\pm\) S.E.M. from 10 independent experiments.

**Fig. 5.** Cyclosporin A and tacrolimus inhibit forskolin- and glucose-induced luciferase gene expression in isolated islets of transgenic mice. Isolated islets were incubated with 10 \mu M forskolin or glucose (20 mM) and cyclosporin A (5 \mu M) or tacrolimus (100 nM), as indicated. Luciferase activity is expressed relative to the respective stimulated luciferase activity in each experiment. The values are presented as means \(\pm\) S.E.M. from five independent experiments.

**Fig. 6.** Tacrolimus and cyclosporin A inhibit glucose-induced expression of the insulin promoter-reporter gene in normal, mature pancreatic islets in a concentration-dependent manner. Isolated islets of the transgenic reporter mice were incubated with 20 mM glucose and increasing amounts of either tacrolimus or cyclosporin A. Luciferase activity is expressed relative to the respective glucose-stimulated luciferase activity in each experiment. Values are presented as means \(\pm\) S.E.M. from six independent experiments.
1) the low IC₅₀ values of cyclosporin A and tacrolimus for inhibition of human insulin promoter activity (35 and 1 nM, respectively; Fig. 6), 2) the lack of effect of cyclosporin A (200 nM) and tacrolimus (20 nM) on RNA synthesis as indicated by [³H]uridine incorporation, and 3) the lack of effect of a compound, rapamycin, that is chemically related to tacrolimus and binds to the same immunophilin, these findings strongly suggest that cyclosporin A and tacrolimus inhibit in a specific manner glucose-stimulated human insulin gene transcription in primary pancreatic islets.

Effect of Cyclosporin A on Human Insulin Promoter-Reporter Gene Expression In Vivo. If the observed actions of cyclosporin A and tacrolimus on human insulin promoter activity in isolated pancreatic islets are meaningful for the in vivo situation, these immunosuppressants should produce similar effects in mice. As a first attempt to investigate this question, human insulin promoter-reporter mice were treated with cyclosporin A. A dose was chosen (10 mg/kg body weight) that is within the range used clinically and has been shown to result in rodents having drug blood levels which are within the therapeutic range (Fehmann et al., 1987). After treatment of reporter mice for 7 days, reporter gene expression in pancreatic islets was decreased by approximately 60% (Fig. 7), which is consistent with the view that cyclosporin A inhibits human insulin promoter activity in pancreatic islets in vivo.

Effect of Glucose on Calcineurin Phosphatase Activity in Pancreatic Islets. Because the stimulation by glucose of human insulin gene transcription in isolated islets was inhibited by the calcineurin phosphatase blockers cyclosporin A and tacrolimus (see above), the effect of glucose on calcineurin phosphatase activity in primary mouse pancreatic islets was investigated. When isolated mouse pancreatic islets were incubated at a low glucose concentration (5 mM), calcineurin phosphatase activity was between 400 and 700 pmol of phosphate/min/mg. A high glucose concentration (20 mM) stimulated calcineurin phosphatase activity approximately 5-fold (Fig. 8). This elevated activity was blocked by cyclosporin A and tacrolimus (Fig. 8).

Discussion

With the exception of some studies on transfected fetal rat islet cells (German et al., 1990) or single rat islet cells (Leibiger et al., 1998), experiments investigating the regulation of insulin gene transcription have been performed in tumor islet β-cell lines (Ohneda et al., 2000). This holds true also for studies on the effect of cyclosporin A and tacrolimus on insulin gene transcription (Redmon et al., 1996; Siemann et al., 1999; Lawrence et al., 2001, 2002). Tumor β-cell lines share many characteristics with normal β-cells and are extremely helpful in the investigation of β-cell biology. However, their properties also differ from normal β-cells. For example, islet β-cell lines do not permit a normal response of the insulin gene to glucose, a major physiological regulator of insulin gene transcription (Nielsen et al., 1985; Marie et al., 1993). Therefore, to study insulin gene transcription in normal, mature pancreatic islet β-cells, a novel approach was used in the present study. We used mice that were transgenic for a human insulin promoter-regulated reporter construct.

The use of reporter mice has been successful for studying the regulation of the transcriptional activity of fragments from other promoters in various tissues both in vivo and in vitro. For example, an NFAT-binding motif directing the expression of a reporter gene in transgenic mice allowed for the identification of NFAT activation in T-cell populations (Verweij et al., 1990). A minimal promoter containing binding sites for nuclear factor-κB-related transcription factors indicated the activation by distinct nuclear factor-κB/Rel proteins in different cell types (Lernbecher et al., 1993). More recently, cAMP response element-reporter mice were used to demonstrate the induction of cAMP response element-
ated gene expression during long-term potentiation in hippocampal slices in vitro (Impey et al., 1996). This reporter mouse approach was used in the present study to investigate insulin gene transcription in normal β-cells. Transgenic mice were generated that carry as a transgene the luciferase reporter gene fused to the human insulin promoter from −339 to +112. Approximately 300 base pairs of the 5'-flanking region of the human insulin gene are known to be sufficient for transgene expression specifically in the β-cells of pancreatic islets in mice (Fromont-Racine et al., 1990). Accordingly, the human insulin promoter directed the transcription of the luciferase reporter gene in pancreatic islets. It was found to confer a normal, physiological glucose response to reporter gene expression in isolated pancreatic islets. Thus, although the human insulin promoter is studied in a murine cell context that might differ from the one provided by human β-cells, these transgenic mice seem to be a valuable model to study human insulin gene transcription in normal mature pancreatic islets.

