Title page

Activation of the dual-leucine-zipper-bearing kinase (DLK) and induction of beta-cell apoptosis by the immunosuppressive drug cyclosporin A*

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Running title page

a) Induction of beta-cell apoptosis by Cyclosporin A and DLK

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d) abbreviations: CBP, CREB binding protein; CREB, cAMP response element binding protein; DLK, dual-leucine-zipper bearing kinase; IRS-2, insulin receptor substrate-2; JIP/IB, JNK interacting protein/islet brain; JNK, c-Jun N-terminal kinase; MKK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; NFAT, nuclear factor of activated T-cells; TORC, transducer of regulated CREB;

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Abstract

Posttransplant 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 beta-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 beta-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 as well as 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 IC50 value for the inhibition of calcineurin activity in beta-cells. These data suggest, that cyclosporin A through inhibition of calcineurin activates DLK, thereby leading to beta-cell apoptosis. This action may thus be a novel mechanism through which cyclosporin A precipitates posttransplant diabetes.

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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 wide spread therapeutic use is marred by a number of side effects, shared by both drugs, among them posttransplant diabetes (Kahan, 1989; European FK506 Multicentre 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 (NFAT) and its translocation to the nucleus thereby inhibiting NFAT-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 CBP and TORC through inhibition of calcineurin activity (Riggins and Clipstone, 2001; Oetjen et al., 2005; Screaton et al., 2004). CREB seems to play a pivotal role for the survival and the function of insulin producing pancreatic beta-cells: Mice expressing a dominant-negative CREB mutant in their beta-cells became diabetic and their beta-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 induced by glucose, cyclic AMP and membrane depolarisation (Oetjen et al., 1994; Eggers et al., 1998; Oetjen et al., 2003 a;b). The inhibition of CREB-dependent human insulin gene transcription by cyclosporin A and tacrolimus in a beta-cell line and in primary mature islets (Oetjen et al., 2003 a;b), leading to beta-cell dysfunction might contribute to the development of posttransplant diabetes. In addition, a beta-cell toxic effect, leading to islet cell damage, exerted by both drugs has been described (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 beta-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

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activate the dual specificity kinases MKK4 and MKK7 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 depolarisation via calcineurin (Mata et al., 1996). Since cyclosporin A and tacrolimus stimulated the phosphorylation of c-Jun by DLK in the beta-cell line HIT, the calcineurin-sensitive phosphorylation of DLK appears to be required for the enzymatic activity of DLK (Oetjen et al., 2006). Noteworthy, 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 beta-cells was investigated.

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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 % FCS, 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 (Stratagen) to balance the amount of DNA per well/6-well-plate. The transfection efficiency using this method is 14 % in HIT cells. For the knock-down of cellular DLK approximately 350,000 cells were transfected by oligofectamine according to the manufacturer's protocol with 50 pmole of either non-specific 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 medium 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-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 14,000xg. 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).

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Immunocytochemistry. HIT cells were cultured on a cover slip. 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 % BSA in PBS). Incubation with an antibody against cleaved caspase-3 (Cell signalling, Danvers, MA, USA) (1:50 dilution) was overnight at 4°C. The fluorescent-labelled anti-rabbit antibody Alexa Fluor 488 (invitrogen, Karlsruhe, Germany) (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). As secondary antibody served a TRITC-labelled anti-mouse antibody (invitrogen, Karlsruhe, Germany). Cleaved caspase-3 positive cells were counted in and expressed as 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). Briefly, the pancreata of mice were mechanically dispersed in Krebs-Ringer buffer supplemented with 5 mM glucose and digested 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 hour after isolation and the islets were harvested after 25 hours 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 lysis buffer/dish (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA,

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10 % glycerol, 1 % Triton X-100, 1 % NP-40, 1 mM $NaVO_4$, 50 mM NaF, 20 mM β-glycero-phosphate, 2 mM PMSF and protease inhibitors), passed 5 times through a 20 G needle, incubated on ice for 30 min and centrifuged at 4°C, 14,000 rpm for 5 min. For the immunoprecipitation the pre-cleared supernatant containing 1000 µg protein was incubated with 60 µl of pre-swollen protein A agarose beads (50 % (vol/vol) 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 PMSF and protease inhibitors). Immunoprecipitated DLK was incubated for 60 min at 30°C with 25 µM ATP, 2 µg of dephosphorylated casein (Sigma, Taufkirchen, Germany), 3 µCi $^{[32]}$P-γ-ATP (GE Healthcare, Freiburg, Germany) in a total volume of 40 µl gently shaking. The reaction was terminated by adding SDS-sample buffer and an aliquot was subjected to SDS-PAGE. 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 programme Quantity One, version 4 from Biorad.

