Nutritional regulation of proteases involved in fetal rat insulin secretion and islet cell proliferation

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Epidemiological studies have indicated that malnutrition during early life may programme chronic degenerative disease in adulthood. In an animal model of fetal malnutrition, rats received an isocaloric, low-protein (LP) diet during gestation. This reduced fetal β-cell proliferation and insulin secretion. Supplementation during gestation with taurine prevented these alterations. Since proteases are involved in secretion and proliferation, we investigated which enzymes were associated with these alterations and their restoration in fetal LP islets. Insulin secretion and proliferation of fetal control and LP islets exposed to different protease modulators were measured. Lactacystin and calpain inhibitor I, but not isovaleryl-L-carnitine, raised insulin secretion in control islets, indicating that proteasome and cysteinyl cathepsin(s), but not μ-calpain, are involved in fetal insulin secretion. Insulin secretion from LP islets responded normally to lactacystin but was insensitive to calpain inhibitor I, indicating a loss of cysteinyl cathepsin activity. Taurine supplementation prevented this by restoring the response to calpain inhibitor I. Control islet cell proliferation was reduced by calpain inhibitor I and raised by isovaleryl-L-carnitine, indicating an involvement of calpain. Calpain activity appeared to be lost in LP islets and not restored by taurine. Most modifications in the mRNA expression of cysteinyl cathepsins, calpains and calpastatin due to maternal protein restriction were consistent with reduced protease activity and were restored by taurine. Thus, maternal protein restriction affected cysteinyl cathepsins and the calpain–calpastatin system. Taurine normalised fetal LP insulin secretion by protecting cysteinyl cathepsin(s), but the restoration of LP islet cell proliferation by taurine did not implicate calpains.

Islet: Calpain: Cathepsin: Proteasome: Taurine: Low-protein diet

Epidemiological studies have indicated that malnutrition during pregnancy and early life may programme chronic degenerative diseases, such as diabetes, obesity and cardiovascular disease, in adult life (Barker et al. 1993; Ravelli et al. 1999). An experimental model of fetal malnutrition is the maternal, isocaloric low-protein (LP) diet in the rat. We have previously shown that the low-protein – 8% (LP) v. 20% (C) – diet resulted in lower body weight, β-cell mass, islet vascularisation, islet cell proliferation and insulin secretion, while raising islet cell apoptosis and sensitivity to cytotoxic agents in 21.5-day-old rat fetuses (Snoeck et al. 1990; Petrik et al. 1999; Cherif et al. 2001; Merezak et al. 2001; Boujendar et al. 2002). Long-term consequences of early protein restriction were observed in the islets of adult offspring with regard to insulin secretion (Dahri et al. 1991; Iglesias-Barreira et al. 1996) and susceptibility to cytokines (Merezak et al. 2004). Long-term consequences also included changes in the glucose concentration in response to an oral glucose tolerance test during pregnancy (Dahri et al. 1995), altered glucose tolerance and insulin resistance, as well as increased glucose uptake by the peripheral tissues with ageing (Ozanne, 2001).

Since amino acids are the building blocks of protein synthesis, protein turnover might slow down in most fetal organs if there were protein restriction. To allow such thrift, cells must reduce their intracellular protease activities. In fact, adult rats subjected to protein restriction had reduced cathepsin and calpain activities in several organs (Benuck et al. 1995), whereas general food restriction altered the proteolytic activities of proteasome as well as the activities of cysteinyl cathepsins (Inubushi et al. 1996; Radak et al. 2002). Furthermore, these proteases intervene in the cell functions addressed in this paper, i.e. cell proliferation, apoptosis and secretion (Johnson, 2000; Sreenan et al. 2001; Perrin & Huttenlocher, 2002), functions that are altered in the LP progeny. They are therefore possible candidates in the mechanisms of LP diet-induced fetal alterations.

Calpains (or Ca2+-activated neutral protease) are a family of cysteinyl proteases. In islets, only μ-calpain...
and the mRNA of an atypical calpain – calpain 10 – have been identified (Ma et al. 2001; Kitahara et al. 1985). Cathepsins are a protease family comprising cysteine, serine and aspartyl proteases (for a review, see Barrett et al. 1998). In particular, cysteine cathepsins have been localised in the secretory granules of the endocrine pancreatic cells (Im et al. 1989). The proteasome (or multicatalytic proteinase complex) represents up to 1% of the total cell protein content (Baumeister et al. 1998).

