

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 isoenergetic, 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 proteases 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 CVD, in adult life (Barker *et al.* 1993; Ravelli *et al.* 1999). An experimental model of fetal malnutrition is the maternal, isoenergetic low-protein (LP) diet in the rat. We have previously shown that the LP – 8% 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-d-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 Ca^{2+} -activated neutral protease) are a family of cysteinyl proteases. In islet cells, only μ -calpain and the mRNA of an atypical calpain – calpain 10 – have been identified (Kitahara *et al.* 1985; Ma *et al.* 2001). Cathepsins are a protease family comprising cysteinyl, serinyl and aspartyl proteases (for a review, see Barrett *et al.* 1998). In particular, cysteinyl 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, 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 β -cell mass, islet cell proliferation, apoptosis and susceptibility of cytokines, as well as insulin secretion.

In the present 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 (control) and isoenergetic 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; (LLnL)), 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 (FBS) and an antibiotic mixture (penicillin 200 U/ml, streptomycin 0.2 mg/ml) from Gibco (Grand Island, NY, USA). Isovaleryl-L-carnitine (IVC) 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 Boehringer Mannheim (Mannheim, Germany), and secondary fluorescein isothiocyanate labelled antibody from DAKO (Glostrup, Denmark). Methanol, ethanol, HCl and borate were analysis grade and purchased from VEL (Leuven, Belgium). Insulin assay (ELISA) kits were purchased from Mercodia (Uppsala, Sweden). Affymetrix GeneChip Rat expression 230A probe array cartridges 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 (Peterborough, Cambridgeshire, UK).

Table 1. Composition of the diets (g/100 g diet)

| | Control | Low-protein |
|----------------------------|---------|-------------|
| Mineral and vitamin mix | 6.85 | 6.95 |
| Casein (88% protein) | 22.0 | 9.0 |
| dl-Methionine | 0.20 | 0.08 |
| Corn starch | 8.0 | 8.0 |
| Cellulose | 5.0 | 5.0 |
| Soyabean oil/safflower oil | 4.3 | 4.3 |
| Cerelease (glucose) | 53.65 | 66.67 |

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 was provided with a 20% protein diet, and a second group (LP) was given an 8% protein diet made isoenergetic 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 control or an isoenergetic 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 killed 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 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 killed 21.5-d-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 in 2 ml aliquots 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 mostly of pancreatic fibroblasts. This results in the neoformation of islet-like structures (called islets later) composed mainly of β cells (Mourmeaux *et al.* 1985) that retain their fetal character (low responsiveness to glucose).

Islets were challenged with LLnL, IVC or lactacystin in FBS-antibiotic mixture-RPMI 1640 medium (10:1:89, by vol.) in the presence of 50 μ M-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 FBS-antibiotic mixture-RPMI 1640 medium (10:1:89, by vol.) 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 μ l acidic ethanol (HCl-EtOH in H₂O 0.55:71:27 v/v) 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 h 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 TdT-mediated dUTP-X nick end labelling 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 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2'-phenylindole dihydrochloride/Mowiol. Samples were analysed in a confocal microscope (MPC1024UV, Biorad, Hemel Hempstead, Hertfordshire, UK). The excitation wavelengths were 488 and 351 nm, and the emission wavelengths were collected using 515LP and 455/30 nm filters. Negative controls were included in each experiment using label solution instead of TdT-mediated dUTP-X nick end labelling reaction mixture. Apoptotic rates were expressed as ratio of apoptotic, tetramethylrhodamine-labelled and total, 4',6-diamidino-2'-phenylindole dihydrochloride-labelled 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 was 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 $\mu\text{g}/\text{ml}$ 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-PBS (1:12 500, by vol.) 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). 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 NTP. Following the *in vitro* transcription 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 (five 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), 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; Affymetrix). 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* Scheffe'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 μ M-LLnL, as well as 0.5 and 1 μ M-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 Ca^{2+} 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 (SEM 0.2) % instead of 0.6 (SEM 0.1) % (*N* nine cultures, *n* sixty-four to seventy-six 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 μ M 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 μ M, but not at 0.5 μ M, 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 in the presence of 0.5 mM-IVC raised control islet cell proliferation by 86 (SEM 3) %; *P*<0.01, 48 h exposure to 10 μ 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*<0.01) 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 control 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

Table 2. Activities of protease modulators used in the present paper (LLnL, lactacystin, IVC) on different proteases (cysteinyl cathepsins, calpains and proteasome)

| | Cysteinyl cathepsins | μ -Calpain | m-Calpain | Calpain 10 | Proteasome |
|-------------|----------------------|----------------|-----------|------------|------------|
| LLnL | ↓↓ | ↓↓ | ↓ | n.d. | ↓ |
| Lactacystin | = | = | = | n.d. | ↓↓ |
| IVC | = | ↑ | ↑↑ | n.d. | = |

↑, activation; ↓, inhibition; =, without effect; n.d., not determined.