Materials. Cyclosporin A (ciclosporin) was provided by Novartis Pharma AG (Basel, Switzerland), tacrolimus (FK506) 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 a $p<0.05$ was considered statistically significant. Values are given as mean ± S.E.M..

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Results

Cyclosporin A-induced beta-cell death. The effect of the immunosuppressive drug cyclosporin A on beta-cell viability was studied in the insulin producing pancreatic islet beta-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 of 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 of cyclosporin A reduced cell viability by 20 % (Fig. 1C). Incubation with 100 nM 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 beta-cell viability in a time- and concentration-dependent manner.

Cyclosporin A-induced beta-cell death shows signs of apoptosis. Necrosis and apoptosis (also called programmed cell death) are the two main forms of cell death (Hengartner, 2000). In order to investigate which kind of cell death was induced by cyclosporin A in beta-cells, internucleosomal DNA fragmentation and activation of caspase-3, being characteristic for 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 treatment. In additional experiments, fragmentation of HIT cell DNA was observed after 48 h treatment also with 3 µM of the immunosuppressive drug (data not shown). The cleavage of the caspase-3 substrate DEVD linked to the fluorophore AFC 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 employed. 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. Fig. 2B depicts a typical microscopy image of cultured HIT cells in transmitted light (upper panel) and the same section in fluorescent light for the detection of cleaved caspase-3. Fig. 2C shows that HIT cells undergo spontaneous apoptosis, since 3 % ± 0.5

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(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 as to 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 (Hengartner, 2000). Apoptotic cells were already detectable after 24 h 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 GAPDH (Fig. 2E). Thus, cyclosporin A impairs beta-cell survival by inducing beta-cell apoptosis in a time- and concentration-dependent manner.

Cyclosporin A and tacrolimus enhance 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. Fig. 3 shows a 3.6-fold ± 0.9 and a 7.4-fold ± 3.7 (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-fold ± 1.7 and 11-fold ± 5.3 (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-fold ± 0.19 and 1.5-fold ± 0.19 (n=5), respectively (supplementary figure 1). Since 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 induces beta-cell apoptosis and enhances cyclosporin A-induced apoptotic beta-cell death. In order to investigate the effect of DLK on beta-cell apoptosis, an expression vector for flag epitope-

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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 beta-cells 18.6-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 beta-cells 4.2 fold (Fig. 5). Cyclosporin A and DLK together raised 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 above 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 when compared to the expression of DLK (100 %; $p<0.05$; n=4). Cyclosporin A (10 µM for 24 h) did not change increase the expression levels of either DLK wild-type or mutant (100% as compared to 88.4 % ±7.3 in the presence of cyclosporin A, and 82.2 % ±7.3 as compared for 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 beta-cell apoptosis as early as within 12 h (Fig. 6A). In the presence of DLK, increasing concentrations of cyclosporin A induced beta-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 beta-cell death. Using the small inference RNA approach the cellular DLK was reduced to 24.35 % ±6.7 (versus 100 % ±6.3 in the presence of non-specific siRNA; $p<0.05$; n=4). This DLK knock-down was sufficient to diminish the apoptosis inducing effect of cyclosporin A (Fig. 7).

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Discussion

Consistent with recent evidence for a pivotal role of calcineurin for beta-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 beta-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 has 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, enhanced caspase-3 activity and decreases full-length caspase-3 and Bcl_{XL} levels in HIT pancreatic islet beta-cells or primary mature islets, all indicating that cyclosporin A-induced beta-cell death includes cyclosporin A-induced beta-cell apoptosis. The DLK appears to play an important role in this apoptotic effect of cyclosporin A since i) cyclosporin A was found to stimulate DLK kinase activity in beta-cells, and ii) the overexpression and thus activation of DLK was found to induce beta-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 murine islets and in the beta-cell line HIT (Oetjen et al., 2006). DLK activity was shown to be regulated at least in part through association with the scaffold protein JIP1/IB (JNK-interacting protein/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 appears 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 (Nihalani et al, 2001; 2003; Leung and Lassam, 2001). Calcineurin

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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 pathways 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 as well as the substrate phosphorylation of DLK was 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 beta-cells, as observed in the present study, through inhibition of calcineurin phosphatase activity. This view is further supported by the fact that also a structurally distinct calcineurin inhibitor, tacrolimus, stimulated DLK kinase activity.

The activation by cyclosporin A of DLK kinase activity appears to be sufficient to induce beta-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 beta-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 beta-cell apoptosis at concentrations that have been shown before to increasingly inhibit calcineurin phosphatase activity in beta-cells (Schwaninger et al., 1995). When taken together, the data of the present study are consistent with the notion that cyclosporin A induces apoptotic beta-cell death through inhibition of calcineurin leading to enhanced DLK activity.

