Processing of pro-islet amyloid polypeptide (proIAPP) by the prohormone convertase PC2

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Abstract Islet amyloid polypeptide (IAPP), 'amylin', is the component peptide of islet amyloid formed in Type 2 diabetes. IAPP is expressed in islet β-cells and is derived from a larger precursor, proIAPP, by proteolysis. An in vitro translation/translocation system was used to separately examine processing of human proIAPP by the β-cell endopeptidases PC2, PC3 or furin. ProIAPP was converted to mature IAPP by PC2 but there was little conversion by furin or PC3. These data are consistent with processing of proIAPP in β-cell secretory granules. Abnormal cellular proteolysis associated with type 2 diabetes could contribute to IAPP amyloidosis.

Key words: Islet amyloid polypeptide; PC2; PC3; Furin; β-Cell; Propeptide processing

1. Introduction

Islet amyloid polypeptide (IAPP), 'amylin', is a component peptide of amyloid fibrils deposited in islets of type 2 diabetic patients [1,2]. IAPP is colocalised with insulin in the secretory granules of islet β-cells and co-secreted in response to β-cell secretagogues [3,4]. IAPP has been identified in all species examined so far [5] and, in common with other secretory peptides, is synthesized as a larger precursor molecule. ProIAPP (67 amino acids in man) is converted to the mature form (37 amino acids) by removal of C- and an N-terminal flanking peptides [6,7]. Both cleavage sites are conserved in mammals and contain pairs of basic amino acids, a common recognition motif for the subtilisin-like family of prohormone convertases [8].

Several members of the prohormone convertase family have been described including PC2, PC3 (also known as PC1), PACE4, furin, PC4, PC5 and PC5/6B [9]. PC2 and PC3 are expressed in neuroendocrine cells where they are involved in the processing of prohormones. PACE4 and furin are expressed in a wide range of cell types where they are thought to cleave propolypeptides within the constitutive secretory pathway. PC4 is expressed in the testis. The expression of PC5 is widespread, but its differential splice product PC5/6B is expressed mainly in the intestine [9].

The subtilisin-like proteases are synthesized as zymogens which undergo maturation during their transit through the secretory apparatus. PC3 is synthesized as an 88 kDa propolypeptide which undergoes rapid autocatalytic maturation under optimal conditions of neutral pH and low calcium, compatible with the milieu within the endoplasmic reticulum [10]. PC2 is synthesized as a 75 kDa propolypeptide which is cleaved to a 68 kDa mature enzyme by a mechanism which occurs at pH 5.5 and millimolar calcium, and which is also thought to be autocatalytic [11]. This maturation event is likely to occur within the later stages of the secretory pathway. Furin is synthesized as a 90 kDa polypeptide which is cleaved to the 80 kDa mature enzyme, an integral membrane protein located within the trans Golgi network [12]. PC2, PC3 and furin are calcium-dependent proteases which are optimally active at acidic (PC2, PC3) or neutral pH (furin).

The endoprotease(s) responsible for the processing of proIAPP is unknown. The fact that IAPP is co-localized with insulin in β-cell granules, and human proinsulin is processed by the combined action of PC2 and PC3 [13] suggests that these enzymes might be involved in proIAPP processing. Alternatively, proIAPP may be processed in a separate compartment, possibly by furin.

The aim of the present study was to determine whether these enzymes were involved in the processing of proIAPP to IAPP. An in vitro transcription translation system derived from *Xenopus* eggs was used [14].

2. Materials and methods

The human IAPP cDNA was obtained from Dr. J.S. Verbeek (University of Utrecht) [15], the human furin cDNA from Dr. G. Matthews (University of Birmingham), and the human PC2 and mouse PC3 cDNAs were from Dr. D.F. Steiner (University of Chicago). cRNAs were prepared using SP6 RNA polymerase in an in vitro transcription system [16]. The preparation and use of the *Xenopus* egg extract translation system was as previously described [14]. Products of translation were radiolabelled using [^{3}H]leucine since mature IAPP lacks methionine residues. Translations were terminated after 2 h by addition of 1 μl of RNase A (10 mg/ml). Further incubations were optimised to ensure activation of the proenzymes and provide optimal conditions for activity against proIAPP. For PC3, incubations were allowed to proceed for a further 4 h to allow for maturation of proPC3 (pH 7.0, 1 mM calcium). The pH was then adjusted to 5.5 and the calcium concentration to 10 mM, and the samples incubated overnight. For PC2, the samples were immediately adjusted to pH 5.5 and 10 mM calcium and the samples incubated overnight. For furin the samples were incubated overnight without any additions, i.e. pH 7.0 and 1 mM calcium. Samples were then analysed by SDS-PAGE on either 10% polyacrylamide, Tris/glycine-buffered (high-M_r; 200–20 kDa) or 13% polyacrylamide, Tris/tricine-buffered (low-M_r, 50–2.5 kDa) gels. The use of two gels permitted the visualisation of the high-M_r enzymes and their precursors in addition to the low-M_r IAPP molecules. These gels were subsequently processed for fluorography.

