Expression of a potent anti-apoptotic protein in MIN6 β-cells using METAFECTENE PRO

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Introduction:

Diabetes refers to a group of serious, chronic metabolic diseases requiring long-term medical attention and affecting 4% to 5% of the global population (King et *al.*, 1998). The number of people affected with diabetes in 134 monitored countries in 2000 was 151 million (Data obtained from "Diabetes Atlas 2000", International Diabetes Federation), a number which is likely to be closer to 177 million world wide according to the World Health Organization. Every year, there are over 800,000 deaths directly due to diabetes, and 4 million more which can be attributed to various complications related to this metabolic disorder.

Type 2 diabetes is the most common form of diabetes is (~95%) and the risk of developing it is known to be driven by genetic and environmental factors including age, geographical area of residence, ethnicity, lifestyle and obesity. The progression of type 2 diabetes is associated with a decrease in β-cell mass (Weir and Bonner-Weir, 2004) which is essentially a consequence of increased apoptosis and/or a decreased proliferation. Therefore, it is clear that the identification of novel molecular targets to enhance β-cell proliferation and/or to protect β-cells from apoptotic signals is of high interest since this could have major therapeutic implications for type 2 diabetes through preventing the progressive loss of β-cell mass. In this regard, we recently identified one factor (called "Fx" in this report for intellectual property purposes) that has been previously shown to regulate apoptosis and proliferation in various cell types. However, nothing is known about its role and function in pancreatic β-cells. Therefore, to determine whether Fx plays a critical role in the regulation of apoptosis in β-cells, we monitored the effect of Fx expression on cis/platinum-induced caspase-3 activation using the insulinoma MIN6 cell line. Transfection of this hard-to-transfect cell line was established using the novel transfection reagent METAFECTENE PRO.

Materials and methods:

Materials: METAFECTENE PRO was obtained from Biontex Laboratories GmbH (Martinsried, Germany). MIN6 cells were a kind gift from Dr. Y. Oka and Professor J.-I. Miyazaki (then at University of Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), L-glutamine, penicillin/streptomycin and N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (DEVD) were purchased from Sigma Aldrich (Dorset, U.K.). Fetal bovine serum (FBS) was from Invitrogen (Paisley, UK), and cis/diaminedichloroplatinum (cis/platinum) was from Merck Biosciences (Nottingham, UK).

Cell culture: MIN6 β-cells were maintained in culture at 37°C in DMEM (25mM glucose) supplemented with 2mM glutamine, 10% FBS, 100 units/ml penicillin and 100µg/ml streptomycin.

Caspase-3 apoptosis assay: The assay was performed as previously reported (Muller et al, 2006). Briefly, MIN6 cells were lysed, the protein content was quantified by the Bradford assay and caspase-3 activity was measured using 100µg total protein by spectrophotometric (405nm) detection of cleavage of the chromophore *p*-nitroanilide from the substrate DEVD.

Experimental procedures / transfection protocol:

Transfection: MIN6 cells (~500,000 cells) were seeded to 60mm tissue culture dishes and maintained in culture in 4ml DMEM (10% FBS) for 3 to 4 days, after which a confluence of ~60-70% was reached. Each transfection was performed as described by the manufacturer (Biontex) except that antibiotics were kept in the medium. Briefly, MIN6 cells were washed once with PBS and maintained in culture with 3 ml serum-free DMEM during the preparation of the DNA/METAFECTENE PRO complexes (~30 minutes). MIN6 cells were then subjected to transfection using 4 μg pEGFP-C2/Fx to assess the transfection efficiency or 4 μg pcDNA3/Fx to study the impact of Fx on cis/platinum-induced apoptosis. Eight hours after transfection the medium was replaced with fresh DMEM supplemented with 10% FBS.

Apoptosis assay: to study the effect of Fx on apoptosis, we used a model in which caspase-3 activation was triggered by the use of cis/platinum. In brief, MIN6 cells were washed once with 4 ml PBS and then maintained in culture overnight in 3 ml DMEM (25mM glucose + 10% FBS) in the absence or presence of 100μM cis-platinum (dissolved in DMSO). The role of Fx in the regulation of apoptosis was assessed by comparing the effect of Fx over-expression on caspase-3 activity in MIN6 cells. To eliminate the possibility that caspase-3 activity could be altered by the transfection procedure, control cells were subjected to transfection using 4 μg of the corresponding empty pcDNA3 plasmid.