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

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

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Footnotes

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Legends to Figures

Fig. 1. Effect of cyclosporin A on the viability of the beta-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 performed. B, concentration-response curve. HIT cells were treated with increasing concentrations of cyclosporin A for 48 h, followed by 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.

Fig. 2. Cyclosporin A-induced beta-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 out 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. The upper panel shows HIT cells in transmitted light, the lower panel depicts the same section in fluorescence light. The arrows point to cleaved caspase-3 (lower panel) and the corresponding cells in transmitted light (upper panel). C, cyclosporin A-induced increase in caspase-3 activation, time-course. HIT cells were treated with 10 µM cyclosporin A for the time indicated (black dots). The white dot represents 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 hours with 5 µM

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cyclosporin A (CsA) or left untreated (-) in the presence of 5 mM glucose. Islets were collected after 25 hours and subjected to immunoblot, using antibodies against full length caspase-3 (upper arrow) and Bcl_{XL} (lower arrow). To check for the same amount of protein in each lane, an antibody recognizing GAPDH was used (lower panel). An image of a typical immunoblot is shown.

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 (upper left panel), stained for flag epitope-tagged DLK in fluorescence light (upper right panel), stained for cleaved casapse-3 in fluorescence light (lower left panel) and an overlay (lower right panel). HIT cells were transiently transfected with the expression vector for flag epitope-tagged DLK and cultured on cover slips. After 48 h cells were fixed and prepared for immunocytochemistry.

Fig. 5 Induction of beta-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 prepared for immunocytochemistry. Values are mean ± S.E.M. of three different experiments, each done in duplicate.

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

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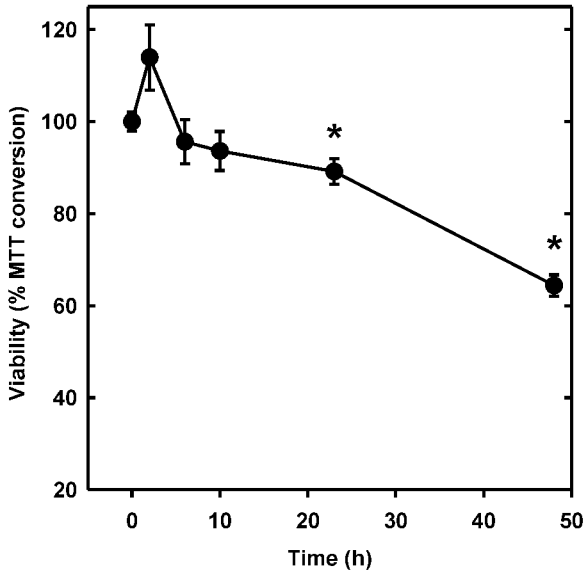
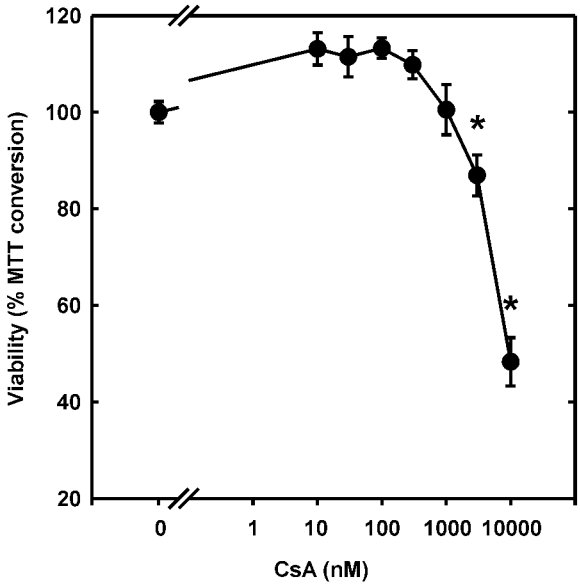
epitope-tagged DLK and treated with 10 µM cyclosporin A for the time indicated. 48 h after transfection cells were fixed and prepared for immunocytochemistry. The white dot and the cross-hatched area represent the cleaved caspase-3 positive cells in the presence of only DLK without treatment with cyclosporin 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 IC50 that is similar to the known IC50 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 (black dots) or treated only with 10 µM cyclosporin A (control, transfected with bluescript) (white dots). 48 h 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.

Fig. 7 Knock-down of cellular DLK diminished to apoptosis inducing effect of DLK. HIT cells were transiently transfected with non-specific 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 non-specific siRNA. Values are mean ± S.E.M. of two different experiments, each done in duplicate. *$p<0.05$ versus control.