3. Results

3.1. Translation of IAPP and furin mRNA

Furin mRNA was translated in the *Xenopus* egg extract and the radiolabelled products analysed on high and low molecular

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	N-IAPP	Islet amyloid polypeptide	C-IAPP
		1 37	
Human	TPIES:::HQVEKR	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY	GKRNAVEVLKREPLNYLPL
Monkey	-----:::------	----------R------T---------D--	----------
Cat	-----:::N-----	----------IR----L-----P--------	---ST-DI-N--------F
Dog	---K-:::--M---	----------RT---L-----P--------	----TI-I-N-G-------
Rat	--VG-GTNP--D--	----------R----L-PV-PP--------	----VA-DPN--S-DF-L-
Mouse	--VR-GSNP-MD--	----------R----L-PV-PP--------	-----AGDPN--S-DF-KV

Fig. 1. Amino acid sequence of proIAPP in 6 mammals. The sequence IAPP 20–29 is conserved in those species in which the molecule forms amyloid fibrils but in rodents there are three proline substitutions. Cleavage sites between the N-(N-IAPP) and C-terminal (C-IAPP) flanking peptides are highly conserved as Lys/Arg. A third dibasic site is present in primate C-IAPP.

weight SDS gels. Two proteins of M_r 100 and 90 kDa were observed following a 2 h translation period (Fig. 2, upper panel). When the translation reaction was allowed to continue overnight, two proteins of 90 kDa and 80 kDa were observed. On the low molecular weight gel, a single band at M_r 10K was present after 2 h translation which increased in intensity with overnight incubation (Fig. 2, lower panel). These results represent the processing of profurin (100 kDa) to 90 kDa and 80 kDa mature forms of the enzyme. The 100 kDa to 90 kDa processing event is consistent with N-terminal cleavage of profurin and the 90 kDa to 80 kDa step could represent either additional N-terminal or C-terminal processing. Other proteins of M_r 18K, and at 12K were visible but these were present in the absence of added mRNA and are likely to be low molecular weight proteins synthesized in the extract which are unrelated to the enzymes or IAPP.

Translation of hIAPP mRNA resulted in the appearance of a 7.5 kDa proIAPP peptide (Fig. 2, lower panel). Coincubation overnight with furin did not alter the intensity of the 7.5 kDa band. There was a small amount of processing to the 4 kDa

