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Activation of the Dual-Leucine-Zipper-Bearing Kinase and Induction of β-Cell Apoptosis by the Immunosuppressive Drug Cyclosporin A[S]

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ABSTRACT

Post-transplant diabetes is an untoward effect often observed under immunosuppressive therapy with cyclosporin A. Besides the development of peripheral insulin resistance and a decrease in insulin gene transcription, a β-cell toxic effect has been described. However, its molecular mechanism remains unknown. In the present study, the effect of cyclosporin A and the dual leucine-zipper-bearing kinase (DLK) on β-cell survival was investigated. Cyclosporin A decreased the viability of the insulin-producing pancreatic islet cell line HIT in a time- and concentration-dependent manner. Upon exposure to the immunosuppressant fragmentation of DNA, the activation of the effector caspase-3 and a decrease of full-length caspase-3 and Bcl_{XL} were observed in HIT cells and in primary mature murine islets, respectively. Cyclosporin A and tacrolimus, both potent inhibitors of the calcium/calmodulin-dependent phosphatase calcineurin, stimulated the enzymatic activity of cellular DLK in an in vitro kinase assay. Immunocytochemistry revealed that the overexpression of DLK but not its kinase-dead mutant induced apoptosis and enhanced cyclosporin A-induced apoptosis to a higher extent than the drug alone. Moreover, in the presence of DLK, the effective concentration for cyclosporin A-caused apoptosis was similar to its known IC_{50} value for the inhibition of calcineurin activity in β cells. These data suggest that cyclosporin A through inhibition of calcineurin activates DLK, thereby leading to β-cell apoptosis. This action may thus be a novel mechanism through which cyclosporin A precipitates post-transplant diabetes.

Cyclosporin A and tacrolimus are clinically important immunosuppressive drugs used after organ transplantation and in the treatment of autoimmune diseases (Ho et al., 1996). However, their widespread therapeutic use is marred by a number of side effects shared by both drugs, among them post-transplant diabetes (Kahan, 1989; European FK506 Multicenter Liver Study Group, 1994; U.S. Multicenter FK506 Liver Study Group, 1994; Jindal et al., 1997; van Hooff et al., 2004). Although both drugs are structurally distinct and bind to their respective intracellular receptors, the immunophilins, they exert their immunosuppressive and some of their undesired effects through inhibition of the calcium/calmodulin-dependent phosphatase calcineurin (Ho et al., 1996). Blocking of calcineurin prevents the dephosphorylation of the nuclear factor of activated T cells and its translocation to the nucleus, thereby inhibiting nuclear factor of activated T cells-dependent gene transcription (Ho et al., 1996.). In addition, both drugs inhibit the transcriptional activity of the cAMP response element-binding protein CREB at the level of its coactivators CREB binding protein and transducer of regulated CREB through inhibition of calcineurin activity (Riggins and Clipstone, 2001; Screaton et al., 2004; Oetjen et al., 2005). CREB seems to play a pivotal role in the survival and the function of insulin-producing pancreatic β cells: mice expressing a dominant-negative CREB mutant in their β cells became diabetic, and their β cells underwent an apoptotic cell death (Jhala et al., 2003). Furthermore, CREB binds to its recognition sites present within the rat insulin I gene and the human insulin gene promoter and stimulates their transcriptional activity in-

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ABBREVIATIONS: DLK, dual leucine-zipper bearing kinase; JNK, c-Jun NH_2-terminal kinase; CREB, cAMP response element binding protein; JIP1, c-Jun NH_2-terminal kinase interacting protein-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; siRNA, short interfering RNA; PBS, phosphate-buffered saline; FK506, tacrolimus.