A


Viability (% MTT conversion)
120
100
80
60
40
20
0
10
20
30
40
50
Time (h)
*
*

B


Viability (% MTT conversion)
120
100
80
60
40
20
0
1
10
100
1000
10000
CsA (nM)
*
*

C

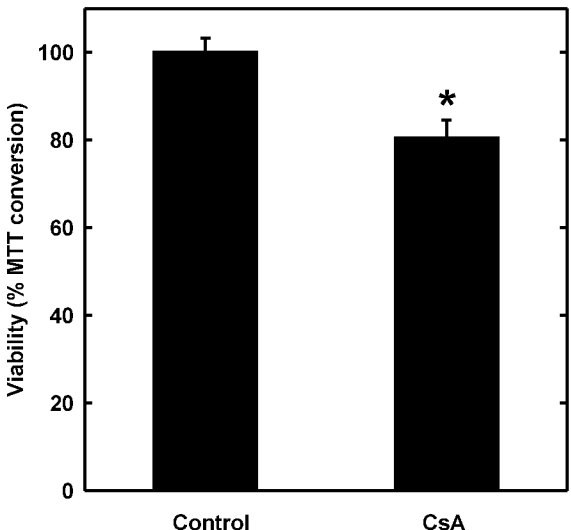

Viability (% MTT conversion)
100
80
60
40
20
0
Control
CsA
*

Fig. 1

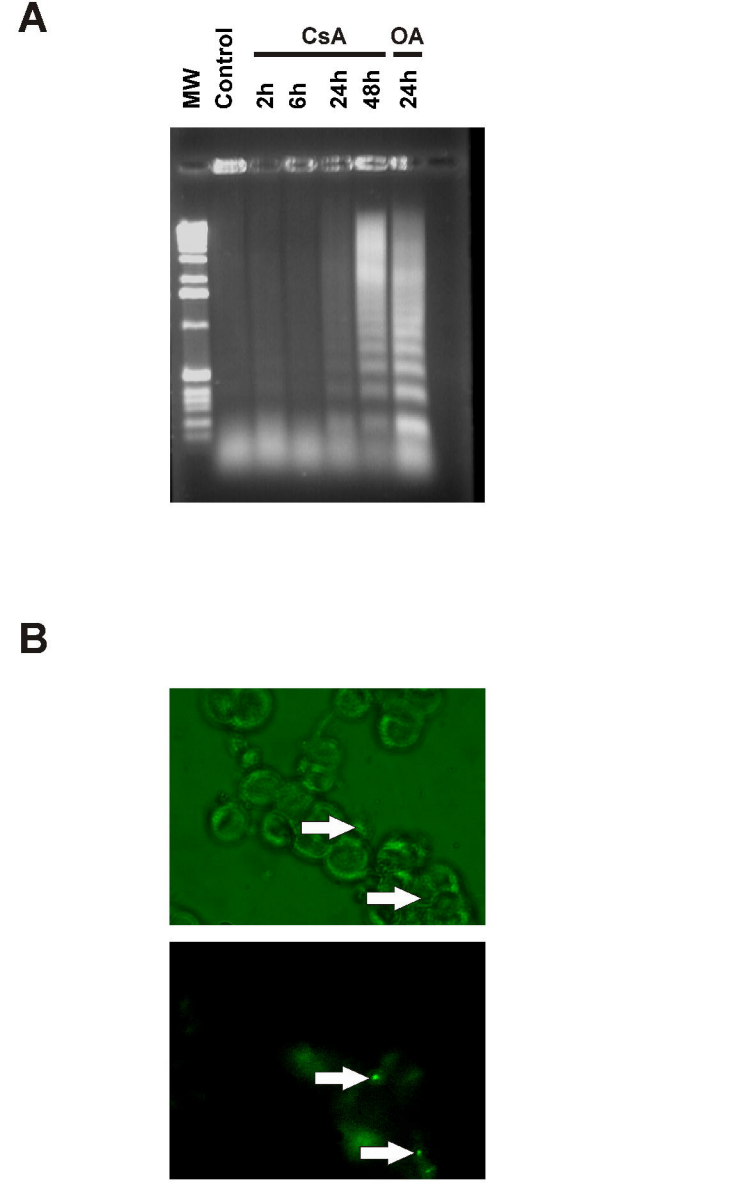

A
MW
Control
CsA
2h
6h
24h
48h
OA
24h
B
C
cleaved caspase-3 positive cells (%)
Time (h)
D
cleaved caspase-3 positive cells (%)
CsA (nM)
E
FL Caspase-3
BclXL
GAPDH
-
CsA

