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Anti-caspase-3 preconditioning increases proinsulin secretion and deteriorates posttransplant function of isolated human islets

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Abstract

Human islet isolation is associated with adverse conditions inducing apoptosis and necrosis. The aim of the present study was to assess whether antiapoptotic preconditioning can improve in vitro and posttransplant function of isolated human islets.

A dose-finding study demonstrated that 200 µmol/L of the caspase-3 inhibitor Ac-DEVD-CMK was most efficient to reduce the expression of activated caspase-3 in isolated human islets exposed to severe heat shock. Ac-DEVD-CMK-pretreated or sham-treated islets were transplanted into immunocompetent or immunodeficient diabetic mice and subjected to static glucose incubation to measure insulin and proinsulin secretion.

Antiapoptotic pretreatment significantly deteriorated graft function resulting in elevated nonfasting serum glucose when compared to sham-treated islets transplanted into diabetic nude mice ($p < 0.01$) and into immunocompetent mice ($p < 0.05$). Ac-DEVD-CMK pretreatment did not significantly change basal and glucose-stimulated insulin release compared to sham-treated human islets but increased the proinsulin release at high glucose concentrations (20 mM) thus reducing the insulin-to-proinsulin ratio in preconditioned islets ($p < 0.05$).

This study demonstrates that the caspase-3 inhibitor Ac-DEVD-CMK interferes with proinsulin conversion in preconditioned islets reducing their potency to cure diabetic mice. The mechanism behind this phenomenon is unclear so far but may be related to the ketone CMK linked to the Ac-DEVD molecule. Further studies are required to identify biocompatible caspase inhibitors suitable for islet preconditioning.

Keywords: Human islet transplantation, apoptosis, caspase-3 inhibitors.

Introduction

Islet transplantation has been established as a significant treatment for patients suffering from life-threatening hypoglycemic episodes (1) and has now reached equivalent function rates when compared to pancreas transplantation alone (2, 3). Nevertheless, the broad application of this treatment on diabetic patients is limited with respect to the percentage of islet preparations that have the potency to induce long-term insulin independence in recipients of islet allografts (4, 5).

The low efficiency of islet transplantation is mainly related to external variables such as ischemia (6) and immediate immune reactions in the recipients (7) but may also be explained by the intrinsic principle of enzymatic islet isolation. This process essentially involves the extraction of islets from their natural environment. After release from the acinar tissue islets are exposed to numerous insults during subsequent steps of islet production. Many of those factors are associated with the induction of apoptosis including removal of islets from the extracellular matrix (8, 9), use of hyperosmolaric media (10, 11), and incubation in a highly artificial and hypoxic environment during pretransplant culture (12, 13).

We previously demonstrated that islets preconditioning with the caspase-3 inhibitor N-acetyl-DEVD-chloromethyl ketone (Ac-DEVD-CMK) increases islet posttransplant survival when determined as recovery of porcine and human insulin after transplantation in nondiabetic mice (14). The aim of the present study was therefore to investigate the potency of Ac-DEVD-CMK to improve the functional capacity of human islets transplanted into diabetic immunodeficient or immunocompetent mice. In addition, we assessed the impact of different caspase inhibitors on glucose-stimulated insulin and proinsulin secretion.

Materials and methods

All animal studies were approved by local ethics committees.

Islet isolation

Human islets were isolated from human multiorgan donors (n = 16) as previously described (15). Purified islet fractions were pooled in CMRL 1066 (PAA, Pasching, Austria) supplemented with 10 mM HEPES, 1 mM pyruvate, 1 mM L-glutamine (PAA), 10% fetal calf serum, 100 U/µg/mL penicillin-streptomycin (Gibco, Life Technologies, Paisley, United Kingdom), and 20 µg/mL ciprofloxacin (Bayer, Leverkusen, Germany) for subsequent sampling, aliquotation and preconditioning at 37°C in humidified atmosphere (5% CO_2).

Islet characterisation

Islet yield was evaluated by converting islets to islet equivalents (IEQ) with an average diameter of 150 µm (16). After overnight incubation at 37°C quantified aliquots of preconditioned or sham-treated islets were washed twice at 1xg in CMRL 1066 and assessed for secretory capacity. Insulin content and glucose stimulated insulin release during 120 min of static incubation in CMRL 1066 containing either 2.8 or 20 mmol/L glucose (17) was measured by an enzyme immunoassay specific for human insulin (Mercodia, Uppsala, Sweden). Potential islet damage was determined simultaneously measuring proinsulin release by means of a specific enzyme immunoassay (Mercodia, Uppsala, Sweden). Viability of preconditioned and sham-treated islets was quantified by a fluorometric assay using fluorescein diacetate (Sigma, Dorset, United Kingdom) and propidium iodide (Sigma) for staining of intact and damaged islet cells, respectively (15). Apoptosis in heat-shocked islets was measured by means of fluorescence microscopy (18) utilizing a double labeling with annexin V (Pharmingen, Oxford, United Kingdom) for staining of external phosphatidylserine exposure and propidium iodide for detection of necrotic cells. Early and late apoptosis were distinguished quantifiying the percentage of annexin V-positive and propidium iodide-negative cells and double positive cells, respectively (19).