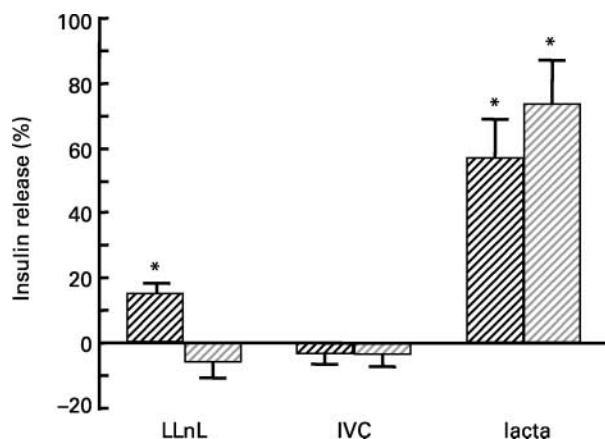


Fig. 1. Fractional insulin release during the last 24 h of culture from fetal control (hatched) and low-protein (white) rat islets treated with LLnL (10 μ M, 48 h), IVC (0.5 mM, 24 h) or lactacystin (lacta, 1 μ M, 48 h). Fractional insulin release from treated islets is expressed as the percentage relative to the fractional insulin release from the corresponding untreated islets. Values are means \pm SEM; $N=3-4$ experiments; $n=9-15$ samples; * $P<0.05$: treated islets v. untreated islets.

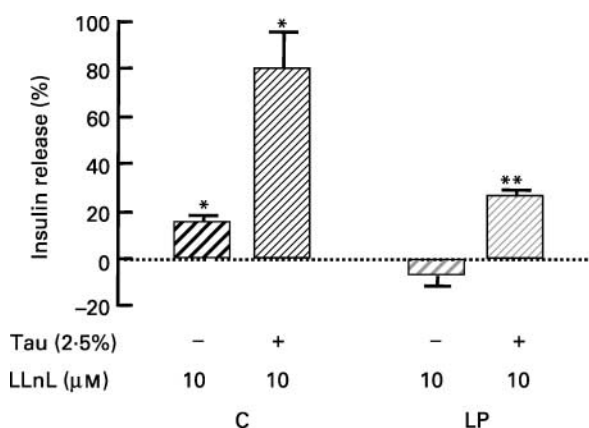


Fig. 2. Effect of maternal 2.5% taurine (Tau) supplementation on the fractional insulin release during the last 24 h of culture of fetal control (C) and low-protein (LP) rat islets treated with LLnL (10 μ M, 48 h). Fractional insulin release from treated islets is expressed as the percentage relative to the fractional insulin release from the corresponding untreated islets. Values are means \pm SEM; $N=3-4$; $n=12-16$; * $P<0.05$, ** $P<0.001$: treated islets v. untreated islets.

units) of all the α and β subunit mRNA 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 μ -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 μ - 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 prohormone thiol protease. The impact of the maternal LP diet and/or taurine supplementation on these

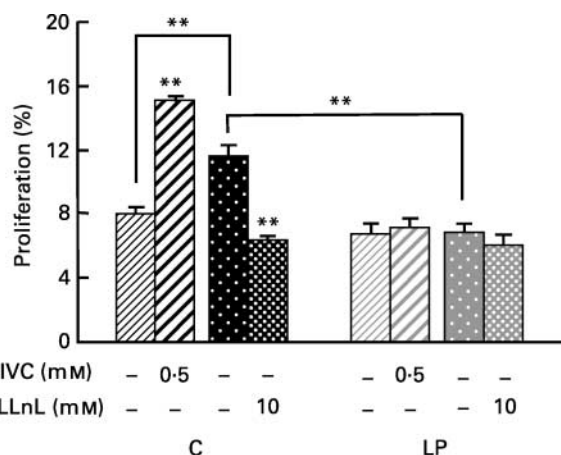


Fig. 3. Proliferation of isolated fetal control (C) and low-protein (LP) rat islets treated with LLnL or IVC. Proliferation rates are means \pm SEM; $N=3-4$ experiments; $n=24-37$ islets; ** $P<0.001$: treated islets v. untreated islets.

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 low protein 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).