Maternal and fetal plasma levels and the islet concentrations of some amino acids were altered by the LP diet (Reusens et al. 1995), especially the sulphur amino acid taurine (2-aminoethane sulphonic acid) (Reusens et al. 1995; Reusens & Remacle, 2001). A nutritional deficit of one single nutrient, i.e. taurine, affected calpain but not cathepsin activity in rat retina (Tsung & Lombardini, 1995; Reusens & Remacle, 2001). The proteasome (or multicatalytic proteinase complex) represents up to 1% of the total cell protein content (Baumeister et al. 1998).

Maternal and fetal plasma levels and the islet concentrations of some amino acids were altered by the LP diet (Reusens et al. 1995), especially the sulphur amino acid taurine (2-aminoethane sulphonic acid) (Reusens et al. 1995; Reusens & Remacle, 2001). A nutritional deficit of one single nutrient, i.e. taurine, affected calpain but not cathepsin activity in rat retina (Tsung & Lombardini, 1985). Supplementation of the maternal LP diet with 2.5% taurine protected the LP offspring not only prenatally (Cherif et al. 1998; Merezak et al. 2001; Boujendar et al. 2002, 2003), but also in the long term (Merezak et al. 2004) against alterations of the 1% of the total cell protein content (Baumeister et al. 1998).

In this paper, we investigated which protease might be associated with the programming of fetal islet functions due to a maternal LP diet and whether these alterations could be prevented by taurine.

Materials and methods

Materials

The 20% protein (C) and isocaloric 8% (LP) diets were purchased from Hope Farms (Woerden, The Netherlands). Their composition, as described previously (Snoeck et al. 1990), is summarised in Table 1. Taurine, collagenase type V, calpain inhibitor I (N-acetyl-leucyl-leucyl-norleucinal), human serum, normal goat IgG, isopropanol and ethidium bromide were purchased from Sigma (St Louis, MO, USA), and RPMI 1640 medium (with glutamax I), Hank’s balanced salt solution, fetal bovine serum and an antibiotic mixture (penicillin 200 U/ml, streptomycin 0.2 mg/ml) from Gibco (Grand Island, NY, USA). Isovaleryl-L-carnitine was a gift from Sigma-Tau (Pisa, Italy). Lactacystin was purchased from Affiniti Probes (Exeter, UK). 5-Bromo-2′-deoxyuridine (BrdU), anti-BrdU antibody and the ‘In Situ Cell Death Detection Kit, TMR red’ were purchased from Affymetrix (Santa Clara, CA, USA), TRizol and the SuperScript II system from Invitrogen (Carlsbad, CA, USA), the BioArray High Yield RNA Transcript Labelling kit, and biotin-labelled CTP and UTP, from Enzo Diagnostics (Farmingdale, NY, USA), RNeasy columns from Qiagen (Hilden, Germany), streptavidin–phycoerythrin conjugate from Molecular Probes (Eugene, OR, USA) and biotinylated anti-streptavidin antibody (goat) from Vector Laboratories (Cambridgeshire, UK).

Animals and diets

All procedures were performed with the approval of the animal ethics committee of the Université Catholique de Louvain. Animals were housed in animal facilities maintained at 25°C on a 14 h light/10 h dark cycle. Nulliparous 3-month-old Wistar rats were time-mated and randomly allocated to one of four groups on the first day of gestation. A control group (C) was provided with a 20% protein diet, and a second group (LP) was given an 8% protein diet made isocaloric by the addition of carbohydrates. The two diets were given throughout gestation. Free access to drinking water was allowed. Two additional groups of animals were fed a C or an isocaloric LP diet supplemented with taurine-enriched (2.5:100, w/v) drinking water. These groups were thus 20% protein + taurine and 8% protein + taurine. The dams were sacrificed at day 21-5 of gestation. The LP fetuses showed a growth retardation of 5–10%, as in our previous experiments (Snoeck et al. 1990; Boujendar et al. 2002). Fertility was not affected by the diets, and litters usually comprised 10–15 pups. Small litters (fewer than seven pups) were not used.

Cell culture and incubation

All cultures, including the digestion of the fetal pancreatic tissue, were carried out in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of the antibiotic mixture. For each parameter studied, at least three independent cultures (N) consisting of fetal islets obtained from the pancreata of the entire litters of three dams were analysed for each nutritional group.

Pancreases from sacrificed 21-5-day-old fetuses were removed aseptically, placed in cold medium and minced. They were digested with collagenase type V (629 U/12 pancreata/ml) by shaking the tubes for approximately 7 min at 37°C. Digestion was stopped by adding cold medium. The tissue suspension was washed twice, gently stirred at room temperature for 60 min and centrifuged at low speed. The digested tissue was resuspended in 1 ml medium/pancreas and distributed per 2 ml in 35 mm Petri dishes.