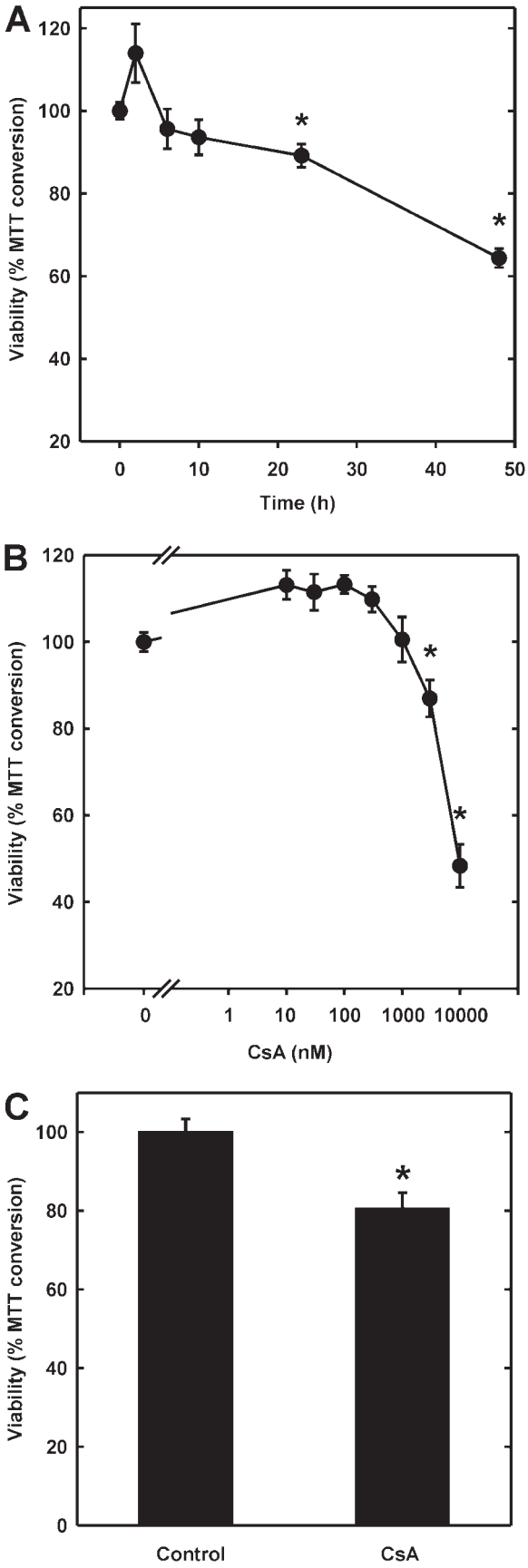

Fig. 1. Effect of cyclosporin A on the viability of the β-cell line HIT. A, time course. HIT cells were treated with 10 μM cyclosporin A for the time indicated, and cell viability using the MTT test was determined. B, concentration-response curve. HIT cells were treated with increasing concentrations of cyclosporin A for 48 h, followed by the MTT test. C, long-term incubation with cyclosporin A. HIT cells were incubated for 5 days with 30 nM cyclosporin A, followed by MTT test. Values are expressed relative to the mean value in each experiment of the control (no treatment). Values are mean ± S.E.M. of three independent experiments, each done in quadruplicate. *, $p < 0.05$ versus control.

duced by glucose, cAMP, and membrane depolarization (Oetjen et al., 1994; Eggers et al., 1998; Oetjen et al., 2003a,b). The inhibition of CREB-dependent human insulin gene transcription by cyclosporin A and tacrolimus in a β-cell line and in primary mature islets (Oetjen et al., 2003a,b), leading to β-cell dysfunction, might contribute to the development of post-transplant diabetes. In addition, a β-cell toxic effect, leading to islet cell damage, exerted by both drugs has been described previously (Drachenberg et al., 1999; Hui et al., 2005). However, the underlying molecular mechanism remains unknown.

The dual leucine-zipper-bearing kinase (DLK) is expressed in diverse tissues, including murine mature islets and β cells; it belongs to the group of the mitogen-activated protein kinases (Holzman et al., 1994; Oetjen et al., 2006). Acting as a triple kinase, DLK was shown to phosphorylate and activate the dual-specificity kinases mitogen-activated protein kinase kinase 4 and mitogen-activated protein kinase kinase 7, resulting in the phosphorylation and activation of the mitogen-activated kinase Jun-N-terminal kinase (JNK) (Merritt et al., 1999). In neuronal and glial aggregates, the phosphorylation state of DLK is regulated by membrane depolarization via calcineurin (Mata et al., 1996). Because cyclosporin A and tacrolimus stimulated the phosphorylation of c-Jun by DLK in the β-cell line HIT, the calcineurin-sensitive phosphorylation of DLK seems to be required for the enzymatic activity of DLK (Oetjen et al., 2006). It is noteworthy that in a neuronal cell line, DLK was shown to induce apoptosis (Xu et al., 2001). Therefore, in the present study, the effect of cyclosporin A and DLK on the survival of β-cells was investigated.

Materials and Methods

Plasmids and Cell Culture. The expression vectors for DLK and DLK K185A have been described before (Mata et al., 1996). HIT-T15 cells (Santerre et al., 1981) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were transfected by Metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol with 2 μg of expression vector or Bluescript (Stratagene, La Jolla, CA) to balance the amount of DNA per well per six-well plate. The transfection efficiency using this method is 14% in HIT cells. For the knockdown of cellular DLK, approximately 350,000 cells were transfected by Oligofectamine according to the manufacturer's protocol with 50 pmol of either nonspecific or specific stealth siRNA (Invitrogen, Karlsruhe, Germany). Cells were treated with cyclosporin A with the indicated concentrations and for the indicated time periods.

Viability Assay. Viability of HIT cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) test (Janjic and Wollheim, 1992). Cells were seeded on 96-well plates and treated with the indicated concentrations and for the indicated time periods. After 48 h or 5 days of culture, 10 μl of MTT solution (5 mg/ml in PBS, final concentration 0.5 mg/ml) was added, and cells were further incubated for 2 h at 37°C. Cells were lysed by 3% SDS followed by the addition of 0.04 N isopropanol-HCl. The metabolism-