Islet antiapoptotic preconditioning

Quantified aliquots of 2000 purified IEQ with a purity ≤ 80% were incubated overnight at 37°C in culture medium supplemented with the cell-permeable and irreversible caspase-3 inhibitor Ac-DEVD-CMK (Calbiochem, Schwalbach, Germany). This inhibitor is specific for caspase-3, and to a minor degree also specific for caspase-6, -7 and -10. To identify the most efficient concentration of this compound, isolated human islets were initially exposed for 80 min to severe heat shock at 43°C prior to overnight recovery at 37°C in the presence of Ac-DEVD-CMK added in a range from 0 to 200 µmol/L. Afterwards, the expression of activated caspase-3 was detected in heat-shocked islets and visualized by means of a monoclonal rabbit-anti-human antibody (Pharmingen) utilizing Western blot technique and densitometry for quantification as previously described in detail (20). The level of caspase-3 protein expression was calculated as arbitrary units (AU) normalized to microgram sample protein. While preconditioning of transplanted islets was exclusively performed with Ac-DEVD-CMK, glucose-stimulated secretion of insulin and proinsulin was assessed after pretreatment utilizing Ac-DEVD-CMK or benzyloxycarbonyl-DEVD-fluoromethyl ketone (Z-DEVD-FMK) (Calbiochem). Sham-treated islets were incubated in culture medium only.

Islet transplantation

Diabetes was induced in male, athymic NMRI nude mice or C57/Bl6j mice (Harlan, Hanover, Germany) by a single intravenous injection of respectively 240 or 190 mg/kg streptozotocin (STZ, Sigma, Deisenhofen, Germany) four days prior to transplantation. The nonfasting serum glucose levels of all STZ-treated mice exceeded 350 mg/dL. Islets aliquoted for in vivo studies were exclusively pretreated with Ac-DEVD-CMK (14). After overnight incubation at 37°C 2000 human IEQ were washed twice at 1xg and transplanted beneath the kidney capsule of diabetic mice.

Blood samples were taken from the tail vein every other day for determination of postprandial serum glucose utilizing a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA, USA). Nonfasting serum glucose levels < 200 mg/dL were defined as normoglycemic and considered as graft function. Graft failure was defined by assessment of two subsequent serum glucose levels exceeding 200 mg/dL. Thirty-two days after transplantation nephrectomy of graft-bearing kidneys was performed to demonstrate return of hyperglycemia.

Data analysis

Analysis of matched samples was carried out by the Friedman test followed by Dunn´s test for multiple comparisons utilizing Prism software 6.0b from Graphpad. Comparison of two experimental groups were performed by the Wilcoxon or Mann-Whitney test for pairwise-matched and independent samples, respectively. Graft function calculated as time of normoglycemia was analysed utilizing the Log-rank test. Significance was expressed as P-value and considered for $p < 0.05$. $P > 0.05$ is termed nonsignificant (NS). For clarity, all values are expressed as mean ± standard error (SEM) rather than the correct non-parametric measures of median and quartiles.

Results

Effect of Ac-DEVD-CMK on terminal effectors of apoptosis

Exposure of isolated human islets for 80 min to severe heat shock at 43°C increased the expression of stress proteins such as HSP-90 and HSP-70 several-fold by 159 ± 17 and 1423 ± 423%, respectively ($p < 0.05$). As exemplarily shown in Fig. 1a, Ac-DEVD-CMK had no effect on the expression of HSP-70, indicating that this caspase inhibitor does not interfere with protein synthesis after sublethal stress. Similar observations were made for HSP-90 (data not shown).

Severe hyperthermic stress resulted in a significant increase of activated caspase-3 expression from 0.041 ± 0.017 AU/µg protein in sham-treated islets to 0.145 ± 0.078 AU/µg in heat-exposed islets equivalent to an increase of 300 ± 39% ($p < 0.05$). Supplementation with different concentrations of Ac-DEVD-CMK revealed that only a concentration of 200 µmol/L was significantly effective to reduce caspase-3 expression to a baseline level of 0.040 ± 0.019 AU/µg equivalent to a reduction of 72 ± 3% compared to heat-shocked islets not treated with Ac-DEVD-

CMK (Fig. 1b). For that reason all subsequent transplant and glucose-stimulation experiments were performed using 200 µmol/L Ac-DEVD-CMK.