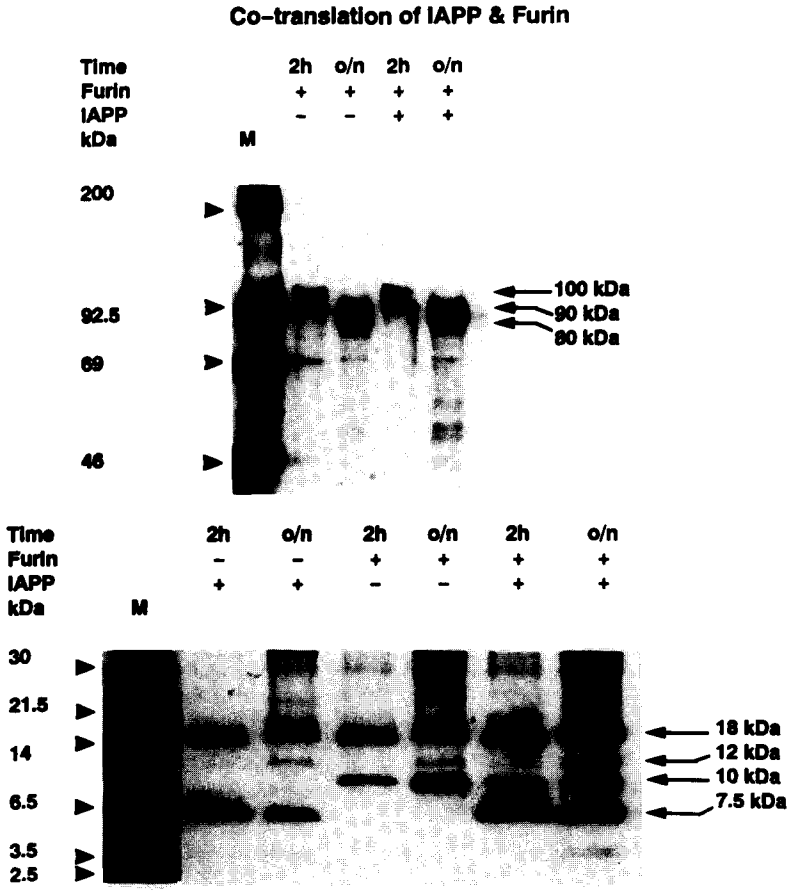

Fig. 2. Co-translation of IAPP and furin cRNAs. IAPP and furin cRNAs were translated in the *Xenopus* egg extract in the combinations indicated. After incubation for 2 h or overnight samples were analysed by Tris/SDS-PAGE and fluorography (upper panel) and Tricine SDS-PAGE and fluorography (lower panel). Position of molecular weight markers (with corresponding M_r in kDa) are indicated on the left.

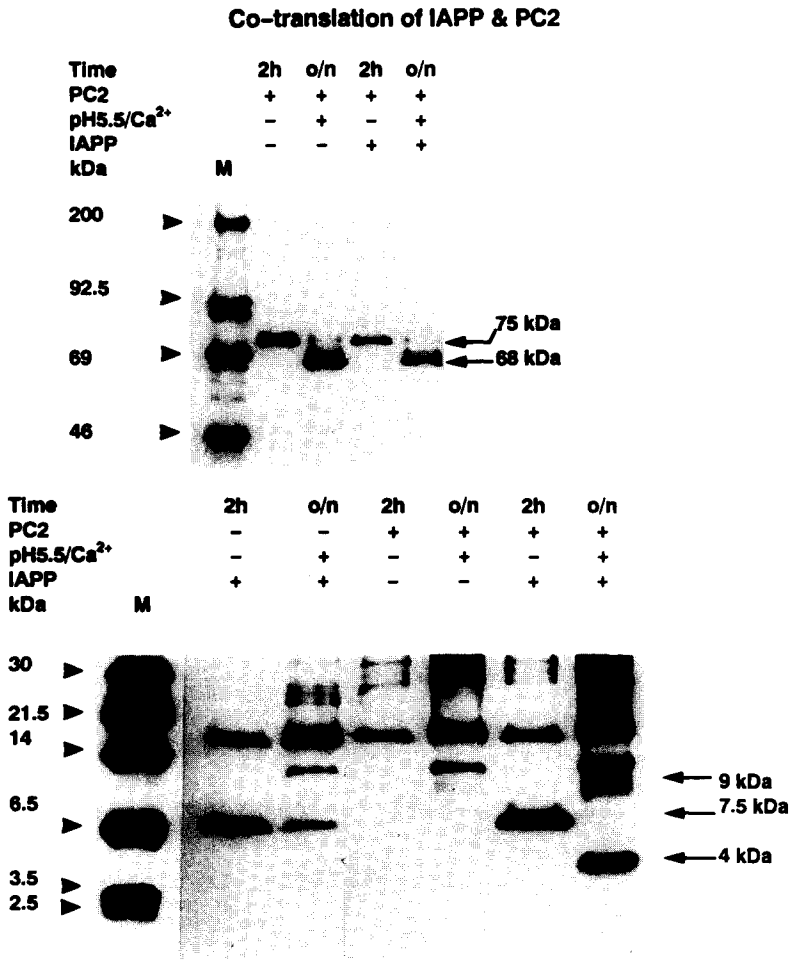

Fig. 3. Co-translation of IAPP and PC2 cRNAs. IAPP and PC2 cRNAs were translated as indicated in the *Xenopus* egg extract. After 2 h the extract was adjusted to pH 5.5 and 10 mM calcium and incubation was continued overnight. Samples were and analysed by Tris/SDS-PAGE and fluorography (upper panel) and Tricine SDS-PAGE and fluorography (lower panel). Position of molecular weight markers (with corresponding M_r in kDa) are indicated on the left.

mature form of iAPP but this was also seen in the absence of furin (Fig. 2, lower panel) and was probably due to endogenous proteolytic activity within the egg extract.