The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Culture media were changed daily after the second incubation day. During the course of culture, islet cells proliferate and differentiate, and progressively aggregate on a layer of non-endocrine cells consisting

<table>
<thead>
<tr>
<th>Table 1. Composition of the diets (g/100 g diet)</th>
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<tr>
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<tr>
<td>Mineral and vitamin mix</td>
</tr>
<tr>
<td>Casein (88% protein)</td>
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<tr>
<td>dl-Methionine</td>
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<tr>
<td>Corn starch</td>
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<tr>
<td>Cellulose</td>
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<tr>
<td>Soyabean oil/safflower oil</td>
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<td>Cerslose (glucose)</td>
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mostly of pancreatic fibroblasts. This results in the neoformation of islet-like structures (called islets below) composed mainly of β cells (Mourmeaux et al. 1985) that retain their fetal character (low responsiveness to glucose).

Islets were challenged with LLlnL, IVC or lactacystin in 10% FBS/1% antibiotic mixture/RPMI 1640 in the presence of 50 μM BrdU on the fifth day of culture. To determine the experimental conditions and analyse the insulin secretion, islets were treated directly with the inhibitors or the activator in the presence of the pancreatic fibroblasts with which they had been cultured, whereas for determining the proliferation and apoptosis rates, islets were hand-picked and washed twice in 10% FBS/1% antibiotic mixture/RPMI 1640 prior to treatment with an inhibitor or activator.

In order to analyse the proliferation rates and apoptosis, treated islets were fixed with methanol for 10 min at 4°C and samples stored at −20°C. For the analysis of fractional insulin release during the last 24 h of culture, incubation media were recovered and islets were harvested separately in 500 μl acidic ethanol (HCl–EtOH in H2O 0.55%–71.27%) and samples stored at −20°C. The proliferation, apoptosis and insulin secretion experiments were carried out at least in triplicate.

For microarray analysis of the fetal islets, cultured islets were incubated during the last 24 hours in RPMI 1640 medium containing 0.5% human serum, hand-picked and rinsed in Hank’s balanced salt solution. Batches of 2000 islets were transferred into TRIzol and snap-frozen at −80°C. For each experimental group, five islet batches were analysed, each batch containing islets from two or three cultures. For these experiments, between ten and fifteen dams were used per group.

**Apoptosis assay**

In order to exclude potential cytotoxicity of the chosen experimental conditions, islets were assayed for apoptosis after treatment with protease inhibitor or activator. For this purpose, fixed islets were rehydrated twice in PBS, resuspended in 50 μl/sample TUNEL reaction mixture and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Samples were washed twice in blocking buffer (PBS/0.1% Triton X-100/0.5% bovine serum albumin) and mounted with 50 μg/ml DAPI/Mowiol. Samples were analysed in a confocal microscope (MPC1024UV, Biorad, Hemel Hempstead, Hertfordshire, UK). The excitation wavelength was 488 nm, and the emission wavelengths were 605/32 nm and 522/35 nm. Proliferation rates represent the ratio of BrdU-labelled proliferating and ethidium bromide-labelled total nuclei. Whenever possible, at least ten islets (n) were analysed for each of the three or four cultures (N) in at least two focal sections separated by 10 μm, thereby counting a total of at least 2500 nuclei.

**Insulin assay**

The insulin released into the culture media and the insulin content of the islets in each culture dish were measured using the Mercodia high-range ELISA kit as per instructions. This method allows the determination of 3–150 ng/ml with variation coefficients within and between assays of about 5%.

Prior to analysing the insulin secretion samples and sonicated insulin content samples, appropriate dilutions were made in the sample buffer provided in the ELISA kits. Samples were analysed using the Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For each culture (N), three or four samples (n) were analysed for each treatment. The total insulin contained in the islets was estimated by adding the amounts of insulin secreted during the last 24 h of culture and the insulin contained in the islets at the end of the experiment. Fractional insulin release was calculated by dividing the amount of secreted insulin by the total insulin content and by expressing it in percentages.

**Proliferation studies**

Fixed, isolated islets were rehydrated in PBS pH 7.4, permeabilised for 1 h at 37°C with 2 M HCl/PBS, neutralised with 0.1 M borate/PBS, washed with PBS and exposed to anti-BrdU antibody (6 μg/ml PBS/0.1% bovine serum albumin) for 1 h at room temperature, washed with PBS and exposed overnight to fluorescein isothiocyanate antibody (1/400 in PBS) in the dark and at room temperature. After rinsing with PBS, cells were labelled with ethidium bromide (20 μl/250 ml PBS) for 15 min in the dark at room temperature, washed with PBS, placed on cover slips and mounted in Mowiol.