Exposure of islets to severe hyperthermia increased the percentage of early apoptosis (annexin V-positive, propidium iodide-negative cells) as well as necrosis (annexin V-negative, propidium iodide-positive) from 6.3 ± 0.3% and 16.1 ± 0.4% in sham-treated islets to 21.2 ± 1.8% ($p < 0.01$) and 19.9 ± 0.6% (*ns*) after heat shock, respectively. This is equivalent to an increase of 339 ± 26% and 124 ± 3%, respectively. In consistency with the expression of activated caspase-3, it was found that a significant reduction of external phosphatidylserine exposure to baseline levels could only be obtained using a concentration of 200 µmol/L of Ac-DEVD-CMK (Fig. 2, blank bars). Simultaneously, the administration of Ac-DEVD-CMK gradually increased the proportion of necrosis in heat-shocked islets from 19.9 ± 0.6% to 24.9 ± 0.8% ($p < 0.05$) (Fig. 2, grey bars). Compared to early apoptosis or necrosis, the proportion of cells undergoing late apoptosis (annexin V-positive, propidium iodide-positive) after heat shock was on a substantially lower level (Fig. 2, black bars). Nevertheless, although the increase of late apoptosis induced by 200 µmol/L of Ac-DEVD-CMK appeared to be marginal it reached statistically relevant significance when compared to 0 µmol/L ($p < 0.01$) and 50 µmol/L ($p < 0.05$) Ac-DEVD-CMK.

Posttransplant islet function

In contrast to the initial dose-finding study, transplant experiments in diabetic nude mice revealed deterioration of graft function after islet preconditioning with Ac-DEVD-CMK. Transplantation of preconditioned human islets resulted in a significant increase of the nonfasting serum glucose (Fig. 3a). The Log-rank test revealed a significant reduction of graft function by 50% when comparing the final graft function of 44.4% in preconditioned islets with 88.9% determined in sham-treated islets ($p < 0.01$, Fig. 3b).

A similar pattern of graft survival was observed after transplantation of preconditioned islets into diabetic immunocompetent mice. Again, islet pretreatment with Ac-DEVD-CMK decreased functional potency of islet grafts resulting in a significant elevation of nonfasting serum glucose as shown in Fig. 4a. While 100% graft function was measured three days after transplantation of sham-treated islets only 50% graft survival could be measured at the same time point in mice transplanted with preconditioned islets (Fig. 4b). However, eight days posttransplant none of preconditioned grafts were surviving while 33.3% of the sham-treated grafts were still functioning (Fig. 4b, $p < 0.05$ by Log-rank test). When the experiment was terminated 14 days after transplantion one out of nine sham-treated recipients had a functioning graft.

Islet viability and in vitro function

The question whether a possible toxicity of Ac-DEVD-CMK as observed after transplantation is related to the chemical structure of the inhibitor or its associated ketone was assessed by viability staining and static glucose stimulation of human islets preconditioned by either Z-DEVD-FMK or Ac-DEVD-CMK (n = 6).

A small but nevertheless significant reduction of viability was measured in islets incubated overnight in Ac-DEVD-CMK (77.6 ± 2.6%, $p < 0.05$) in comparison to sham-treated controls (83.9 ± 0.4%) and islets pretreated with Z-DEVD-FMK (81.3 ± 1.4%, *NS* vs. sham-treatment).

The effects of the different caspase inhibitors on insulin secretion were limited except that a significant increase of basal insulin secretion was measured when isolated islets were preconditioned with Z-DEVD-FMK ($p < 0.05$, Table 1). No significant effect of preconditioning on glucose stimulation index or intracellular insulin content was observed (Table 1).

The assessment of proinsulin secretion revealed that all caspase inhibitors tested had an impact on islet insulin processing particularly during glucose stimulation. At 20 mmol/L glucose a higher proinsulin release was found in all preconditioned islets compared to sham-treated islets reaching statistical significance only after pretreatment with Ac-DEVD-CMK ($p < 0.05$ vs. sham-treated, Table 2). This resulted in a significantly higher proinsulin stimulation index in Ac-DEVD-CMK-preconditioned islets compared to sham-treated controls and Z-DEVD-FMK pretreated islets ($p < 0.05$, Table 2). In comparison to sham-treated controls, that were characterised by a significantly increased insulin-to-proinsulin ratio at 20 mmol/L glucose ($p < 0.05$ vs. basal, Table 2), the insulin-to-proinsulin ratio of Z-DEVD-FMK pretreated islets remained unchanged after switching from basal to high glucose concentration or decreased significantly in Ac-DEVD-CMK preconditioned islets ($p < 0.05$ vs. basal, Table 2).

Discussion

The number of adverse factors affecting islet integrity during pancreas processing performed for islet isolation and transplantation is substantial. The process of organ procurement is usually initiated after confirmation of brain death which induces a cascade of proinflammatory events in the pancreas and other peripheral organs (21, 22). The massive release of cytokines after brain death can simultaneously induce necrosis and apoptosis resulting in a substantial loss of islet morphological and functional integrity (23, 24). Prolonged cold ischemia is another variable that is associated with a reduction of islet viability (6, 25). It was demonstrated that the detrimental effects of brain death are aggrevated when retrieved tissue is exposed to prolonged periods of cold ischemia (26). This includes also the expression of proapoptotic key molecules such as Bax and caspases (27, 28). When islet isolation is performed, the enzymatic digestion of the pancreas results in the disconnection of islets from vasculature and the destruction of the extracellular matrix (29, 30). The disruption of vital cell-matrix interactions results in the activation of proapoptotic pathways (31, 32). During enzymatic pancreas dissociation, usually performed at 37°C, islets are

exposed to an anoxic milieu which can be regarded as warm ischemia thus rapidly depleting ATP and potentiating cellular death pathways (33). At the end of the isolation procedure, purified human islets are characterized by the overexpression of proapoptotic mediators (28, 34, 35) which may further increase during pretransplant free-floating culture (8, 36).