3.2. Translation of PC2 and IAPP mRNA

Translation of PC2 mRNA for 2 h generated a single product of 75 kDa (Fig. 3, upper panel), which corresponds to proPC2. Following an overnight incubation at pH 5.5 and 10 mM calcium, the 75 kDa protein was processed to a 68 kDa protein. These results are consistent with our previous findings that proPC2 undergoes autocatalytic maturation to the 68 kDa mature form of the enzyme under these conditions [11]. On the low molecular weight gel the cleaved proPC2 propeptide was visible as a diffuse band of approximately M_r 9 kDa (Fig. 3, lower panel). Coincubation of PC2 and hIAPP overnight resulted in a clear processing of proIAPP (7.5 kDa) to mature IAPP (4 kDa).

3.3. Translation of PC3 and IAPP mRNA

As previously described [10], proPC3 (88 kDa) was rapidly converted to the 80 kDa mature enzyme in a reaction that was optimal at neutral pH and low calcium (Fig. 4, upper panel). After 6 h, when almost all of the PC3 was in the mature form, conditions were changed to suit the optimal activity of the mature enzyme, i.e. pH 5.5 and 10 mM calcium. Under these conditions the 80 kDa mature enzyme was stable (Fig. 4, upper panel). However, the 8 kDa cleavage fragment of proPC3 (visible on the low molecular weight gel as a band at 8–10 kDa) underwent further cleavage to a 4 kDa peptide (Fig. 4, lower panel). When PC3 and hIAPP mRNA were coincubated overnight, the 7.5 kDa proIAPP was unaffected. Although, any mature IAPP would have been masked by the presence of the 4 kDa product of the PC3 propetide there was no reduction of the intensity of the proIAPP band in the presence of PC3 (Fig. 4, lower panel).

4. Discussion

The major finding of this study was that proIAPP (7.5 kDa) was converted to a mature 4 kDa product by PC2 but not, or at least relatively inefficiently, by PC3 or furin. Processing at the C-terminal junction therefore involves cleavage by PC2