The analysis of proliferation rates was carried out in a confocal microscope (MPC1024UV, Biorad, Hemel Hempstead, Hertfordshire, UK). The excitation wavelength was 488 nm, and the emission wavelengths were 605/32 nm and 522/35 nm. Proliferation rates represent the ratio of BrdU-labelled proliferating and ethidium bromide-labelled total nuclei. Whenever possible, at least ten islets (n) were analysed for each of the three or four cultures (N) in at least two focal sections separated by 10 μm, thereby counting a total of at least 2500 nuclei.

**Microarray analysis**

Islet batches stored in TRIzol were extracted with chloroform at room temperature for 5 min; the aqueous phase was treated with a glycogen–isopropanol mixture, extracted overnight at −80°C and RNA-pelleted at 4°C. RNA was further purified by centrifugation in the presence of 75% ethanol–TRIzol at 4°C, air-dried at room temperature, resuspended in nuclease-free water and stored at −80°C. Quality control of the RNA samples was performed by acrylamide gel electrophoresis.

**cRNA preparation and in vitro transcription**

For each sample, 5–6 μg total RNA was used as starting material for the cDNA preparation. The first- and second-strand cDNA synthesis was performed using the SuperScript II System according to the manufacturer’s instructions except for using an oligo-dT primer containing a T7 RNA polymerase promoter site. Labelled cRNA was prepared
using the BioArray High Yield RNA Transcript Labelling Kit. Biotin-labelled CTP and UTP were used in the reaction together with unlabelled NTPs. Following the IVT reaction, the non-incorporated nucleotides were removed using RNeasy columns.

Array hybridisation and scanning
cRNA 15 μg was fragmented at 94°C for 35 min in a fragmentation buffer containing 40 mM Tris-acetate pH 8.1, 100 mM KOAc and 30 mM MgOAc. Prior to hybridisation, the fragmented cRNA in a 6xSSPE-T hybridisation buffer (1 M NaCl, 10 mM Tris pH 7.6, 0.005% Triton) was heated to 95°C for 5 min and subsequently to 45°C for 5 min before loading onto the Affymetrix GeneChip Rat Expression 230A probe array cartridge. (n = 5 in each group). The probe array was then incubated for 16 h at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station. The probe array was exposed to ten washes in 6xSSPE-T at 25°C followed by four washes in 0.5xSSPE-T at 50°C. The biotinylated cRNA was stained with a streptavidin–phycoerythrin conjugate, final concentration 2 mg/ml in 6xSSPE-T for 30 min at 25°C, followed by ten washes in 6xSSPE-T at 25°C. An antibody-amplification step followed, using normal goat IgG as the blocking reagent, final concentration 0.1 mg/ml, and biotinylated anti-streptavidin antibody (goat), final concentration 3 mg/ml. This was followed by a staining step with a streptavidin–phycoerythrin conjugate, final concentration 2 mg/ml (Molecular Probes, Eugene, OR, USA), in 6xSSPE-T for 30 min at 25°C and 10 washes in 6xSSPE-T at 25°C. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (Hewlett Packard GeneArray Scanner G2500A). The readings from the quantitative scanning were analysed by the Affymetrix Gene Expression Analysis Software.

Statistics
Statistical evaluations were made using ANOVA followed by post hoc Sheffe’s tests. Differences with a P-value < 0.05 were considered significant.

Results
Set-up of incubation conditions
The choice of the protease modulators was dictated by their ability to cover the range of proteases we were interested in (Table 2). We chose the proteasome-specific lactacystin (Fenteany & Schreiber, 1998; Lin et al. 1998) and the m-calpain activator IVC, since μ-calpain responds to IVC in cells that lack m-calpain (Kitahara et al. 1985; Pontremoli et al. 1990). We also selected the non-specific calpain inhibitor I (LLnL) because it inhibits cysteinyl cathepsins and μ-calpain, and, to a much lesser extent, m-calpain and the proteasome (Rock et al. 1994).