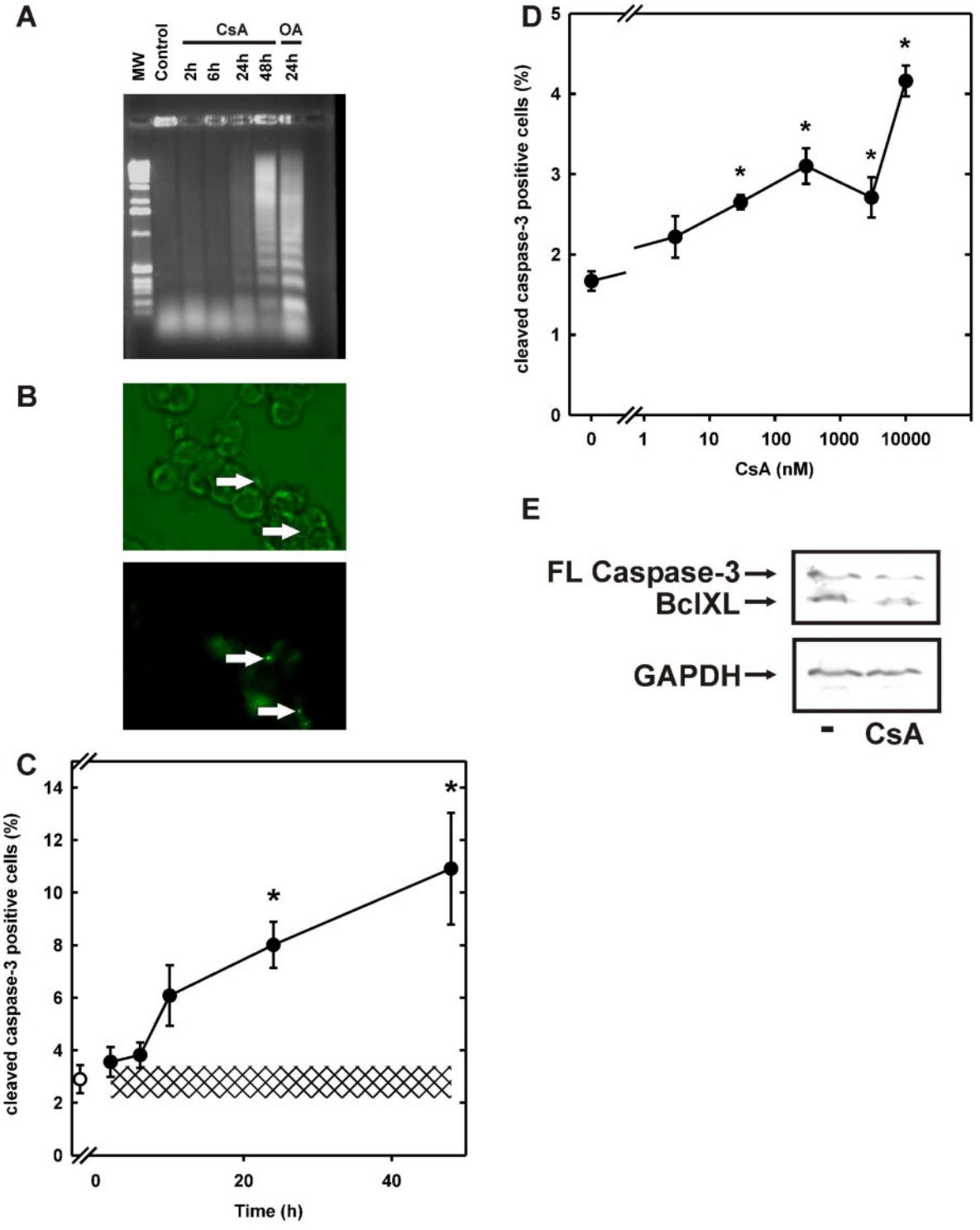

Fig. 2. Cyclosporin A-induced β-cell death shows signs of apoptosis. A, cyclosporin A-induced DNA fragmentation. HIT cells were treated with 10 μM cyclosporin A for the time indicated, cells were harvested after 48 h culture. Depicted is one of three agarose gel showing the fragmentation of HIT cell DNA. OA, treatment of cells with okadaic acid (50 nM) for 24 h; control, no treatment; MW, molecular weight marker B, immunocytochemistry of cleaved caspase-3 to examine caspase-3 activation; typical microscopy image of HIT cells. Top, HIT cells in transmitted light; bottom, the same section in fluorescence light. The arrows point to cleaved caspase-3 (bottom) and the corresponding cells in transmitted light (top). C, cyclosporin A-induced increase in caspase-3 activation, time course. HIT cells were treated with 10 μM cyclosporin A for the time indicated (●). ○, the percentage of cleaved caspase-3-positive cells without treatment. Values are mean ± S.E.M. of three different experiments, each done in duplicate. *, $p < 0.05$ versus control. D, cyclosporin A-induced increase in caspase-3 activation, concentration-response curve. HIT cells were treated for 48 h with increasing concentrations of cyclosporin A as indicated. Values are mean ± S.E.M. of three different experiments, each done in duplicate. *, $p < 0.05$ versus control E, cyclosporin A-induced apoptosis in primary islets. Approximately 500 isolated primary murine islets per group were treated for 24 h with 5 μM cyclosporin A (CsA) or were left untreated (−) in the presence of 5 mM glucose. Islets were collected after 25 h and subjected to immunoblot using antibodies against full-length caspase-3 (top arrow) and Bcl_{XL} (bottom arrow). To check for the same amount of protein in each lane, an antibody recognizing glyceraldehyde-3-phosphate dehydrogenase was used (bottom]). An image of a typical immunoblot is shown.

dependent conversion of the tetrazolium salt MTT into magenta-colored formazan was measured at 550 nm in a microplate reader.

Internucleosomal DNA Fragmentation. HIT cells were treated as indicated and harvested. Cell pellets were stored overnight in 70% ethanol/PBS at −20°C. For the extraction of low molecular weight DNA, the cell pellets were incubated in citric acid phosphate buffer (0.1 M citric acid/ 0.2 M phosphate buffer) for 30 min at room temperature and subsequently centrifuged at 14,000*g*. DNA within the supernatant was treated with RNase and proteinase K and separated by electrophoresis on a 0.8% agarose gel (Krautheim et al., 2000).

Immunocytochemistry. HIT cells were cultured on a coverslip. When indicated, cells were transiently transfected and treated with cyclosporin A. After 48 h, cells were washed twice with PBS, fixed in 100% methanol (−20°C), washed three times in PBS, treated with 0.1% fresh sodium borohydride dissolved in PBS and with blocking buffer (10% horse serum, 1% bovine serum albumin in PBS). Incubation with an antibody against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) (1:50 dilution) was overnight at 4°C. The fluorescent-labeled anti-rabbit antibody Alexa Fluor 488 (Invitrogen) (1:50 dilution) served as secondary antibody. For the detection of transfected DLK or its mutant, a murine monoclonal antibody against the Flag epitope was used (Sigma, Taufkirchen, Germany) (dilution 1:50). A tetramethylrhodamine B isothiocyanate-labeled anti-mouse antibody (Invitrogen) served as secondary antibody. Cleaved caspase-3-positive cells were counted in and expressed as a percentage of either all cells examined (not-transfected cells, Bluescript-transfected cells) or Flag-tagged DLK-expressing cells (cells transfected with DLK wild type or its mutant). In each group, approximately 300 cells were counted manually.