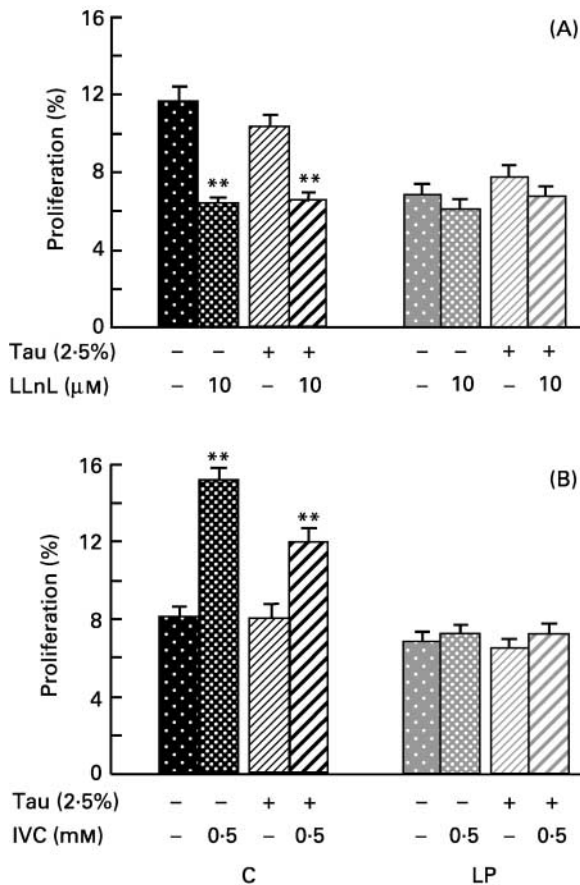


Fig. 4. Effect of maternal 2.5% taurine (Tau) supplementation on the proliferation of fetal control (C) and low-protein (LP) rat islet cells treated with (A) LLnL or (B) IVC. Proliferation rates are means \pm SEM; $N=3-4$ experiments; $n=21-37$ islets; ** $P<0.001$: treated islets *v.* untreated islets.

The low protein 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

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

| Gene | C | | LP | | LP + Tau | |
|-------------|--------|------|---------------------|------|---------------------|------|
| | Mean | SEM | Mean | SEM | Mean | SEM |
| Cathepsin H | 268.6 | 8.0 | 238.3 ^a | 10.1 | 276.4 | 25.0 |
| Cathepsin L | 1020.2 | 31.1 | 1255.8 ^a | 62.6 | 1060.4 | 67.6 |
| Calpain 1 | 154.4 | 5.2 | 160.3 | 5.3 | 182.9 ^{aa} | 4.1 |
| Calpain 2 | 272.3 | 28.3 | 192.6 ^a | 10.2 | 195.7 ^a | 21.1 |
| Calpain 10 | 131.5 | 5.7 | 113.9 ^a | 4.4 | 130.8 | 7.0 |
| Calpastatin | 40.3 | 12.8 | 84.4 ^a | 6.3 | 67.1 | 6.3 |

C, control; LP, low protein; Tau, taurine.

^{a,aa}Mean values within a column with unlike superscript letters were significantly different

^a $P<0.05$; ^{aa} $P<0.001$.

Data are means from the five independent cultures and are expressed as fluorescence intensity of the signal.

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 responded to calpain inhibitors but was unaffected by proteasome inhibitors. It was also unaffected by an inhibitor specific for cathepsin B (Sreenan *et al.* 2001; Zhou *et al.* 2003), but this does not, however, rule out regulation by another cysteinyl cathepsin. This 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 feature a mature glucose-responsive insulin secretion (Cherif *et al.* 1998, 2001). The protease(s) involved in fetal insulin secretion might be cysteinyl 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 cysteinyl cathepsin-like protease may be involved in the lower insulin secretion of the LP islets. Cysteinyl 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 low protein diet affects the expression of cathepsin, calpain and calpastatin mRNA

In addition to the susceptibility of fetal islet cysteinyl cathepsin and calpain, but not proteasome, activities to the maternal LP diet, we also collected preliminary evidence for changes in the corresponding mRNA. 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 cysteinyl 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 cysteinyl 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 mRNA 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.* (1985) 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 re-investigating 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 low protein 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.* 2002, 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 cysteinyl cathepsin activity in control and LP islets and thereby restored insulin secretion. Since taurine did not modify RNA levels of cysteinyl 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 RNA of the cathepsin–cystatin system that were altered by the LP diet, i.e. cathepsins H and L, without altering the expression of RNA 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, cysteinyl 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 cysteinyl cathepsin activity.

Contrary to its effect on cysteinyl 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 mRNA 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 (Tsung & Lombardini, 1985). In that paper, however, an immediate effect of taurine was examined. In addition,

the cathepsin analysed was not a cysteinyl cathepsin, and retinal cells contain m-calpain and not μ -calpain like pancreatic islet cells (Persson *et al.* 1993; Azuma *et al.* 2000).

In the present paper, we show that proteases, in particular cysteinyl 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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