The appropriate experimental conditions were established by analysing the impact of LLnL, IVC or lactacystin on the proliferation of control islet cells. The lowest doses tested, i.e. 10 μM LLnL, as well as 0.5 and 1 μM concentrations of lactacystin, were sufficient to reduce islet cell proliferation, provided that exposure to these inhibitors lasted for 48 h. A 24 h challenge of islets with 0.5 mM IVC was sufficient to increase their proliferation rate strongly. The different incubation times needed are due to the different modes of action of these protease modulators. LLnL and lactacystin act on the cell cycle (Choi et al. 1997; Chen et al. 2000); therefore, more than 24 h were necessary for a sufficient number of islet cells to be inhibited. IVC might act more rapidly because it raises the affinity of calpain for Ca²⁺ and its autocatalytic conversion rate (Pontremoli et al. 1990).

Neither control nor LP islet cell survival was affected by exposure to any of these protease modulators (results not shown). Consistent with our previously published data (Merezak et al. 2001), the basal apoptotic rate of LP islet cells was higher than in controls, reaching 1.5 ± 0.2% instead of 0.6 ± 0.1% (N = 9 cultures, n = 64–76 islets).

Fractional insulin release
The fraction of insulin released into the RPMI 1640 culture medium during the last 24 h of culture was quantified (Fig. 1). LLnL 10 μM raised control insulin release by 15% (SEM 3%; P < 0.02), whereas LP islets did not respond at all. Fractional insulin release from both control and LP islets was not influenced by 0.5 mM IVC. Lactacystin at 1 μM, but not at 0.5 μM, was effective in raising control and LP insulin secretion by 57% (SEM 12%; P < 0.03) and 74% (SEM 14%; P < 0.02), respectively.

Maternal 2.5% taurine supplementation enhanced the sensitivity of fractional control insulin release even further and restored the sensitivity of LP insulin release to LLnL (Fig. 2). It raised control insulin release by 81% (SEM 16%; P < 0.02) and LP insulin release by 27% (SEM 3%; P < 0.01).

Proliferation of islet cells
Proliferation of isolated control islets responded to both IVC and LLnL (Fig. 3) in opposite ways. Whereas 24 h

| Table 2. Activities of protease modulators used in this paper (LLnL, lactacystin, IVC) on different proteases (cysteinyl cathepsins, calpains and proteasome) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Cysteiny1 cathepsins | μ-Calpain | m-Calpain | Calpain 10 | Proteasome |
| LLnL | | | | |
| Lactacystin | = | = | n.d. | |
| IVC | = | = | n.d. | |

1. activation; |, inhibition; =, without effect; n.d., not determined.
in the presence of 0·5 mM IVC raised control islet cell proliferation by 86% (SEM 3%; \( P, 0·01 \)), 48 h exposure to Q17 10 m M LLnL reduced control islet cell proliferation by 43% (SEM 5%; \( P, 0·01 \)). The most striking feature of LP islet cell proliferation was the fact that it responded to neither the calpain inhibitor nor the calpain activator (Fig. 3).

The proliferation rate of untreated control islet cells in the IVC experiments was lower than in the LLnL experiments (Fig. 3). Since islets were cultured for 24 h in the presence of BrdU in the former, but cultured for 48 h with BrdU in the latter, proliferation rates were thus measured on the sixth day of culture in the IVC experiments but on the seventh day of culture in the LLnL experiments. The difference (\( P<0·001 \)) observed between the proliferation rates of untreated control islets therefore represents the proliferation during the seventh day of culture. It was not observed in the case of LP islets: LP islet cells seemed to have exhausted their proliferation capacity after the sixth day of culture. Indeed, whereas on the sixth day of culture untreated LP islet cells did not proliferate significantly less than controls (\( P=0·059 \)), on the seventh day of culture their proliferation rate was significantly lower than the proliferation rate of untreated controls (\( P<0·01 \)). Since the proteasome did not seem to be affected by the LP diet, as indicated in the insulin experiment, we did not include lactacystin in our proliferation experiments.

Supplementation of the maternal diet with 2·5% taurine did not influence the response of C islet cell proliferation to LLnL, although it slightly reduced the response to IVC (Fig. 4). It was also ineffective in restoring a response of LP islet cell proliferation to LLnL and IVC.

