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II - Insulin processing in mitochondria

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Abstract Our objective was to know how insulin is processing in mitochondria; if IDE is the only participant in mitochondrial insulin degradation and the role of insulin degradation on IDE accumulation in mitoplasts. Mitochondria and its fractions were isolated as described by Greenwalt. IDE was purified and detected in immunoblot with specific antibodies. High insulin degradation was obtained through addition to rat's diet of 25 g/rat of apple and 10 g/rat of hard-boiled eggs, 3 days a week. Mitochondrial insulin degradation was assayed with 5 % TCA, insulin antibody or Sephadex G50 chromatography. Degradation was also assayed 60 min at 37 °C in mitochondrial fractions (IMS and Mx) with diet or not and without IDE. Degradation in fractions precipitated with ammonium sulfates (60–80 %) were studied after mitochondrial insulin incubation (1 ng. insulin during 15 min, at 30 °C) or with addition of 2.5 mM ATP. Supplementary diet increased insulin degradation. High insulin did not increase mitoplasts accumulation and did not decrease mitochondrial degradation. High insulin and inhibition of degradation evidence insulin competition for a putative transport system. Mitochondrial incubation with insulin increased IDE in matrix as observed in immunoblot. ATP decreased degradation in Mx and increased it in IMS. Chromatography of IMS demonstrated an ATP-dependent protease that degraded insulin, similar to described by Sitte et al. Mitochondria participate in insulin degradation and the diet increased it. High insulin did not accomplish mitochondrial decrease of degradation or its accumulation in mitoplasts. Mitochondrial incubation with insulin increased IDE in matrix. ATP suggested being a regulator of mitochondrial insulin degradation.

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Abbreviations

IDE	Insulin-degrading enzyme
OM	Outer membrane
IMS	Inter-membrane space
M	Mitoplasts
IM	Inner membrane
Mx	Matrix
NEM	N-ethylmaleimide
TCA	Trichloroacetic acid

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Introduction

After binding to cell surface receptors, insulin is internalized and degraded. Although insulin degradation has extensively been studied, the site(s) of degradation, the enzyme(s) involved and the physiological importance of insulin degradation have remained unclear (Duckworth et al. 1998). Substantial evidence suggests that the primary cellular insulin-degrading activity is dependent on insulin-degrading enzyme (IDE), a protease from the family metallo-endoprotease (Makarova and Grishin 1999). Although IDE was first described in cytosol, its subcellular localization was demonstrated in various organelles like endosomes (Hamel et al. 1991), peroxisomes (Hamel et al. 2001), nucleus (Udrisar et al. 2005), endoplasmic reticulum (Cesar Vieira et al. 2011) and mitochondria (Leissring et al. 2004). It has been proven that the majority of insulin degradation takes place in endosomes and plasma membranes, but other studies have also shown mitochondria as a place for insulin degradation (Hare 1978; Desautels and Goldberg 1982).

Recently, in confocal studies directed to study SIRT4, it was described that insulin was inside the mitochondria (Haigis et al. 2006). In previous study, we observed that IDE facilitates mitochondrial insulin transfer and its accumulation in mitoplasts (Camberos et al. 2014). These studies were developed at 25 °C to avoid insulin degradation, because at 37 °C mitochondrial insulin accumulation could not be demonstrated by electron microscopy (Goldfine et al. 1981).

In agreement with those findings, we proposed that the absence of insulin accumulation at 37 °C was due to incremented insulin degradation. For these reasons we studied the mitochondrial insulin degradation at 30 °C to obtain some equilibrium between mitochondrial insulin accumulations and its degradation. Koppen and Langer described 14 mitochondrial proteases (Koppen and Langer 2007); 5 are present in the matrix, 5 in the inner membrane and 4 in the intermembrane space. Four of all proteases are ATP-dependent; therefore, ATP can be used to check whether or not this enzyme(s) participates in insulin degradation.

It is known that mitochondria are inducible organelles whose enzymes are increased by numerous factors including different nutrients and the diet (Lombardo and Chicco 2006; Pighin et al. 2003; Hein et al. 2010; Hao et al. 2007; Shen et al. 2008). Due to these findings we started to feed rats with a supplementary diet looking for an increment of insulin degradation. Our objective was to know how insulin is processing in mitochondria; if IDE is the only participant in mitochondrial insulin degradation and the role of insulin degradation on IDE accumulation in mitoplasts. To reach this objective we studied degradation at 30 °C in mitochondria and mitoplasts, and at 37 °C in mitochondrial fractions IMS and Mx.