The caspase cascade seems to be the rate limiting step for apoptosis (37). Pretreatment of freshly isolated islets with caspase inhibitors appears as a reasonable option to optimize islet graft function after transplantation as proven in human islets preconditioned by means of the broad spectrum caspase inhibitor N-benzyloxycabonyl-Val-Asp-FMK (Z-VD-FMK) and successfully grafted in diabetic nude mice (38). Nevertheless, as anti-caspase treatment was continued in the recipients it is difficult to compare this study with the present one. Another approach, similar to the present one, demonstrated that diabetic nude mice can successfully be cured by human islets treated for two days of culture with the caspase-3 inhibitor Z-DEVD-FMK prior to transplantation (39). In contrast, a reduction in posttransplant islet function was noted by pretreating porcine islets with the caspase-3 inhibitor Ac-DEVD-CMK prior to transplantation into immunodeficient or immunocompetent diabetic mice (40). The finding of the present study that islet preconditioning with Ac-DEVD-CMK increases proinsulin release may provide an explanation for our results previously obtained in pretreated pig islets. The exact mechanism behind this phenomenon remains to be clarified but the increase of necrotic cell death that we measured in preconditioned human islets confirm previous experiments in canine and human islets revealing that treatment with caspase-3 inhibitors can cause a shift from apoptotic to necrotic cell death (41). This transformation is associated with mitochondrial dysfunction, increased formation of radical oxygen species and depletion of ATP due to increased activation of PARP, the main substrate of caspase-3 (42).

In agreement with the present study, it was previously demonstrated that an increased glucose-stimulated proinsulin release predicts human islet dysfunction after transplantation into diabetic nude mice (43). Remarkably, the magnitude of proinsulin measured after glucose challenge in our experiments reached nearly the same level that had been observed after acute islet damage caused by the instant blood mediated immune reaction (44). This finding suggests an acute toxicity of Ac-DEVD-CMK. However, the reduction of the insulin-to-proinsulin ratio during acute glucose challenge reached significance after treatment with Ac-DEVD-CMK but not with Z-DEVD-FMK which may partially explain the discrepancy between our study and the report of Nakano et al. (39). So far, we can only speculate whether the interference of Ac-DEVD-CMK with proinsulin conversion is related to its associated chloromethyl-ketone CMK as indicated by experiments in Balb/c mice treated with another CMK-linked protease inhibitor, N-alpha-tosyl-L-lysyl-chloromethyl-ketone. This compound exerted acute toxicity when mice were treated with a single injection of 59 mg/kg which is equivalent to a theoretic concentration ranging from 40 to 67 µmol/L CMK (45). The proportion of CMK that was used in the present study corresponds to a concentration of 34 µmol/L which is within the upper range for chronic effects. The CMK-related toxicity can be partially

explained by its glutathione-depleting effect found in different tissues (46). This may be of particular importance for islets which are characterized by a specifically low radical-scavenging capacity (47, 48) simultaneously suffering from proinflammatory conditions as a consequence of effective caspase-3 inhibition (42).

Based on the present findings we conclude that human islet antiapoptotic preconditioning utilizing Ac-DEVD-CMK reduces the metabolic potency of pretreated islets to cure diabetic mice. The deterioration of graft function is associated with reduced viability and enhanced release of proinsulin as confirmed by glucose stimulation in vitro. The mechanisms behind this phenomenon are not fully understood but appeared to be partially related to the associated chloromethyl-ketone CMK. Nevertheless, further studies are required to clarify the proinflammatory effects of Ac-DEVD-CMK and to identify biocompatible caspase inhibitors suitable for islet preconditioning.

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Tables

Table 1 Effect of DEVD-based caspase-3 Inhibitors on glucose-stimulated insulin release and intracellular insulin content

	Insulin Release (pmol/IEQ/120 min)		Stimulation Index	Insulin Content (pmol/IEQ)
	2.8 mM Glucose	20 mM Glucose		
Sham-treated	0.056 ± 0.012	0.185 ± 0.028[a]	3.83 ± 0.79	2.07 ± 0.46
Z-DEVD-FMK	0.114 ± 0.026[b]	0.267 ± 0.034[a]	2.71 ± 0.43	1.47 ± 0.40
Ac-DEVD-CMK	0.081 ± 0.018	0.196 ± 0.035[a]	2.69 ± 0.26	1.83 ± 0.42

[a]$p < 0.05$ vs. basal insulin release (Wilcoxon test); [b]$p < 0.05$ vs. sham-treated controls. Data are expressed as means ± SEM of six individual experiments.