### Microarray analysis

In an attempt to further characterise the LP diet-induced alterations of fetal islets as well as the impact of taurine supplementation, microarray analysis was carried out. Our preliminary data reveal the presence of moderate to high levels (400–2000 fluorescence units) of all the \( \alpha \) and \( \beta \) subunit mRNAs forming the 20S proteasome, as well as varying mRNA levels of different subunits forming the regulatory cap for the ATP- and ubiquitin-dependent proteolytic activity of the 26S proteasome. Moderate to low levels of calpain 1 (catalytic subunit of \( \mu \)-calpain), calpain 2 (catalytic subunit of m-calpain), calpain 8, calpain 10 and the calpain inhibitor calpastatin were expressed (Table 3), in addition to high levels (above 1000 fluorescence units) of calpain small subunit 1 mRNA (regulatory subunit of \( \mu \)- and m-calpain). With regard to the cathepsin–cystatin system, fetal islets expressed high mRNA levels (above 1000 fluorescence units) of cathepsins B and L, and of cystatins B, C and N, as well as low levels of cathepsin S (fluorescence units). They expressed only moderate levels of cathepsins H and D (400–600 fluorescence units), as well as low levels of cathepsins C, E, K, S and Y. We did not, however, detect mRNA of the cathepsin-like...
The impact of the maternal LP diet and/or taurine supplementation on these proteolytic systems is summarised in Table 3. In this table, only those enzymes and endogenous inhibitors that are altered by the diet are included. Cathepsin H mRNA was decreased and L mRNA raised by the LP diet, and both were restored by taurine. Calpain 2 and 10 mRNA was reduced, whereas calpastatin mRNA was raised by the LP diet. Although taurine supplementation did not affect any mRNA levels in the control islets (results not shown), it restored calpain 10 and only partially restored calpastatin mRNA levels in LP islets. In addition, although calpain 1 mRNA was not affected in islets exposed only to the LP diet, it was increased in taurine-supplemented LP islets. Finally, cathepsin H and L mRNA levels were both restored by maternal taurine supplementation.

Discussion

The LP diet silences a cysteinyl protease involved in fetal islet cell proliferation

The proliferation of untreated LP islet cells was lower, in agreement with previous reports (Petrik et al. 1999, and references cited therein). It was unaffected by LLnL and IVC, although control islet cell proliferation responded to both. In other words, μ-calpain and/or calpain 10 activity, which may intervene in islet cell proliferation, appears to be lost in fetal LP islet cells. It might be the reason for the higher cyclin D1 content and the longer G1 phase of the LP islet cell cycle (Petrik et al. 1999) and the ensuing lower proliferation capacity. Since the endogenous calpain inhibitor calpastatin was not detected in β cells (Kitahara et al. 1985), some other disturbed regulatory mechanism(s), such as inactivation by oxidative stress (Guttmann & Johnson, 1998), intracellular localisation (Spencer & Tidball, 1996) or simply absence of calpain protein, may be responsible for the loss of calpain activity in LP islet cells. These data do not exclude the possibility that a decreased cysteinyl cathepsin activity also participates in the reduced response of LP islet cell proliferation to LLnL by lengthening the cell cycle (Johnson, 2000).

Indeed, at least one cysteinyl cathepsin – cathepsin L – participates as a progression factor in cell proliferation (Ishidoh & Kominami, 1998), and a cathepsin B- or cathepsin L-like protease active during the G1 and S phases regulates G1/S progression (Fu et al. 1998).

The LP diet silences a cysteinyl cathepsin involved in fetal insulin secretion

Fetal insulin secretion reacted differently from proliferation to lactacystin, IVC and LLnL. In controls, insulin secretion was not affected by the specific calpain activator IVC, but it responded to the non-specific calpain inhibitor LLnL and to the proteasome-specific inhibitor lactacystin. This suggests that proteasome and a cysteinyl protease – probably a cathepsin but not μ-calpain – intervene in fetal insulin secretion. The potential involvement of proteasome and cathepsins in insulin secretion had already been considered but rejected in adult mouse islets, since their insulin secretion was unaffected by proteasome-specific inhibitor lactacystin. This suggests that proteasome and a cysteinyl protease – probably a cathepsin but not μ-calpain – intervene in fetal insulin secretion. The apparent contradiction with our data may originate in the high calpain inhibitor concentrations used and the different nature of the islets. Indeed, whereas these two studies analysed adult islets, our study targeted fetal islets that do not

Table 3. Genes from proteolytic systems modified by the low-protein (LP) diet and restored by taurine (Tau)