Methods

Materials Porcine insulin was provided by Dr. Anderson (Laboratorio Beta, Buenos Aires, Argentina). $Na^{125}I$ (17.4 Ci/mg specific activity) was obtained from DuPont (Boston, MA, USA). DEAE-SephadexA50, Sephadex G15, Sephadex G25, Sephadex G50 Superfine and Sephadex G200 were from *Pharmacia* Diagnostics AB (Uppsala, Sweden). Percoll PLUS was from GE Healthcare Bio-Sciences (Upsala, Sweden). Phenyl Agarose, Dithiothreitol (DTT), N-ethylmaleimide (NEM), 1,10-Phenantroline (OP), Phenylmethylsulfonyl Fluoride (PMSF), Apyrase, Digitonin and Coomassie brilliant Blue R were manufactured by Sigma Chemical Company (St. Louis, MO, USA). Reagents for SDS-PAGE and immunoblot were purchased from BioRad (Richmond, CA, USA). All drugs were of analytical grade.

Mitochondria isolation Mitochondrias were isolated from rat liver as described by Rickwood et al (Rickwood et al. 1987). The last pellet was diluted in 20 mM HEPES, 0.25 M mannitol, 5 mM K_2PO_4, 5 μM $MgCl_2$, 1 mM DTT, 50 μM Coenzyme A, 50 μM Cocarboxilase, 1 mg/ml bovine albumin fatty-acid free, pH 7.4 (buffer mitochondria) for the studies.

The outer membrane plus intermembrane space (OM + IMS) and mitoplast (M) were obtained by treatment with digitonin, as described by Greenwalt (Greenawalt 1974). The outer membrane (OM) was separated from the intermembrane space (IMS) by centrifugation during 60 min at 100,000×*g*. The inner membrane (IM) and matrix (Mx) were obtained after processing the mitoplast: 3 times of freeze/thawing in liquid nitrogen and 5 times sonication during 5 s, 100 watts, at 4 °C in Ultrasonics sonicator (Branson Ultrasonic Corporation, Danbury, USA) followed by centrifugation during 60 min at 100,000×*g*. All fractions were stored at −70 °C.

Incubation conditions Isolated mitochondria were incubated in batch with buffer mitochondria for recovery (15 min, 30 °C with mild oxygenation). Two types of experiments were effectively carried out.

The reaction was developed in batches for Control (no IDE added) or with IDE (Experimental) at 30 °C. These batches were calculated to contain in 250 μl (volume per tube) a concentration of 1.2 μg of IDE, ~50000 cpm ^{125}I-insulin + 1 ng native insulin and ~1 mg of mitochondria. This volume was distributed in tubes at the indicated times from basal up to 300 s. (basal entails a period of 3 to 4 s). To stop insulin degradation the tubes were placed in ice bath with addition of 25 μl of concentrated NEM, in buffer mitochondria without cofactors (NEM buffer) to reach a final concentration of 2 mM NEM.

When the experiments were developed in tubes, all drugs were added in a cold bath. Two hundred and fifty μl was the final volume (Control or IDE) with ~50000 cpm ^{125}I-insulin/

tube and increased concentration of native insulin (1, 10, 100, 1000, 10000 ng/tube) without or with 0.1 mM NEM. The tubes were warmed during 15 s until they reached an internal temperature of 30 °C, and they were incubated 60 s at 30 °C. In order to stop the reaction after incubation, the tubes were placed in a cold bath (2 °C) and 25 μl of 2 mM NEM was added to each tube.

Enzyme purification and degradation assay IDE was extracted from muscles as described (Camberos et al. 2001; Camberos and Cresto 2007) and fractions with degradation activity were stored at −70 °C. Protein concentration was assayed by absorbance at 280 nm in a spectrometer (Spectronic 3000, array; Markham, Ontario, Canada) or using Bradford's method.

^{125}I-porcine insulin was labeled with the Chloramine-T method and purified with preparative starch gel electrophoresis as previously described (Cresto et al. 1981). The final purification was by chromatography in Sephadex G50 Superfine (50 ml column) and eluted with 20 mM HEPES, 7.4 pH and 1 mg/ml bovine albumin fatty-acid free. Insulin degradation was determined with precipitation of 5 % trichloroacetic acid (TCA), insulin antibody excess (SAI 4; 1/20000) or chromatography. Controls of labeled insulin had more than 95 % of intact insulin; hence, experimental results were corrected for this value.