Fig. 2

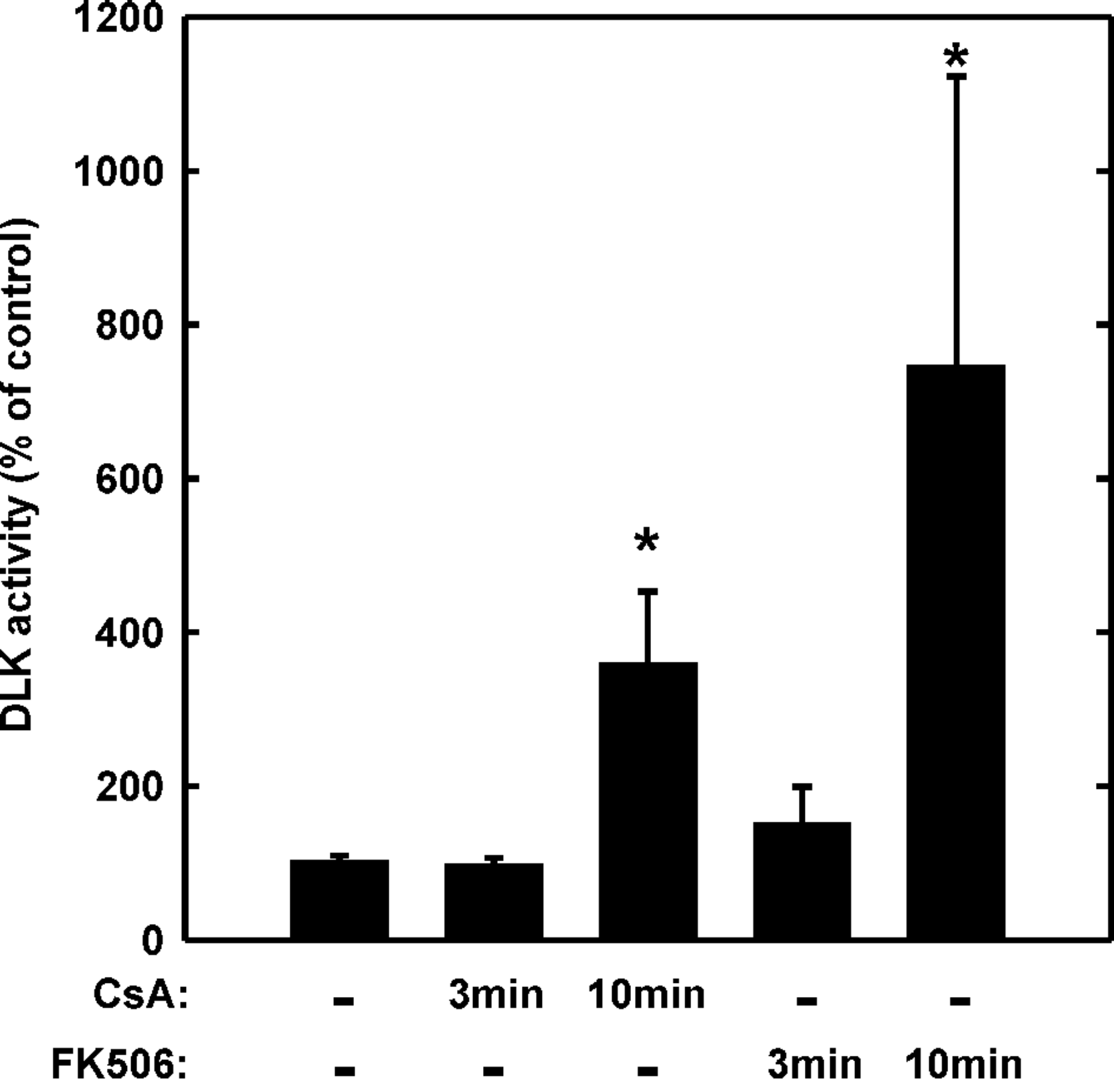

1200
1000
800
600
400
200
0
DLK activity (% of control)
*
*
CsA:
-
3min
10min
-
-
FK506:
-
-
-
3min
10min