Islet Isolation, Culture, and Immunoblot. Pancreatic islets were isolated as described previously (Lacy and Kostianovsky, 1967). In brief, the pancreata of mice were mechanically dispersed in Krebs-Ringer buffer supplemented with 5 mM glucose and digested for 3 to 4 min at 37°C with collagenase P (Roche Diagnostics, Mannheim, Germany). Tissue was shaken; the islets were sedimented twice and were hand-picked. The purity of islets is estimated to be 95%. Isolated islets were incubated in a humidified atmosphere of 95% air/5% CO_2 for 1 h in RPMI 1640 medium containing 5 mM glucose and supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cyclosporin A (5 μM) was added 1 h after isolation, and the islets were harvested after 25 h and subjected to immunoblot assay. Approximately 500 islets were used per lane.

Immunoprecipitation and ex Vivo in Vitro Kinase Assay. HIT cells cultured on 6-cm dishes were treated with cyclosporin A (5 μM) or tacrolimus (167 nM) for the indicated time periods. Cells were lysed in 250 μl of lysis buffer per dish (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% Nonidet P-40, 1 mM $NaVO_4$, 50 mM NaF, 20 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors), passed five times through a 20-gauge needle, incubated on ice for 30 min, and centrifuged at 4°C, 14,000 rpm, for 5 min. For the immunoprecipitation, the precleared supernatant containing 1000 μg of protein was incubated with 60 μl of preswollen protein A agarose beads (50% v/v) and 10 μl of the antibody against the C terminus of DLK (Holzman et al., 1994) for 5 h at 4°C. Beads were washed four times with kinase buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $NaVO_4$, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors). Immunoprecipitated DLK was incubated for 60 min at 30°C with 25 μM ATP, 2 μg of dephosphorylated casein (Sigma), 3 μCi [γ-^{32}P]ATP (GE Healthcare, Freiburg, Germany) in a total volume of 40 μl while gently shaking. The reaction was terminated by adding SDS-sample buffer, and an aliquot was subjected to SDS-polyacrylamide gel electrophoresis. The phosphorylation of DLK and casein was detected by a PhosphorImager. For the determination of the amount of immunoprecipitated DLK, an immunoblot was performed, and the optical density of the band corresponding to immunoprecipitated DLK was evaluated using the program Quantity One, version 4, from Bio-Rad Laboratories (Hercules, CA).

Materials. Cyclosporin A (ciclosporin) was provided by Novartis Pharma AG (Basel, Switzerland), and tacrolimus (FK506) was provided by Fujisawa (Osaka, Japan). A stock solution of cyclosporin A (10 mg/ml) was prepared in ethanol with 20% Tween 80 and further diluted in RPMI. Tacrolimus was solved in ethanol. Controls received the solvent only.

Statistical Analysis. Statistical analysis was done by the Student's *t* test, whereby $p < 0.05$ was considered statistically significant. Values are given as mean ± S.E.M.

Results

Cyclosporin A-Induced β-Cell Death. The effect of the immunosuppressive drug cyclosporin A on β-cell viability was studied in the insulin-producing pancreatic islet β-cell line HIT by the MTT test. After 24 h of treatment with 10 μM cyclosporin A, HIT cell viability was reduced by 10% (Fig. 1A). Prolonged treatment for 48 h with the immunosuppressant further decreased the viability of HIT cells (Fig. 1A). As indicated by the concentration-response curve, 48-h treatment of the cells with 3 and 10 μM cyclosporin A reduced the viability of the cells by 10 and 50%, respectively (Fig. 1B). In addition, incubation of the cells for 5 days with 30 nM cyclosporin A reduced cell viability by 20% (Fig. 1C). Incubation with 100 and 300 nM cyclosporin A for the same periods decreased viability to 84 ± 2.4 and 86% ± 3.6 ($p<0.05$ versus control; $n = 6$), respectively. Thus, cyclosporin A decreased β-cell viability in a time- and concentration-dependent manner.

Cyclosporin A-Induced β-Cell Death Showed Signs of Apoptosis. Necrosis and apoptosis (also called programmed cell death) are the two main forms of cell death (Hengartner, 2000). To investigate which kind of cell death was induced by cyclosporin A in β cells, internucleosomal DNA fragmentation and activation of caspase-3, being characteristic of apoptosis, were studied (Hengartner, 2000). As shown in Fig. 2A, DNA fragmentation in HIT cells started 24 h after treatment with 10 μM cyclosporin A and was more pronounced after 48 h of treatment. In additional experiments, fragmentation of HIT cell DNA was observed after 48-h treatment also with 3 μM immunosuppressive drug (data not shown). The cleavage of the caspase-3 substrate DEVD linked to the fluorophore amino-4-trifluoromethyl coumarin was enhanced 1.7-fold in extracts of HIT cells treated with 10 μM cyclosporin A (data not shown). To investigate an apoptosis-inducing effect of cyclosporin A at the cellular level, immunocytochemical methods were used. Cleavage of caspase-3 at Asp-175 is considered a hallmark of apoptosis (Hengartner, 2000). Therefore, an antibody recognizing caspase-3 cleaved at Asp-175 was used to detect apoptotic cells. Figure 2B depicts a typical microscopy image of cultured HIT cells in transmitted light (top) and the same section in fluorescent light for the detection of cleaved caspase-3. Figure 2C shows that HIT cells undergo spontaneous apoptosis, because 3% ± 0.5 ($n = 4$) of the cells stained positive for cleaved caspase-3. Treatment with cyclosporin A (10 μM) for the indicated time increased the number of cleaved caspase-3-positive cells 3.1-fold after 48 h (Fig. 2C). The difference between the reduction of viability (Fig. 1A) and the number of cleaved caspase-3-positive cells (Fig. 2C) might be due to caspase-3-independent cell death (Hengart-