Table 2 Effect of DEVD-based caspase-3 inhibitors on glucose-stimulated proinsulin release and insulin-t proinsulin ratio

	Proinsulin Release (pmol/IEQ/120 min)			Insulin/Proinsulin Ratio	
	2.8 mM Glucose	20 mM Glucose	Stimulation Index	2.8 mM Glucose	20 mM Glucose
Sham-treated	0.0023 ± 0.0002	0.0039 ± 0.0004[a]	1.74 ± 0.15[c]	27.4 ± 9.1	48.5 ± 8.8[a,c]
Z-DEVD-FMK	0.0045 ± 0.0012	0.0122 ± 0.0052[a]	2.47 ± 0.41[c]	29.5 ± 6.2	36.6 ± 8.3
Ac-DEVD-CMK	0.0028 ± 0.0004	0.0131 ± 0.0021[a,b]	4.58 ± 0.41	30.5 ± 8.6	15.4 ± 2.8[a]

[a]$p < 0.05$ vs. basal release (Wilcoxon test); [b]$p < 0.05$ vs. sham-treated controls; [c]$p < 0.05$ vs. Ac-DEVD-CMK.

Data are expressed as means ± SEM of six individual experiments.
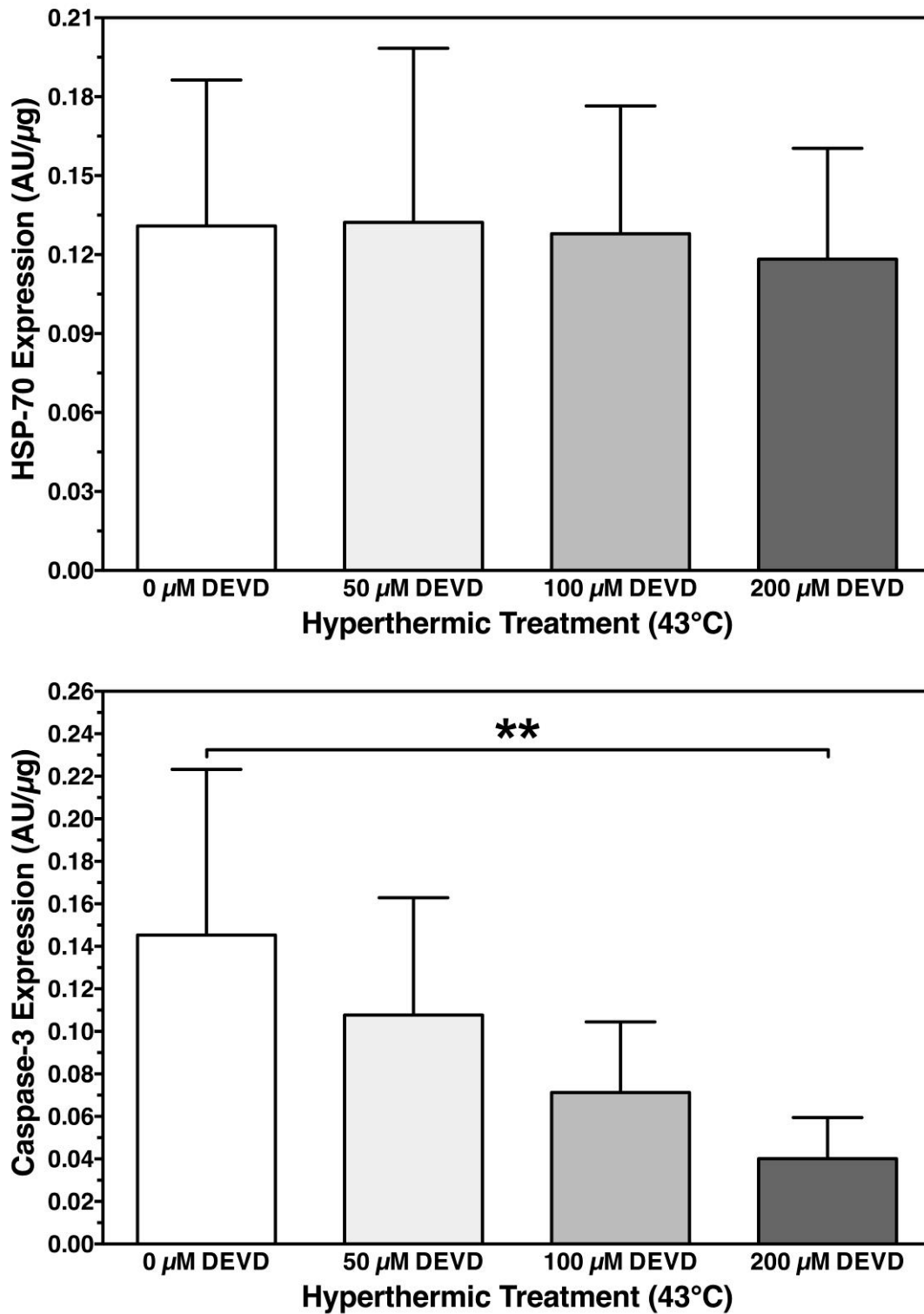
Figure legends