<table>
<thead>
<tr>
<th>Gene</th>
<th>C (Mean SEM)</th>
<th>LP (Mean SEM)</th>
<th>LP + Tau (Mean SEM)</th>
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<tbody>
<tr>
<td>Cathepsin H</td>
<td>268.6 ± 8.0</td>
<td>238.3 ± 10.1*</td>
<td>276.4 ± 25.0</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>1020.2 ± 31.1</td>
<td>1255.8 ± 62.6*</td>
<td>1060.4 ± 67.6</td>
</tr>
<tr>
<td>Calpain 1</td>
<td>154.4 ± 5.2</td>
<td>160.3 ± 5.3</td>
<td>182.9 ± 4.1**</td>
</tr>
<tr>
<td>Calpain 2</td>
<td>272.3 ± 28.3</td>
<td>192.6 ± 10.2*</td>
<td>195.7 ± 21.1</td>
</tr>
<tr>
<td>Calpain 10</td>
<td>131.5 ± 5.7</td>
<td>113.9 ± 4.4*</td>
<td>130.8 ± 7.0</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>40.3 ± 12.8</td>
<td>84.4 ± 6.3*</td>
<td>67.1 ± 6.3</td>
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C, control. *P < 0.05, **P < 0.001. Data are means from five independent cultures and are expressed as fluorescence intensity of the signal.
feature a mature glucose-responsive insulin secretion (Cherif et al. 1998, 2001). The protease(s) involved in fetal insulin secretion might be cysteiny l cathepsins B, H and/or L (Im et al. 1989).

The response of insulin secretion to the non-specific calpain inhibitor LLnL was lost in LP islets, whereas their response to the specific proteasome inhibitor lactacystin was normal. This suggests that proteasome activity is not altered and that inactivation of a cysteiny l cathepsin-like protease may be involved in the lower insulin secretion of the LP islets. Cysteiny l cathepsins might be inactivated in LP islets due to lower expression, oxidation (Lockwood, 1997) or increased levels of cystatin B, the endogenous cathepsin inhibitor found in the secretory granules of β cells (Watanabe et al. 1988). Since the LP diet affects exocytotic machinery (Cherif et al. 2001) and cathepsin-like activity, our results provide a hint as to how the LP diet disturbs fetal insulin secretion.

The LP diet affects the expression of cathepsin, calpain and calpastatin mRNAs

In addition to the susceptibility of fetal islet cysteiny l cathepsin and calpain, but not proteasomes, activities to the maternal LP diet, we also collected preliminary evidence for changes in the corresponding mRNAs. Consistent with the results obtained for insulin secretion, proteasome mRNA was not affected by the LP diet. In addition, taurine supplementation did not influence proteasome mRNA either. The fact that the cysteiny l cathepsin activity involved in insulin secretion was lost in fetal LP islets, despite a normal expression of the strongly expressed cathepsin B mRNA coupled to an increased expression of the equally strongly expressed cathepsin L mRNA, points towards post-translational regulation. Since cystatin B mRNA levels are similar in the four groups of islets, this suggests that the inhibition of cysteiny l cathepsin activity in fetal LP islets is not caused by an increased expression of cystatin B at this level. Unlike these enzymes found in the secretory granules of the β cells, mRNA of the lysosomal cathepsin D, an aspartyl cathepsin, is not affected by protein restriction (results not shown).

Consistent with lost calpain activity in the LP islets, the mRNA levels of calpain 2 and calpain 10 were reduced and the mRNA level of calpastatin was increased, although calpain 1 mRNA expression was normal. Calpain 1 and 2 mRNAs code for the catalytic subunits of μ-calpain and m-calpain, respectively. The regulatory subunit of both enzymes is encoded by a separate mRNA that was not affected by maternal protein restriction or taurine supplementation. The actual presence of calpain 10 protein needs to be verified in our fetal islets. In general, only scarce information may be found on the presence of this protein in the literature. Ma et al. (2001) detected calpain 10 protein in different rat tissues and showed an age-dependent presence of the protein but unfortunately did not include the pancreas in their study. Indirect evidence for calpain 10 activity in islets was provided in a recent paper (Johnson et al. 2004).

Kitahara et al. (1984) were unable to detect m-calpain (synthesised from calpain 2 mRNA) or calpastatin in adult rat islets, whereas we detected their mRNA in fetal islets. It might, therefore, be worthwhile reinvestigating this absence of m-calpain and calpastatin proteins in rat islets. It is conceivable that the presence of both m-calpain and calpastatin might be specific to the fetal islets.