ner, 2000). Apoptotic cells were already detectable after 24 h of treatment with the drug (2.5-fold increase) (Fig. 2C). Incubation of HIT cells for 48 h with increasing concentrations of cyclosporin A, starting with 30 nM, enhanced the number of apoptotic cells (Fig. 2D). Albeit in this set of experiments, the number of cleaved caspase-3-positive cells was lower, 10 μM cyclosporin A caused a similar increase in the number of apoptotic cells. Furthermore, treatment of primary mature murine islets with cyclosporin A reduced the content of full-length caspase-3 to 27.8% ± 9.9 and of the antiapoptotic protein Bcl_{XL} to 39.1% ±19 (n = 3) (Hengartner, 2000) without changing the amount of glyceraldehyde-3-phosphate dehydrogenase (Fig. 2E). Thus, cyclosporin A impairs β-cell survival by inducing β-cell apoptosis in a time- and concentration-dependent manner.

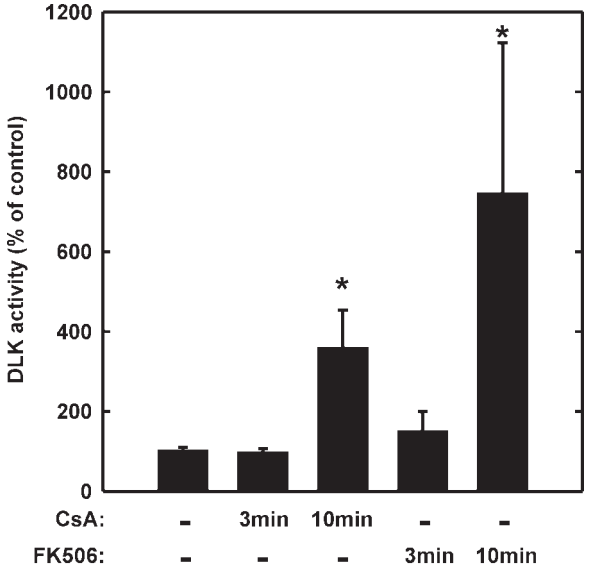

Fig. 3. Cyclosporin A and tacrolimus enhance DLK kinase activity. HIT cells were treated with 5 μM cyclosporin A (CsA) or 167 nM tacrolimus (FK506) for 3 and 10 min as indicated. Cellular DLK was immunoprecipitated, and an in vitro kinase assay was performed using casein as substrate. Phosphorylation of casein is expressed relative to the phosphorylation of casein in the absence of treatment. Values are mean ± S.E.M. of three different experiments, each done in duplicate. *, $p < 0.05$ versus control.

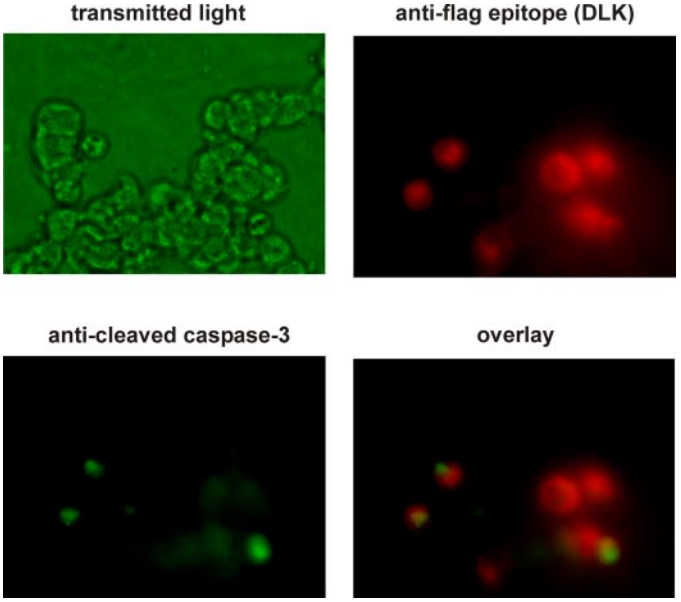

Fig. 4. Double immunocytochemistry for DLK expression and caspase-3 activation as indicated by cleaved caspase-3 levels; typical microscopy image showing the same section in transmitted light (top left), stained for Flag epitope-tagged DLK in fluorescence light (top right), stained for cleaved caspase-3 in fluorescence light (bottom left) and an overlay (bottom right). HIT cells were transiently transfected with the expression vector for Flag epitope-tagged DLK and cultured on coverslips. After 48 h, cells were fixed and prepared for immunocytochemistry.