Fig. 3

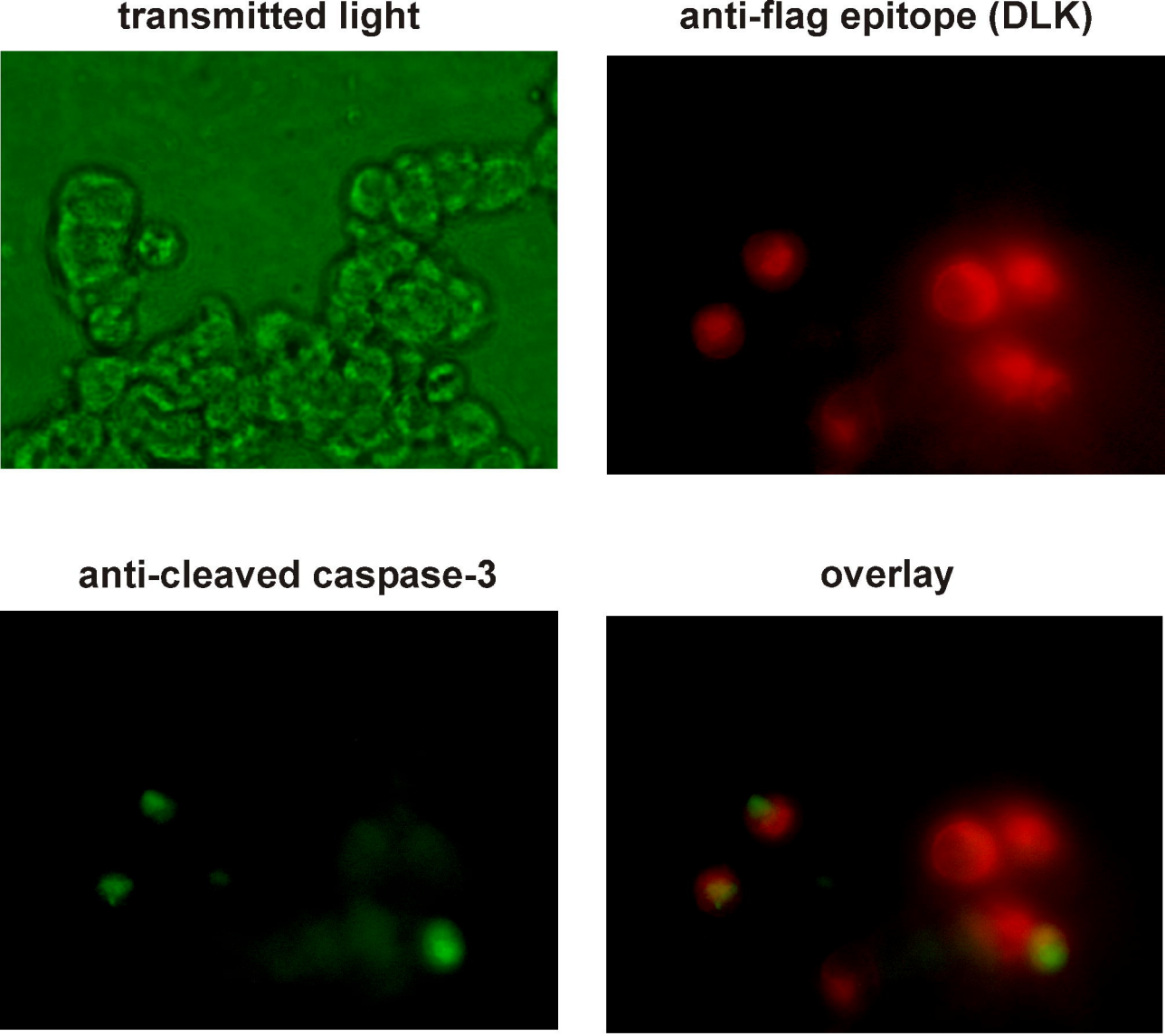

transmitted light
anti-flag epitope (DLK)
anti-cleaved caspase-3
overlay

Fig. 4

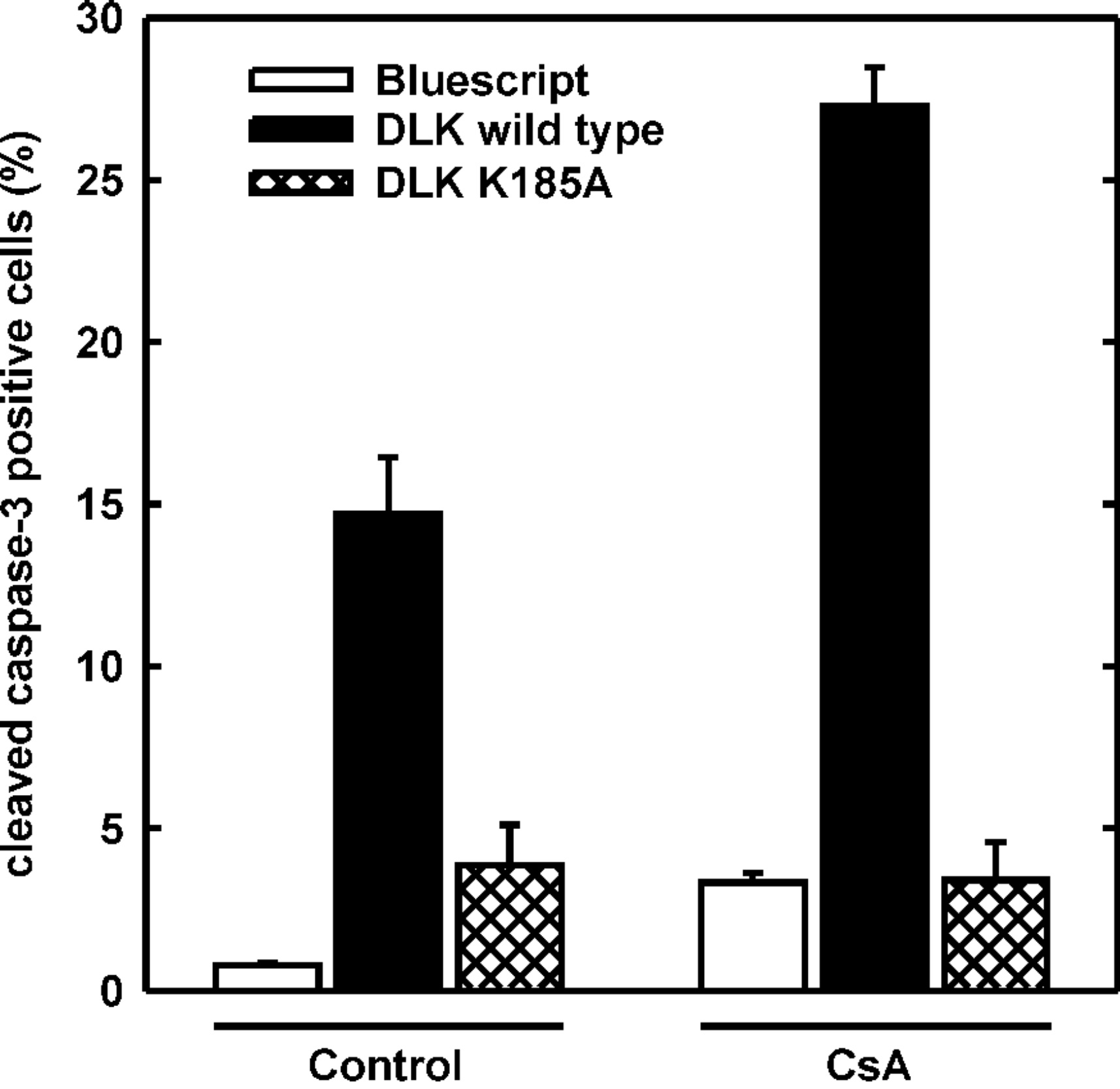

Bluescript
DLK wild type
DLK K185A
cleaved caspase-3 positive cells (%)
30
25
20
15
10
5
0
Control
CsA