Nutritional intervention with taurine protects against LP diet-induced alterations of fetal endocrine pancreas development

Taurine appears to participate in the development of the endocrine pancreas by an unknown mechanism. Indeed, supplementation of the maternal diet with 2.5% taurine prevented the alterations of the β-cell mass caused by the LP diet by correcting β-cell proliferation and apoptosis, and the vascularisation of the endocrine pancreas (Boujendar et al. 2001, 2003). It also prevented the deficiency of fetal insulin release (Cherif et al. 1998). In the present paper, we show that maternal taurine supplementation dramatically increased the response of insulin release to LLnL in controls and, moreover, restored the responsiveness of LP islets. Since taurine supplementation occurred only in utero and control and LP islets were cultured in the same medium in the absence of taurine, not only may a direct effect of this amino acid on islets be excluded, but an influence of taurine on islet development is also revealed at the same time. The question that remains is how taurine raised cysteiny l cathepsin activity in control and LP islets and thereby restored insulin secretion. Since taurine did not modify RNA levels of cysteiny l cathepsins or cystatins in control islets, but increased their responsiveness to the inhibitor LLnL, this points towards post-translational regulation. In addition, taurine corrected the expression of those RNAs of the cathepsin–cystatin system that were altered by the LP diet, i.e. cathepsins H and L, without altering the expression of RNAs that were not affected by the LP diet, including cathepsin B, cystatin B and cathepsin D.

Taurine has been shown to increase glucose utilisation in erythrocytes (Nandhini & Anuradha, 2003). On the other hand, cysteiny l cathepsins are inhibited, while their inhibitor cystatin B is increased, under high glucose conditions (Makita et al. 1998; Nishimura et al. 2000). Since the taurine content is reduced in LP plasma and islets, and maternal taurine supplementation restored plasma taurine levels (Reusens et al. 1995), it is conceivable that taurine supplementation also normalises taurine levels in β cells, which would result in a restored glucose metabolism in these cells. This could in turn normalise cysteiny l cathepsin activity.

Contrary to its effect on cysteiny l cathepsin(s) and insulin secretion, taurine supplementation did not restore calpain activity involved in the proliferation of fetal LP islets. This suggests that the lack of calpain activity in LP islets is due not to mechanisms that can be corrected by taurine during development, but rather to the absence of the protein itself. The functional importance of the normalisation by taurine of the mRNAs of calpains and calpastatin, as well as the increased calpain 1 expression, remains unclear. At first glance, our findings seem to contradict a study in which taurine affected calpain, but not cathepsin, activity in the retina of young adult rats.

Cherif et al. (synthesised from calpain 2 mRNA) or calpastatin in...
(Tsung & Lombardini, 1985). In that paper, however, an immediate effect of taurine was examined. In addition, the cathepsin analysed was not a cysteiny l cathepsin, and retinal cells contain m-calpain and not μ-calpain like pancreatic islet cells (Persson et al. 1993; Azuma et al. 2000). In this paper, we show that proteases, in particular cysteiny l protease activity, can be modulated in the offspring by maternal malnutrition. This may contribute to disturbed fetal islet proliferation and secretion, and thus to long-term programming facilitating diabetes later in life.

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References


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Q21

Q20


Author Queries

JOB NUMBER: 1313

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Q1 I have assumed that FITC is fluorescein isothiocyanate and put both instances of FITC in full. Is this the correct version?
Q2 I have changed 2·5% to a ratio to match journal style. Is this a correct ratio?
Q3 Town for Vector Laboratories please.
Q4 ‘distributed per 2 ml’ – is this ‘in 2 ml aliquots’?
Q5 LLnL and IVC to be in full here as well as abbreviated. Also applies to Table 2 and figure captions, and needs to be added to abbreviations list.
Q6 10% FBS/1% antibiotic mixture/RPMI 1640 needs to be rewritten as a ratio as per journal style please, cf. ethanol–water–methanol (4:5:1, by volume).
Q7 0·55% to 71·27% - is this v/v?
Q8 TUNEL in full please in both places.
Q9 I have assumed that BSA in bovine serum albumin. If this full version is incorrect, please correct in both places.
Q10 DAPI in full please in both places.
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Q13 IVT in full please.
Q14 In 6x and 0·5x, is the ‘x’ a multiplication sign?
Q15 Place of manufacture for Hewlett Packard Gene Array Scanner please.
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Q18 Kitahara et al is 1985 in the ref list and elsewhere in the text, but 1984 here. Should it be 1985?
Q19 Boujendar et al 2001 – should this be 2002? If not, please provide the missing reference.
Q20 Benuck – please provide Banay-Schwartz’s initials.
Q21 Johnson et al and Merezak et al – are there any updates for these two references?

Q22 Reusens et al 1995 – please clarify the publisher and place of publication.
Q23 I have assumed that C is the controls and T taurine. Is this correct? (I’ve also changed T to Tau in line with the figures.)
Q24 Why is (a) in brackets for Calpain 2, LP + T?
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