Cyclosporin A and Tacrolimus Enhanced DLK Kinase Activity. In a neuronal cell line, the overexpression of DLK increased apoptotic cell death (Xu et al., 2001). To investigate whether DLK becomes activated by cyclosporin A and tacrolimus, HIT cells were treated with these drugs, cellular DLK was immunoprecipitated, and an in vitro kinase assay was performed. Figure 3 shows a 3.6 ± 0.9-fold and a 7.4 ± 3.7-fold (n = 6) increase in DLK kinase activity by cyclosporin A and tacrolimus, respectively, after 10-min treatment (Fig. 3). In addition, an increase in the autophosphorylation of DLK was observed (6.7 ± 1.7 and 11 ± 5.3-fold (n = 5) by cyclosporin A and tacrolimus, respectively) (data not shown). Furthermore, a 60-min treatment with lower concentrations of CsA and tacrolimus stimulated DLK kinase activity 1.6 ± 0.19 and 1.5 ± 0.19-fold (n = 5), respectively (supplementary Fig. S1). Because inhibition of calcineurin is common to both structurally distinct drugs, this finding suggests that inhibition of calcineurin phosphatase results in the activation of DLK.

DLK Induced β-Cell Apoptosis and Enhanced Cyclosporin A-Induced Apoptotic β-Cell Death. To investigate the effect of DLK on β-cell apoptosis, an expression vector for Flag epitope-tagged DLK or its kinase-dead mutant was transiently transfected into HIT cells. Double immunocytochemistry was used to detect DLK-overexpressing cells and cells undergoing apoptosis as indicated by cleaved caspase-3 (Fig. 4). DLK enhanced the number of apoptotic β-cells 18.6-

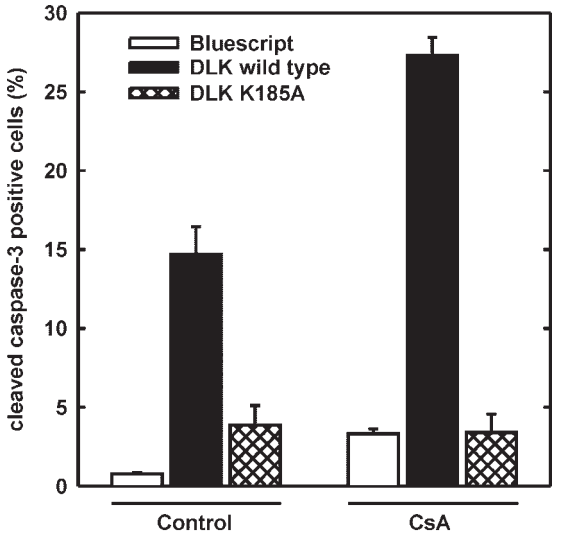

Fig. 5. Induction of β-cell apoptosis by DLK and its synergistic enhancement by cyclosporin A. The enhancement by cyclosporin A of DLK-induced apoptosis is more than additive and depends on its kinase activity. HIT cells were transiently transfected with the indicated plasmids and treated for 24 h with cyclosporin A (10 μM) as indicated. Cells were fixed 48 h after transfection and were prepared for immunocytochemistry. Values are mean ± S.E.M. of three different experiments, each done in duplicate.

fold, whereas its kinase-inactive mutant, DLK K185A, elicited an only 4.9-fold increase (Fig. 5), indicating that the apoptosis-inducing effect of DLK depends on its kinase activity. Treatment with cyclosporin A enhanced the number of apoptotic β cells 4.2-fold (Fig. 5). Cyclosporin A and DLK together increased the number of apoptotic cells to a higher extent than each treatment alone (34.5-fold) (Fig. 5). In contrast, the number of apoptotic cells in the presence of the drug and the DLK mutant was not greater than the level reached by cyclosporin A or the DLK mutant alone (Fig. 5). In immunoblots, the expression of the DLK mutant was 82.2% ± 4.6 compared with the expression of DLK (100%; $p < 0.05$; $n = 4$). Cyclosporin A (10 μM for 24 h) did not increase the expression levels of either DLK wild type or mutant (100% compared with 88.4% ±7.3 in the presence of cyclosporin A, and 82.2% ±7.3 compared with 80.4% ± 9 in the presence of cyclosporin A for DLK and DLK mutant, respectively; $p > 0.05$, $n = 4$). Time course experiments indicated that cyclosporin A enhanced DLK-induced β-cell apoptosis as early as within 12 h (Fig. 6A). In the presence of DLK, increasing concentrations of cyclosporin A induced β-cell apoptosis with an effective concentration of 30 nM (Fig. 6B). This effective concentration is similar to half-maximal inhibitory concentration of cyclosporin A for the inhibition of calcineurin phosphatase activity in HIT cells and in primary mature pancreatic islets (Schwaninger et al., 1995; Oetjen et al., 2003a). These data suggest that cyclosporin A, through inhibition of calcineurin, enhances DLK-induced apoptotic β-cell death. Using the small interfering RNA approach the cellular DLK was reduced to 24.35% ± 6.7 (versus 100% ± 6.3 in the presence of nonspecific siRNA; $p < 0.05$, $n = 4$). This DLK knockdown was sufficient to diminish the apoptosis-inducing effect of cyclosporin A (Fig. 7).

Discussion

Consistent with recent evidence for a pivotal role of calcineurin for β-cell function and survival (Oetjen et al., 2003a,b; Heit et al., 2006), previous studies have shown that the calcineurin inhibitors and immunosuppressive drugs cyclosporin A and tacrolimus have a toxic effect on insulin-producing pancreatic islet β cells (Jindal et al., 1997). For example, in pancreatic biopsies from patients receiving either tacrolimus or cyclosporin A cytoplasmic swelling, vacuolization and abnormal immunostaining for insulin have been observed (Drachenberg et al., 1999). In addition, 48-h treatment of human isolated islets with tacrolimus decreased their viability (Hui et al., 2005). The present study confirms these reports and shows furthermore that cyclosporin A causes DNA fragmentation, increases cleaved caspase-3 levels, enhances caspase-3 activity, and decreases full-length caspase-3 and Bcl_{XL} levels in HIT pancreatic islet β cells or primary mature islets, all indicating that cyclosporin A-induced β-cell death includes cyclosporin A-induced β-cell apoptosis. The DLK seems to play an important role in this apoptotic effect of cyclosporin A because 1) cyclosporin A was found to stimulate DLK kinase activity in β-cells, and 2) the overexpression and thus activation of DLK was found to induce β-cell apoptosis that was further enhanced by cyclosporin A.