Insulin degradation in mitochondria and mitoplasts To know if enzyme(s) were released from incubated mitochondria, insulin degradation in the mitochondrial wash was studied at 30 °C with the addition ^{125}I-insulin.

Mitochondrial insulin degradation was carried out through chromatography of incubation buffer, eluted with 1 M acetic acid, 150 mM NaCl, 0.25 % BSA, and radioactivity was counted. Insulin degradation in mitoplasts was determined through chromatography after their solubilization (2 h in ice bath with 2 % Triton X100). Chromatography in Sephadex G50 Superfine (25 ml column) was eluted as described and the profiles of chromatography were used for comparison of degradation. To enhance the differences, we performed the same experiment of insulin degradation at lower temperature (25 °C) to compare mitochondrial insulin accumulation and degradation.

Insulin and its degradation products were counted, characterized by its elution position and its immunoreactivity. Each peak area was calculated and expressed as a percentage of total area. Radioactivity was counted in a gamma counter with an efficiency of 83.2 % (Packard Instrument Company, Inc., Downers Grove, Illinois, USA).

Insulin degradation studies with sub-mitochondrial fractions These studies were performed in rats with or without supplementary diet but without IDE. Isolated mitochondria and its fractions were obtained as described and we assayed the insulin degradation during 60 min at 37 °C in IMS and Mx; both fractions (on diet or not) were precipitated at 60 and 80 % ammonium sulfate to compare both conditions. The intention was to concentrate IDE (30–60 %) and differentiate the enzyme from other proteases (60–80 % ammonium sulfate concentration).

- Control - IMS and Mx fractions were obtained from isolated mitochondria of rats on diet or not. In all cases IMS and Mx were precipitated with ammonium sulfate, and the 30–60 % was followed by other precipitation at 80 %.
- Insulin - Isolated mitochondria from rats without or with supplementary diet were incubated during 15 min at 30 °C with 1 ng insulin/mg mitochondrial proteins, and insulin degradation was assayed in IMS and Mx after precipitation.
- ATP - Mx and IMS were precipitated from rats supplemented or not with the diet, and insulin degradation was measured after addition of 2.5 mM ATP to each fraction.

IDE from hepatic cytosol (100000×*g*), was obtained after 30–60 % ammonium sulfate precipitation from rats without diet.

Insulin accumulation in mitochondria and mitoplasts

We calculate the insulin accumulation in mitochondria and mitoplasts as the ratio of insulin peak area to total area (expressed as percentage) in the chromatography profile.

SDS-PAGE and immunoblot Each isolated fractions were boiled in sample buffer during 3 min followed to SDS-PAGE in 7.5 % polyacrylamide gel slabs (10–45 μg/protein by line), stained with Coomassie blue or transferred to nitrocellulose membranes. IDE was detected in immunoblot with 1/500 immunopurified carboxy-terminal specific rabbit anti-IDE p15 or 1/750 monoclonal anti-IDE antibody 9B12 (kindly provided by Richard A. Roth, Stanford University, Stanford, CA) as described (Camberos et al. 2001; Camberos and Cresto 2007). Densitometer studies were performed with ScnImage software (based on NIH-Image on the Macintosh platform). Pre-staining standards were used as molecular weight controls.

Degradation studies with intermembrane space When we studied degradation in the 80 % precipitation of IMS, the fraction was desalted through filtration in Sephadex G15 (25 ml column) and eluted with 10 mM TRIS buffer, 50 mM NaCl, 1 mM PMSF and 1 mM DTT, pH 7.4. Insulin degradation in elution was made with the addition or not of 10 mM ATP, and the peaks with increased degradation were

joined, concentrated and subject to other chromatography in the same conditions. This procedure was repeated twice more.

Degradation was studied during 60 min at 37 °C with ions (10 mM MgCl2 + MnCl2) in the proteins eluted from Sephadex G15. Peaks that showed increased insulin degradation with ATP were selected, concentrated, analyzed by SDS-PAGE and stained.

Other analytical procedures Lactate-dehydrogenase (LDH) was assayed using commercially available kit (718807, Boehringer). Catalase was measured by H_2O_2 removal at 240 nm (mU/mg proteins) (Aei 1983). Proteins were determined using Bradford's methods (Bradford 1976).