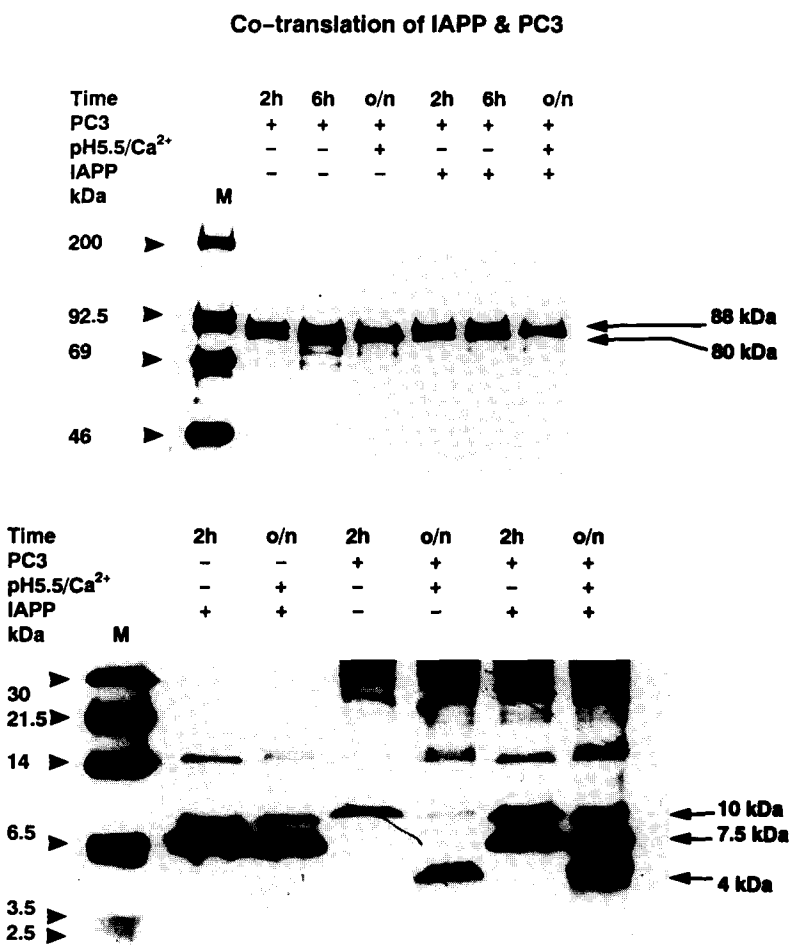

Fig. 4. Co-translation of IAPP and PC3 cRNAs. IAPP and PC3 cRNAs were translated as indicated in the *Xenopus* egg extract. After 2 h and 6 h incubation, samples were taken for analysis and the rest of the extract was adjusted to pH 5.5 and 10 mM calcium. Incubation was then continued overnight. Samples were analysed by Tris/SDS-PAGE and fluorography (upper panel) and Tricine SDS-PAGE and fluorography. Position of molecular weight markers (with corresponding M_r in kDa) are indicated on the left.

between the R and N residues within the sequence TYGKRNAV. At the N-terminal junction, cleavage occurs between the R and K residues within the sequence VEKRKCNT. The C-terminal cleavage site is similar to other PC2 recognition sequences [17], whereas the cleavage between basic pairs of residues that occurs at the N-terminal site is not a common PC2 recognition site. The finding that PC2 rather than furin is involved in proIAPP processing suggests that these events occur within a secretory granule compartment where the pH and calcium concentration are compatible with the requirements for PC2 enzyme activity.

Cleavage of human proinsulin requires the combined activity of both PC2 and PC3 [10]. It is first cleaved by PC3 at the B chain/C peptide junction ($KTRR^{32}$) followed by PC2 at the C peptide/A chain junction ($LQKR^{65}$). Proglucagon on the hand is processed in the α cells solely by PC2 [18]. Interestingly, a patient with a primary defect of PC3 activity has been described [19]. This patient had a history of insulin resistance and hormone disorders associated with elevated plasma concentrations of intact proinsulin and 64,65 split proinsulin which was compatible with a defect in PC3 processing at the B chain/C peptide junction. The patient also exhibited defects in the processing of the ACTH precursor (POMC); however, there was no evidence for aberrant processing of proglucagon [19]. Circulating proIAPP was not estimated but the results from our study would suggest that processing of IAPP (like that of proglucagon) would be unaffected in this patient.

The mechanism of amyloid deposition is not well understood. NIDDM is associated with increased proportions of intact proinsulin and intermediates of proinsulin proteolysis in the circulation and with islet amyloid [20,21]. Human insulinomas also have amyloid deposits and associated defects in proinsulin processing [22]. These observations suggest that impaired prohormone processing within the secretory pathway of the β cell may be related to the deposition of IAPP in insoluble fibres. One possibility is that the maturation or activity of PC2 may be affected by abnormalities in control of the ionic environment within the secretory pathway. Such abnormalities may also affect the intracellular sorting to constitutive or regulated secretory pathways [23]. It is worth noting that overexpression of IAPP in COS-1 cells that lack the regulated pathway resulted in intracellular amyloid formation [24]. COS-1 cells do not express pC2 and so it is unlikely that any proIAPP processing occurred in the transfected cells. This suggests that proIAPP processing is not essential for fibrillogenesis. Extracts of islet and insulinoma amyloid contain largely mature IAPP but there

is evidence of proIAPP in some deposits [25,26]. It is therefore possible that proIAPP could have a role in forming an initial nidus for progressive fibrillogenesis of IAPP in both diabetes and insulinomas.