The DLK is a mitogen-activated protein kinase kinase kinase; it is widely expressed in neural tissues, including the brain and the peripheral nervous system (Holzman et al., 1994; Hirai et al., 2005). Using mice embryos with a disruption of both DLK alleles, this kinase was shown to regulate axon growth and neuronal migration of the developing cerebral cortex (Hirai et al., 2006). DLK is also expressed in

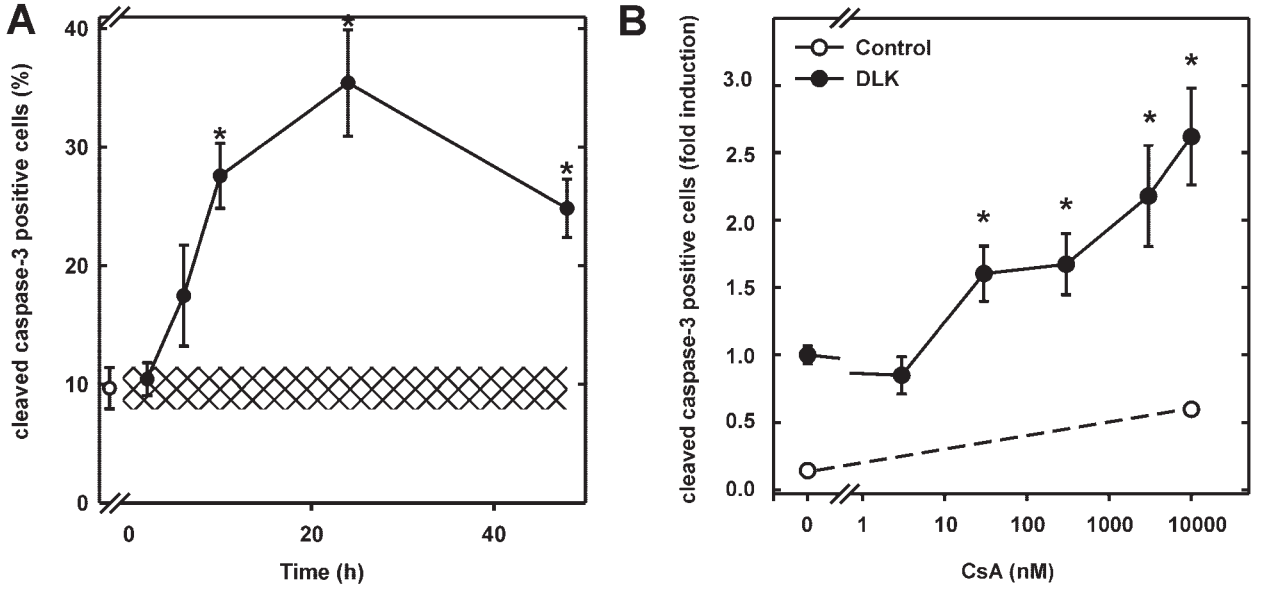

Fig. 6. Time course (A) and concentration-response curve (B) for the enhancement by cyclosporin A of DLK-induced apoptosis. A, HIT cells were transiently transfected with the expression vector for Flag epitope-tagged DLK and treated with 10 μM cyclosporin A for the time indicated. Forty-eight hours after transfection, cells were fixed and prepared for immunocytochemistry. The open circles and the cross-hatched area represent the cleaved caspase-3-positive cells in the presence of only DLK without treatment with cyclosporin A. Values are mean ± S.E.M. of three different experiments, each done in duplicate. *, $p < 0.05$ versus control. B, in the presence of DLK, cyclosporin A induces apoptosis with an IC_{50} value that is similar to the known IC_{50} value of cyclosporin A for the inhibition of calcineurin in HIT cells. HIT cells were transiently transfected with an expression vector of Flag epitope-tagged DLK and incubated for 24 h with increasing concentrations of cyclosporin A (●) or treated only with 10 μM cyclosporin A (control, transfected with Bluescript) (○). Forty-eight hours after transfection, cells were fixed and prepared for immunocytochemistry. Values are relative to the number of cleaved caspase-3 plus DLK-positive cells in the absence of cyclosporin A. Values are mean ± S.E.M. of three different experiments, each done in duplicate. *, $p < 0.05$ versus control.