Fig. 1 Protein expression of **(a)** HSP-70 and **(b)** activated caspase-3 in isolated human islets exposed for 80 min to severe heat shock at 43°C prior to overnight recovery at 37°C in the presence of different concentrations of Ac-DEVD-CMK. Statistical analysis revealed ****$p < 0.01$** comparing caspase-3 protein expression at 0 µmol/L and 200 µmol/L Ac-DEVD-CMK as indicated in **(b)**. Data are expressed as means ± SEM of islet isolations from five different donors.
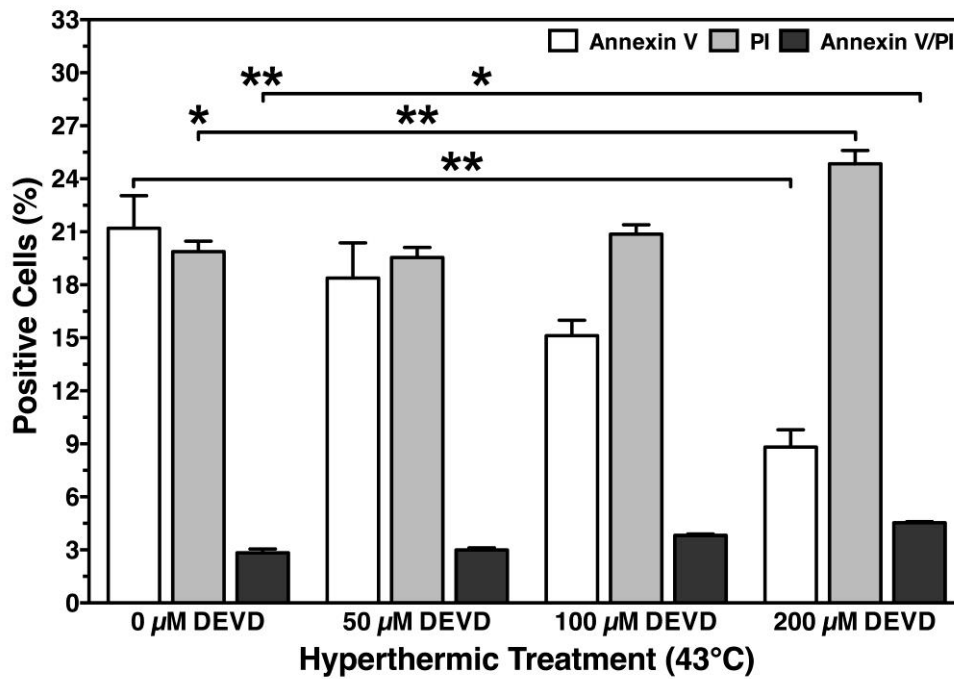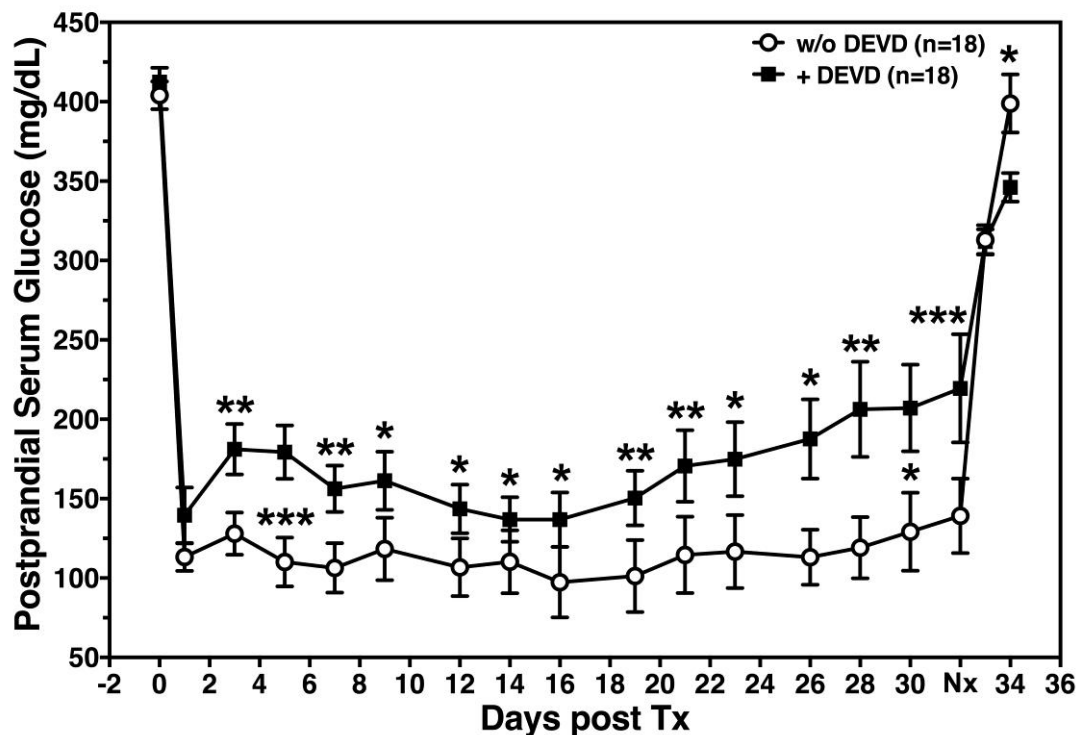
Fig. 2 Percentage of annexin V-positive (blank bars), propidium iodide-positive (grey bars) and double positive (black bars) cells representing respectively early apoptotic, necrotic and late apoptotic cells in isolated human islets exposed for 80 min to severe heat shock at 43°C