Results and discussion:

METAFECTENE PRO efficiently transfects MIN6 cells

To assess the transfection efficiency of METAFECTENE PRO and determine the appropriate DNA:METAFECTENE PRO ratio that generates the highest rate of transfection in MIN6 cells, we tested 3 different ratios using 4 μg of purified pEGFP-C2/Fx. As shown in Figure 1 upper panel, a 1:1 pEGFP-C2/Fx:METAFECTENE PRO ratio gave the best results with a transfection efficiency of approximately 40-50%. Such a high level of lipid-mediated transfection in MIN6 cells has never been obtained previously in our laboratory and is comparable to data obtained when transfection was performed by electroporation. Electroporation is however more stringent and generates a high level of cell death (personal observation). Finally, it is worth noting that increasing the pEGFP-C2/Fx:METAFECTENE PRO ratio from 1:1 to 1:3 did not further improve the reagent's efficiency using the MIN6 cell line.

A two-rounds transfection strategy improves METAFECTENE PRO efficiency

Since no obvious cell death could be observed after transfection, we then tested whether we could further improve the transfection efficiency by performing a second round of transfection using the experimental conditions that were used during the first round. Interestingly, carrying out a second round of transfection 24 hours after the first transfection protocol resulted in a clear increase in the number of EGFP positive cells (figure 1 lower panel). Again, this effect was apparently not associated with any increase in cell death. Counting the EGFP/Fx-positive cells showed that the efficiency of transfection was increased to approximately 90% at a 1:1 pEGFP-C2/Fx:METAFECTENE PRO ratio.

METAFECTENE PRO does not trigger apoptosis and Fx reduces cis/platinum-induced caspase-3 activation

Functional assessment of the role of Fx in β-cell apoptosis was determined using MIN6 cells transfected with pcDNA3/Fx by the two rounds transfection protocol. As shown in Figure 2, over-expression of Fx in MIN6 cells significantly reduced cis/platinum-induced caspase-3 activity by ~45% (lanes 3 and 6). Once again, it is also important to note that the two-rounds transfection procedure involving METAFECTENE PRO did not, by itself, trigger apoptosis (lanes 2 and 5).

Conclusion / summary:

Using METAFECTENE PRO, we obtained a high efficiency of transfection, no apparent cell death and no apoptosis. This enabled us to carry out a preliminary characterization of the role played by Fx in the regulation of apoptosis in MIN6 β-cells.

References:

Diabetes Atlas 2000, International Diabetes Federation. Brussels, IDF, July 2000.

King H, Aubert RE and Herman WH, Diabetes Care, 1998, 21:1414-1431

Muller D, Jones PM and Persaud SJ, FEBS Letter, 2006, 580:6977-6980

Weir GC and Bonner-Weir S, Diabetes, 2004, 53:S16-S210

Appendix: Tables and/or figures:

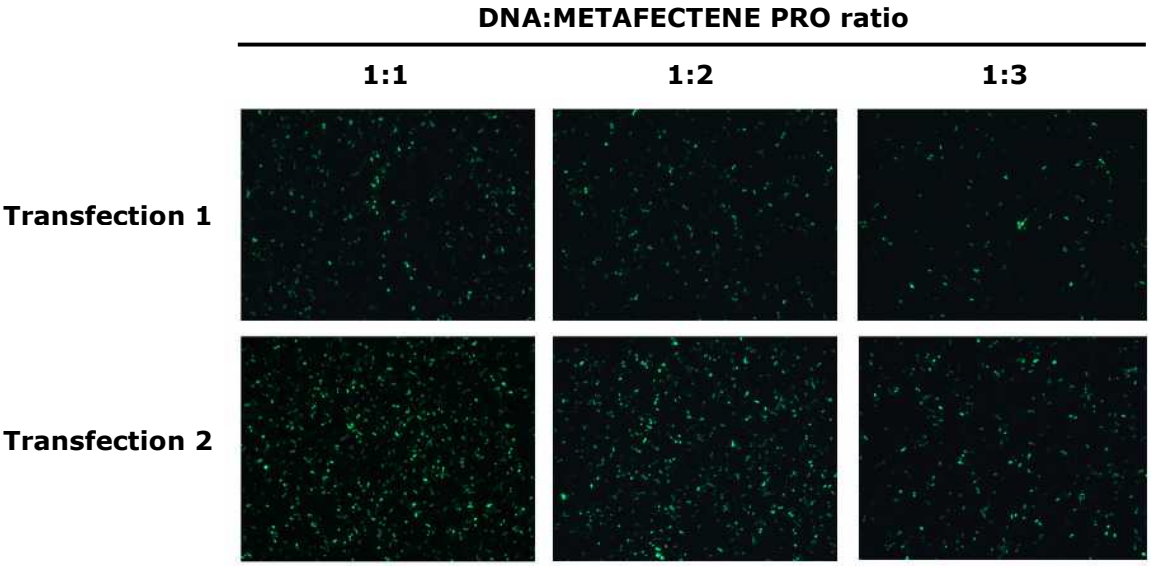

Figure 1: Transfection efficiency of METAFECTENE PRO. MIN6 cells were subjected to transfection using 4 µg pEGFP-C2/Fx and 4 µl (ratio 1:1), 8 µl (ratio 1:2) or 12 µl (ratio 1:3) METAFECTENE PRO. The transfection procedure was performed either once (Transfection 1; upper panels) or twice (Transfection 2; lower panels) with a 24 h interval. The medium was replaced with fresh DMEM (25mM glucose + 10% FBS) 8 h after transfection and the transfection efficiency was monitored 24 h later using a Nikon Eclipse TE2000-U inverse epifluorescence microscope.

pcDNA3:METAFECTENE PRO	–	+	+	–	–	–
pcDNA3/Px:METAFECTENE PRO	–	–	–	–	+	+
100 μM cis/platinum	–	–	+	–	–	+

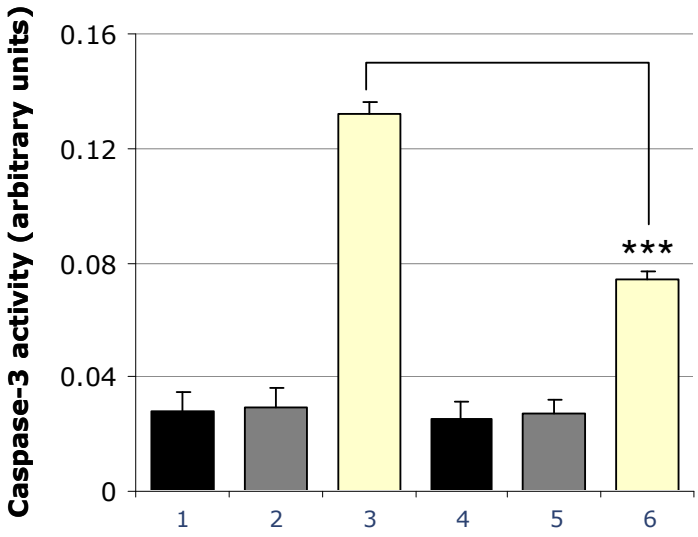

Figure 2: Effect of pcDNA3/Fx on apoptosis using METAFECTENE PRO. MIN6 cells were not transfected (lanes 1 and 4) or subjected to two rounds of transfection using 4 μg pcDNA3 (lanes 2 and 3) or pcDNA3/Fx (lanes 5 and 6) and 4 μl METAFECTENE PRO (ratio 1:1). Then, 24 h after the second transfection, the cells were treated either with 100μM cis/platinum (lanes 3 and 6) or with the same final vehicle concentration of DMSO (lanes 1, 2, 4 and 5) in the presence of 10% FBS and 25mM glucose. Caspase-3 activity was determined 16 h after the addition of cis/platinum to the medium. Results are means + SEM (n = 3) and are representative of two independent experiments (***$p<0.01$).