murine islets and in the β-cell line HIT (Oetjen et al., 2006). DLK activity was shown to be regulated at least in part through association with the scaffold protein JNK-interacting protein (JIP1)/islet brain (Nihalani et al., 2001, 2003). Under basal conditions, JIP interacts with monomeric, catalytically inactive DLK (Nihalani et al., 2001, 2003). Phosphorylation of tyrosine residues of JIP1 by the Src family kinases seems to strengthen the interaction between JIP and DLK, thus maintaining DLK in its inactive state (Nihalani et al., 2007), whereas the phosphorylation of JIP1 on Thr-103 by JNK leads to the dissociation of DLK from JIP (Nihalani et al., 2003). DLK then homodimerizes via its leucine zipper and becomes catalytically active, presumably through autophosphorylation, and results in the activation of JNK (Leung and Lassam, 2001; Nihalani et al., 2001, 2003). Calcineurin might regulate DLK activity by dephosphorylation of DLK itself, impairing its autophosphorylation (Mata et al., 1996; Oetjen et al., 2006). Signals activating JNK may thus be amplified by induction of DLK activity. A model has been proposed whereby apoptotic stimuli promote the stabilization of JNK pathway components like DLK and JIP, leading to a self-amplifying feed-forward loop mechanism, thereby contributing to cell death (Xu et al., 2005). In the present study, the autophosphorylation and the substrate phosphorylation of DLK were stimulated by cyclosporin A and tacrolimus, suggesting that inhibition of calcineurin enhances the phosphorylation of DLK and its enzymatic activity. Therefore, it is most likely that cyclosporin A increases DLK autophosphorylation and kinase activity in HIT β cells, as observed in the present study, through inhibition of calcineurin phosphatase activity. This view is further supported by the fact that a structurally distinct calcineurin inhibitor, tacrolimus, stimulated DLK kinase activity.

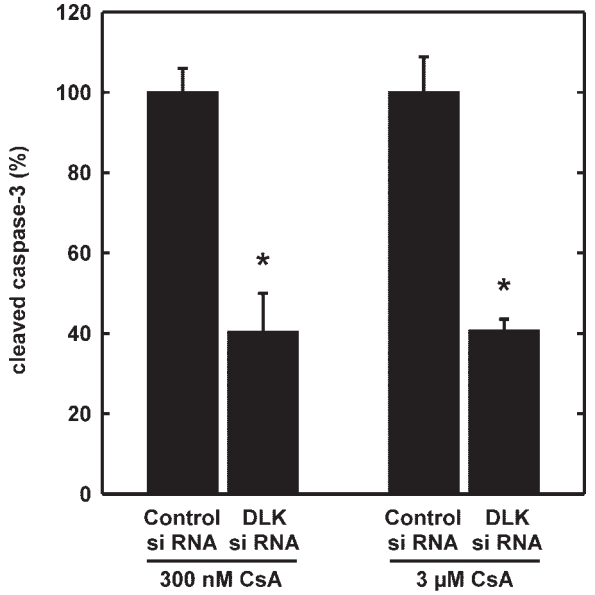

Fig. 7. Knockdown of cellular DLK diminished to apoptosis-inducing effect of DLK. HIT cells were transiently transfected with nonspecific siRNA or for DLK-specific siRNA by Oligofectamine and treated for 48 h with cyclosporin A in the indicated concentrations. Cells were harvested, and immunoblots against the cleaved caspase-3 were performed. The optical density of the cleaved caspase-3-representing band in the presence of DLK-specific siRNA is expressed relative to the optical density of the cleaved caspase-3-representing band in the presence of nonspecific siRNA. Values are mean ± S.E.M. of two different experiments, each done in duplicate. *, $p < 0.05$ versus control.

The activation by cyclosporin A of DLK kinase activity seems to be sufficient to induce β-cell apoptosis. DLK has been shown before to induce apoptosis in a neuronal cell line (Xu et al., 2001). In the present study, the overexpression of DLK but not its kinase-dead mutant was found to markedly induce β-cell apoptosis. The overexpression of DLK, through aggregation and autophosphorylation, confers DLK activity (Nihalani et al., 2000), which is further enhanced by inhibition of calcineurin (Oetjen et al., 2006). Consistent with this view, cyclosporin A enhanced DLK-induced β-cell apoptosis at concentrations that have been shown before to increasingly inhibit calcineurin phosphatase activity in β cells (Schwaninger et al., 1995). When taken together, the data of the present study are consistent with the notion that cyclosporin A induces apoptotic β-cell death through inhibition of calcineurin, leading to enhanced DLK activity.

The pathways through which DLK produces β-cell apoptosis remain to be defined. However, DLK has been shown to inhibit depolarization-induced activity of the transcription factor CREB and its coactivator CREB binding protein in β cells (Oetjen et al., 2006). CREB regulates the transcription of the β-cell survival-promoting insulin receptor substrate-2 gene and the antiapoptotic Bcl-2 gene (Jambal et al., 2003; Jhala et al., 2003). Furthermore, the down-regulation of CREB in β cells of mice results in β-cell apoptosis and diabetes mellitus (Jhala et al., 2003). Thus, inhibition of CREB transcriptional activity may be one mechanism of DLK-induced β-cell apoptosis.

Post-transplant diabetes mellitus is a severe side effect under therapy with the immunosuppressive drugs cyclosporin A and tacrolimus, considering that immunosuppressive therapy has to last for a lifetime and considering the long-term complications of diabetes such as cardiovascular diseases with myocardial infarction, stroke, or renal failure (Kahan, 1989; European FK506 Multicenter Liver Study Group, 1994; U.S. Multicenter FK506 Liver Study Group, 1994; Jindal et al., 1997). Several mechanisms seem to be involved. Like type 2 diabetes mellitus, post-transplant diabetes is believed to be due to insulin resistance (Lohmann et al., 2000) and to a decrease in β-cell function and mass (Van Hooff et al., 2004). The inhibition by cyclosporin A of calcineurin in β-cells may precipitate post-transplant diabetes through inhibition of glucose-induced insulin gene transcription (Oetjen et al., 2003a), leading to decreased insulin biosynthesis and β-cell function. The present study now suggests an additional novel mechanism, namely the activation of DLK kinase activity, leading to β-cell apoptosis and a decrease in β-cell mass. β Cell-specific blockade of DLK, if such inhibitors became available, might be an approach to retard the development of post-transplant diabetes under immunosuppressive therapy with cyclosporin A and tacrolimus.

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