Isolated pancreatic islets from these mice were used to investigate the effect of the immunosuppressants cyclosporin A and tacrolimus on insulin gene transcription in normal, mature islet cells. Cyclosporin A and tacrolimus were found to inhibit human insulin promoter-directed gene expression after stimulation by both forskolin and glucose. The responses to forskolin and glucose follow, at least partially, different regulatory pathways that may include, among others, the transcription factors CREB and PDX1/NFAT, respectively (Fehmann and Habener, 1992; Docherty and Clark, 1994; Ohneda et al., 2000; Lawrence et al., 2001, 2002). The fact that cyclosporin A and tacrolimus inhibit both pathways suggests that both involve a cyclosporin A- and tacrolimus-sensitive step. Consistent with this view, CREB- and NFAT-mediated transcription have been shown to be cyclosporin A- and/or tacrolimus-sensitive in β-cell lines (Schwaninger et al., 1993, 1995; Lawrence et al., 2001, 2002). Tacrolimus has been shown before to inhibit insulin gene transcription at high concentrations in tumor β-cell lines cultured in a high glucose concentration (Redmon et al., 1996; Lawrence et al., 2001). The present study now demonstrates that both cyclosporin A and tacrolimus specifically inhibit glucose-induced human insulin promoter-regulated gene expression in normal, mature islet cells and at low concentrations. Our data indicate that the potency of tacrolimus that is needed to inhibit glucose-induced human insulin gene transcription in normal mature islet cells is approximately 100- and 1000-fold higher than its potency to inhibit glucose-induced transcription of the human insulin gene in the tumor cell line HIT (Redmon et al., 1996) and the rat insulin I gene in the tumor cell line INS-1 (Lawrence et al., 2001), respectively. This high potency in normal islets suggests that the inhibition of insulin gene transcription by the immunosuppressants may involve calcineurin and may be clinically important.

It has been shown previously that in pancreatic islet cells, the IC_{50} values of inhibition of calcineurin activity are approximately 1 and 30 nM for tacrolimus and cyclosporin A, respectively (Schwaninger et al., 1993, 1995). This is consistent with the IC_{50} values of these drugs for the inhibition of calcineurin activity in other tissues (Clippstone and Crabtree, 1992; O’Keeffe et al., 1992). Because cyclosporin A and tacrolimus are chemically distinct and bind to distinct intracellular receptors (immunophilins), they do not share activities other than the inhibition of calcineurin phosphatase activity (Clippstone and Crabtree, 1992; O’Keeffe et al., 1992; Ho et al., 1996). Thus, the combined use of these drugs is a powerful tool to evaluate the role of calcineurin. The present study demonstrates for the first time an inhibition of insulin gene transcription by both drugs with IC_{50} values similar to those for the inhibition of calcineurin in normal islets. These data strongly suggest that cyclosporin A and tacrolimus inhibit human insulin gene transcription through the inhibition of calcineurin. Glucose has the ability to depolarize β-cells and to increase the intracellular free calcium concentration (German et al., 1990). This is likely to activate calcineurin, which is a calcium/calmodulin-dependent protein phosphatase (Clippstone and Crabtree, 1992; O’Keeffe et al., 1992; Ho et al., 1996). Indeed, increasing the concentrations of glucose in the incubation medium from 5 to 20 mM was found in the present study to enhance calcineurin phosphatase activity in primary mouse pancreatic islets approximately 5-fold. Together with the potent inhibition by cyclosporin A and tacrolimus, the stimulation by glucose of calcineurin phosphatase activity strongly supports the view that calcineurin phosphatase activity is an essential part of glucose signaling to the human insulin gene in normal mature islets.

The clinical use of the immunosuppressive drugs cyclosporin A and tacrolimus is associated with impaired glucose tolerance as a major side effect (see above). The underlying mechanism is unclear. The present study shows that cyclosporin A and tacrolimus inhibit human insulin gene transcription in normal islet cells at concentrations that are known to inhibit calcineurin phosphatase activity. Because the immunosuppressive effects of cyclosporin A and tacrolimus are believed to result from the inhibition of calcineurin (Ho et al., 1996), our findings suggest that the concentrations of tacrolimus and cyclosporin A which cause immunosuppression are indistinguishable from those which cause the inhibition of human insulin gene transcription in normal mature β cells. Our data thereby suggest that the inhibition of insulin gene transcription is clinically important and is a mechanism of the diabetogenic effects of cyclosporin A and tacrolimus. Consistent with this view, a dose of cyclosporin A that falls within the therapeutic range inhibited human insulin promoter-reporter gene expression in vivo.

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References