prior to overnight recovery at 37°C in the presence of different concentrations of Ac-DEVD-CMK. Statistical analysis revealed *$p < 0.05$ and **$p < 0.01$ comparing proportion of early apoptotic, necrotic or late apoptotic cells after treatment with 0 µmol/L and 50 µmol/L versus 200 µmol/L Ac-DEVD-CMK. Data are expressed as means ± SEM of islet isolations from six different donors.
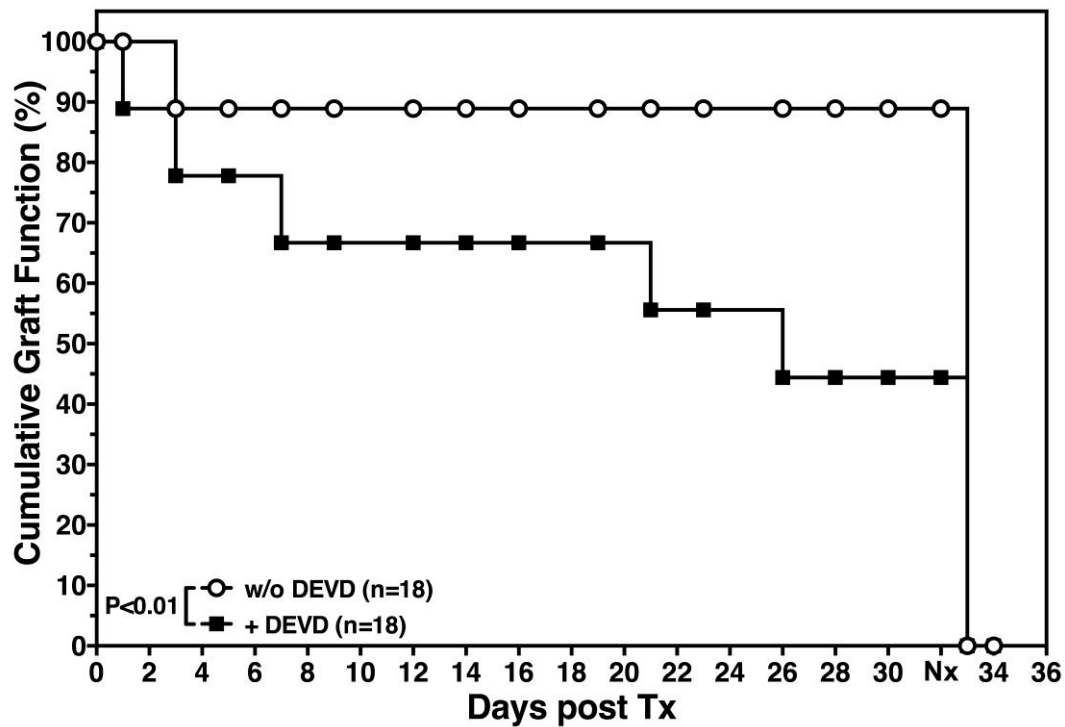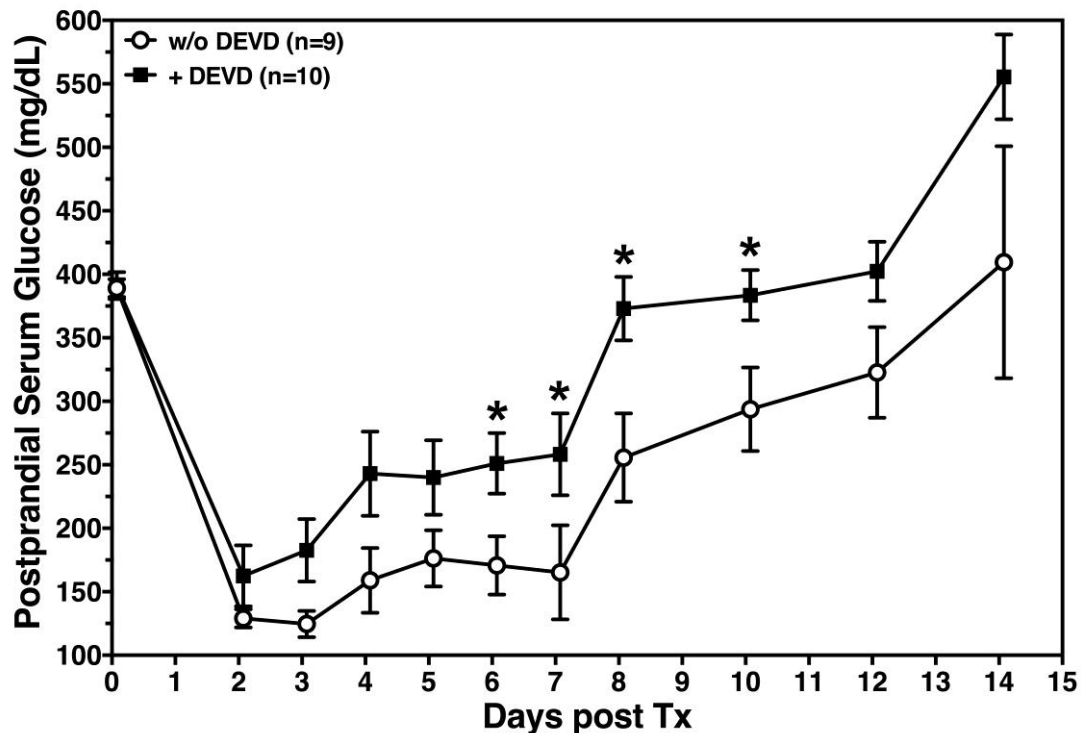
Fig. 3 Postprandial serum glucose levels **(a)** and cumulative graft function **(b)** of sham-treated (blank circles) or Ac-DEVD-CMK-pretreated (black squares) human islets transplanted beneath the kidney capsule of diabetic NMRI nude mice. Graft nephrectomy (Nx) was performed 32 days post transplantation. Statistical analysis by Mann-Whitney test revealed ***p < 