Fig. 5

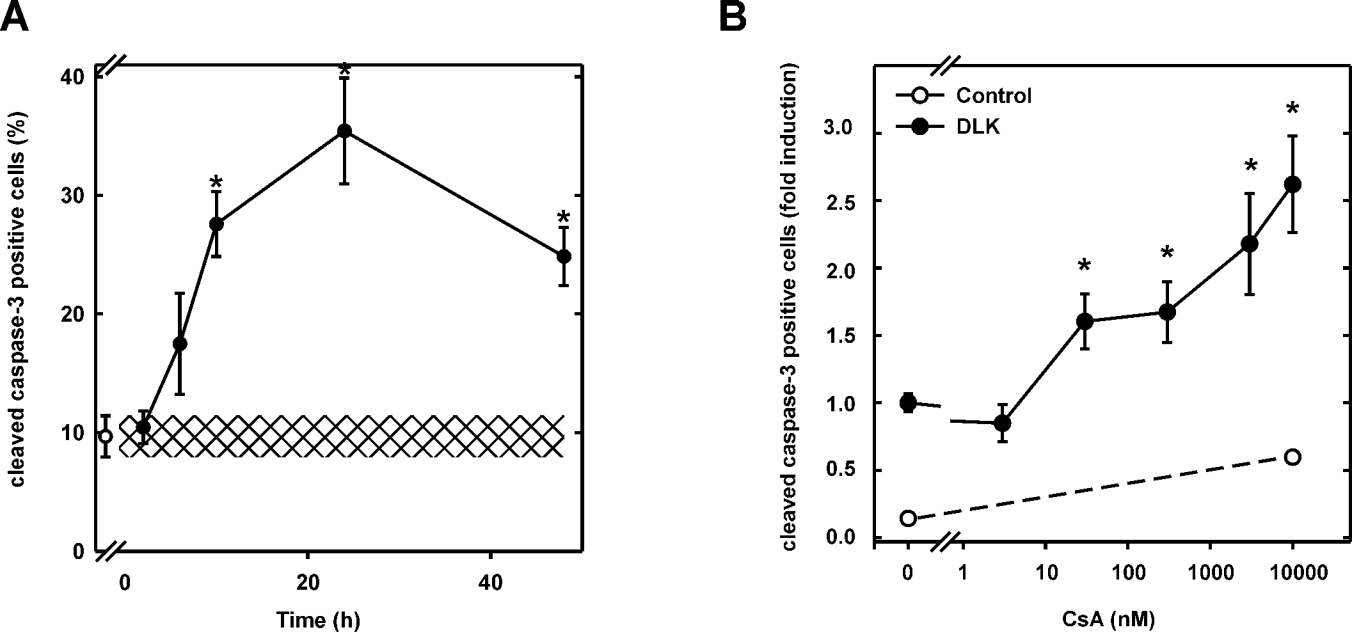

A
cleaved caspase-3 positive cells (%)
40
30
20
10
0
0
20
40
Time (h)
*
*
*
B
cleaved caspase-3 positive cells (fold induction)
Control
DLK
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
10
100
1000
10000
CsA (nM)
*
*
*
*