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References

[1] Clark, A., Cooper, G.J., Lewis, C.E., Morris, J.F., Willis, A.C., Reid, K.B. and Turner, R.C. (1987) Lancet 2, 231–234.
[2] Westermark, P., Wernstedt, C., Wilander, E., Hayden, D.W., O'Brien, T.D. and Johnson, K. (1987) Proc. Natl. Acad. Sci. (USA) 84, 3881–3885.
[3] Clark, A., Lloyd, J., Novials, A., Hutton, J.C. and Morris, J.F. (1991) Diabetologia 34, 449–451.
[4] Sanke, T., Hanabusa, T., Nakano, Y., Oki, C., Okai, K., Nishimura, S., Kondo, M. and Nanjo, K. (1991) Diabetologia 34, 129–132.
[5] Nishi, M., Chan, S.J., Nagamatsu, S., Bell, G.I. and Steiner, D.F. (1989) Proc. Natl. Acad. Sci. (USA) 86, 5738–5742.
[6] Ohagi, S., Nishi, M., Bell, G.I., Ensinck, J.W. and Steiner, D.F. (1991) Diabetologia 34, 555–558.
[7] Betsholtz, C., Christmanson, L., Engström, U., Rorsman, F., Jordan, K., O'Brien, T.D., Murtaugh, M., Johnson, K.H. and Westermark, P. (1990) Diabetes 39, 118–122.
[8] Hutton, J.C. (1994) Diabetologia 37, S48–S56.
[9] Halban, P.A. and Irminger, J.C. (1994) Biochem. J. 299, 1–18.
[10] Shennan, K.I.J., Taylor, N.A., Jermany, J.L., Matthews, G. and Docherty, K. (1995) J. Biol. Chem. 270, 1402–1407.
[11] Matthews, G., Shennan, K.I.J., Seal, A.J., Taylor, N.A., Colman, A. and Docherty, K. (1994) J. Biol. Chem. 269, 588–592.
[12] Creemers, J.W.M., Siezen, R.J., Roebroek, A.J.M., Ayoubi, T.A.Y., Huylebroeck, D. and Van De Ven, W.J.M. (1993) J. Biol. chem. 268, 21862–21834.
[13] Bailyes, E.M., Shennan, K.J.J., Seal, A.J., Smeekens, S.P., Steiner, D.F., Hutton, J.C. and Docherty, K. (1992) Biochem. J. 285, 391–394.
[14] Matthews, G. and Colman, A. (1991) Nucleic Acids Res. 19, 6405–6412.
[15] Mosselman, S., Höppener, J.W.M., Lips, C.J.M. and Jansz, H.S. (1989) FEBS Lett. 247, 154–158.
[16] Shennan, K.I.J., Smeekens, S.P., Steiner, D.F. and Docherty, K. (1991) FEBS Lett. 284.
[17] Bennett, D.L. and Hutton, J.C. (1994) in: Molecular Biology of Diabetes, Part 1 (B. Draznin and D. LeRoith, eds.). Humana Press, Towota, NJ, pp. 213–248.
[18] Rouillé, Y., Westermark, G., Martin, S.K. and Steiner, D.F. (1994) Proc. Natl. Acad. Sci. USA 91, 3242–3246.
[19] O'Rahilly, S., Gray, H., Humphreys, P.J., Krook, A., Polonsky, K., White, A., Gibson, S., Taylor, K. and Carr, C. (1995) N. Engl. J. Med. 333, 1386–1390.
[20] Levy, J.C., Clark, P.M., Hales, C.N. and Turner, R.C. (1993) Diabetes 42, 162–169.
[21] Kahn, S.E., D'Alessio, D.A., Schwartz, M.W., Fujimoto, W.Y., Ensinck, J.W., Taborsky Jr., G. and Porte Jr., D. (1990) Diabetes 39, 634–638.
[22] Clark, A., Morris, J.F., Scott, L.A., McLay, A., Foulis, A.K., Bodkin, N.L. and Hansen, B.C. (1990) in: Amyloid and Amyloidosis 1990 (Natvig, J.B. et al. eds) Kluwer Academic Press, London, pp. 453–456.
[23] Rhodes, C.J. and Alarcón, C. (1994) Diabetes 43, 511–517.
[24] O'Brien, T.D., Butler, P.C., Kreutter, D.K., Kane, L.A. and Eberhardt, N.L. (1995) Am. J. Pathol. 147, 609–616.
[25] Westermark, P., Engström, U., Westermark, G.T., Johnson, K.H., Permerth, J. and Betsholtz, C. (1989) Diabetes Res. Clin. Pract. 7, 219–226.
[26] Clark, A., de Koning, E.J.P., Baker, C.A., Charge, S.B.P. and Morris, J.F. (1993) Diabetologia 36, Suppl. 1 A136.