0.001, **p < 0.01* and *p < 0.05* comparing serum glucose levels by day. Data are expressed as means ± SEM of islet isolations from four different donors **(a).** Analysis of cumulative graft function by Log-rank test revealed *p < 0.01* as indicated in **(b)**.
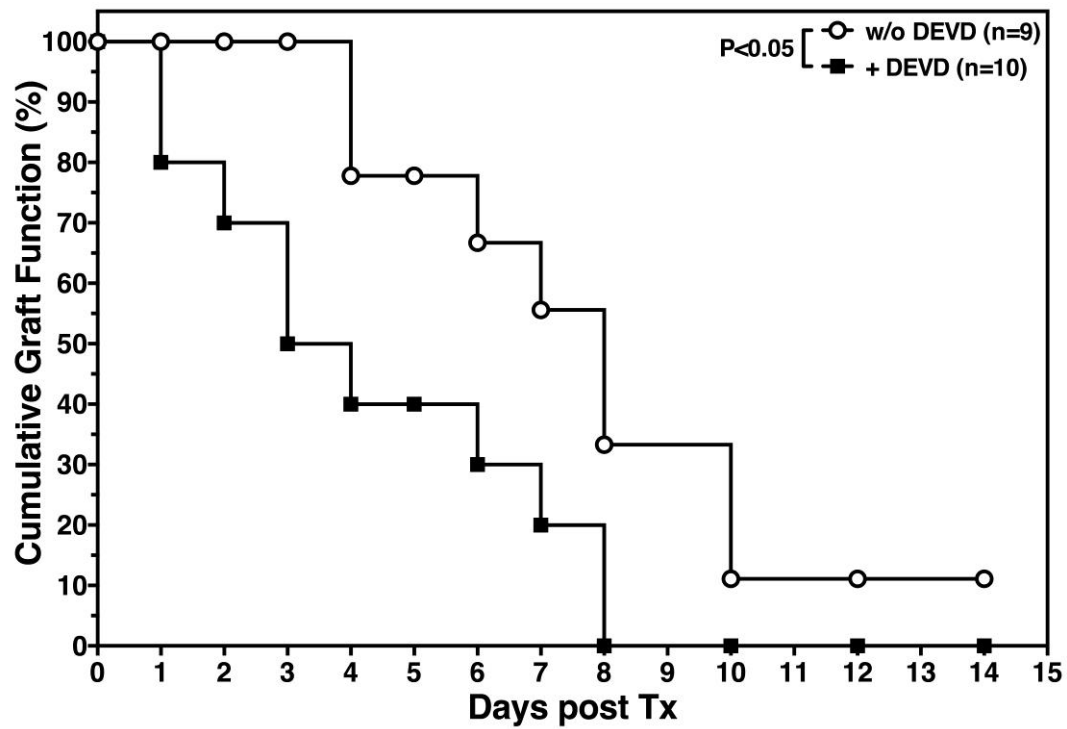
Fig. 4 Postprandial serum glucose levels **(a)** and cumulative graft function **(b)** of sham-treated (blank circles) or Ac-DEVD-CMK-pretreated (black squares) human islets transplanted beneath the kidney capsule of diabetic C57/Bl6j mice. Statistical analysis by Mann-Whitney test revealed *$p < 0.05$* comparing serum glucose levels by day. Data are expressed as means ± SEM of islet isolations from three different donors **(a).** Analysis of cumulative graft function by Log-rank test revealed *$p < 0.05$* as indicated in **(b)**.