Fig. 6

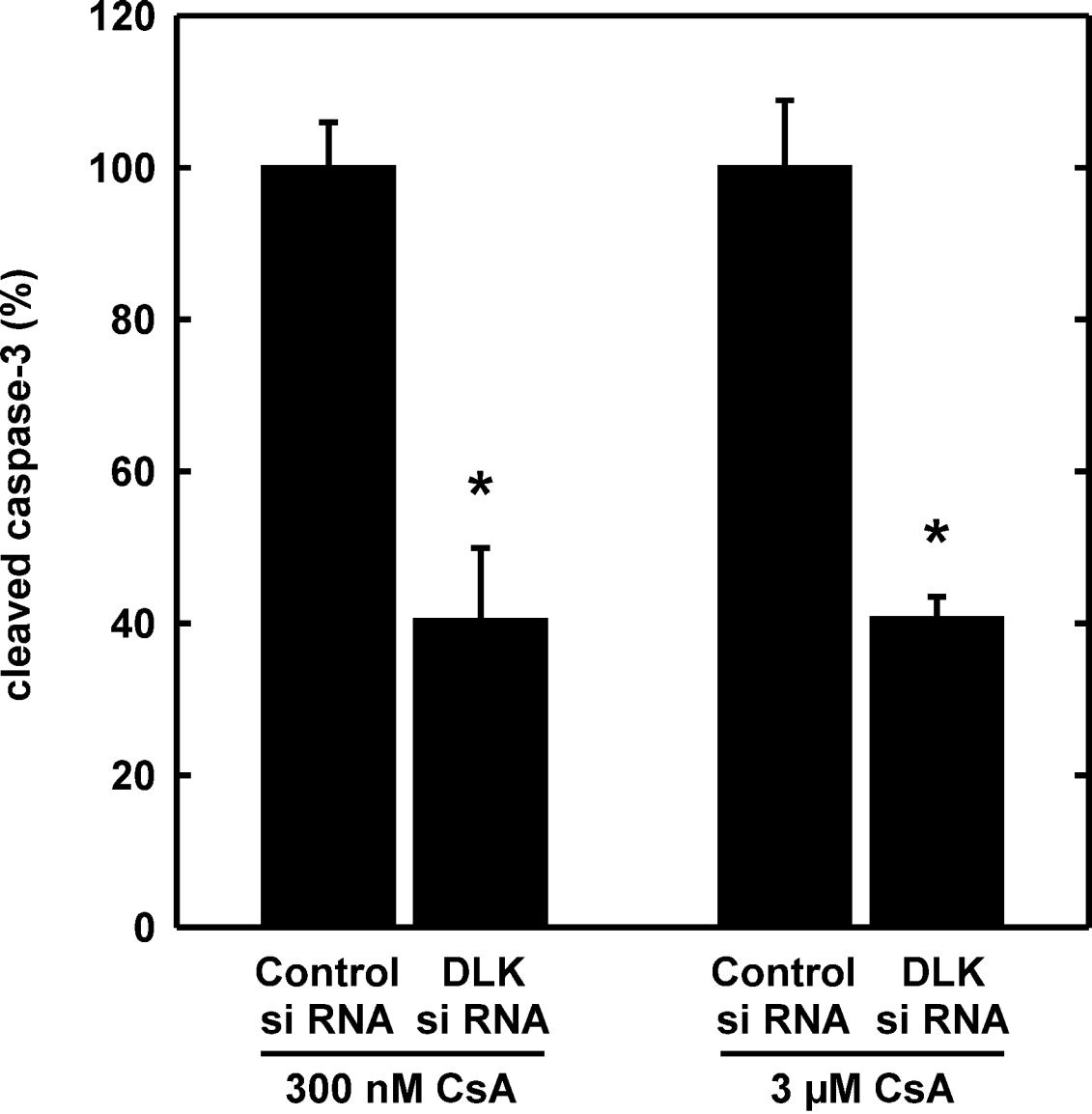

120
100
80
60
40
20
0
cleaved caspase-3 (%)
*
*
Control si RNA
DLK si RNA
Control si RNA
DLK si RNA
300 nM CsA
3 μM CsA

Fig. 7