Statistic Results are expressed as the Mean ± SEM. Some experiments were expressed as percent changes of its own basal to allow comparisons between Control and IDE. The statistical significance has always been calculated with the absolute values. We used the "Unpaired t test" from Prism 4 (GraphPad Software for Windows, San Diego, Ca, USA) to determine the significance between groups.

Supplementary information

Rat diet The experimental protocol was approved by the Institutional Committee of Care and Use of Laboratory Animals of the Hospital Garrahan (CICUAL). Male Wistar rats were kept in proper conditions in the animal facility at the Endocrinology Research Center. Mitochondrial insulin degradation was measured by chromatography in rats fed with commercial food. In order to establish conditions for high insulin degradation, we started to supplement commercial food with 25 g/rat of apple and 10 g/rat of hard-boiled eggs, 3 days a week, and we thoroughly studied mitochondrial insulin degradation (60 s at 30 °C). We used this dietary supplement for all experiments unless otherwise required.

Results

Insulin degradation in mitochondria at 30 °C Insulin degradation at 30 °C in mitochondrial wash was not detectable in the basal, and after 60 s of incubation it was 0.1 % (n: 7). We used more active conditions to verify if mitochondrial wash would have some enzymatic release during the experiments, incubating the same material 15 min at 37 °C and we found an insulin degradation of 0.36 %.

Chromatography of mitocondrial insulin degradation in rats with supplementary diet showed increment of degradation with the time (Fig. 1). In order to assess these results we studied degradation in mitochondria purified with Percoll from rats after a prolonged period of time (3 month) with the same diet. Chromatography of mitochondrial degradation at 30 °C (basal and 300 s) showed total insulin degradation in both cases (not shown). To obtain a quantification approach of endogenous insulin degradation by mitochondrial rat IDE, we studied at 30 °C the insulin degradation with addition of Bacitracin (without IDE) because Bacitracin competes with insulin for binding to IDE. Bacitracin decreased by 27 % the mitochondrial insulin degradation in 60 s of incubation time (Fig. S1, A), and this partial inhibition suggests that other enzyme(s) could participate in mitochondrial insulin degradation. To objectivize the participation of IDE we studied mitochondrial insulin degradation with high insulin concentrations (10000 ng/mg mitochondria) in Control (no IDE) and IDE addition (1.2 μg/IDE), and increasing concentrations of N-ethylmaleimide, a potent inhibitor of IDE. As observed in Fig. S1, B mitochondrial insulin degradation was reduced although not completely with 1 mM NEM, suggesting the

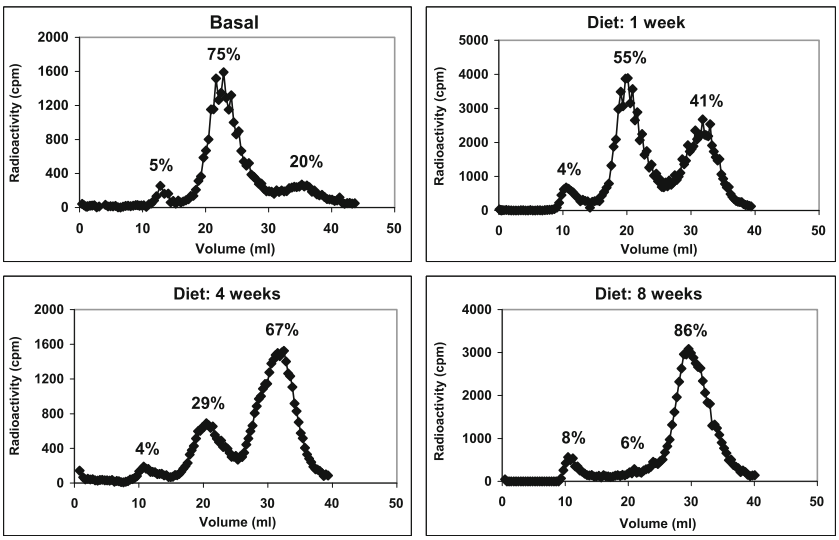


Fig. 1 Chromatography of mitochondrial insulin degradation of rats with supplementary diet. The diet of rats was supplemented with 25 g/rat of apple and 10 g/rat of hard-boiled eggs, 3 days a week. The diet was extended over more than 1 month. Insulin degradation was measured after 60 s of mitochondrial incubation as described in Supplementary Information. Peak 1: aggregated insulin. Peak 2: free insulin. Peak 3: degraded insulin. The supplementary diet increased degradation when maintained during prolonged time

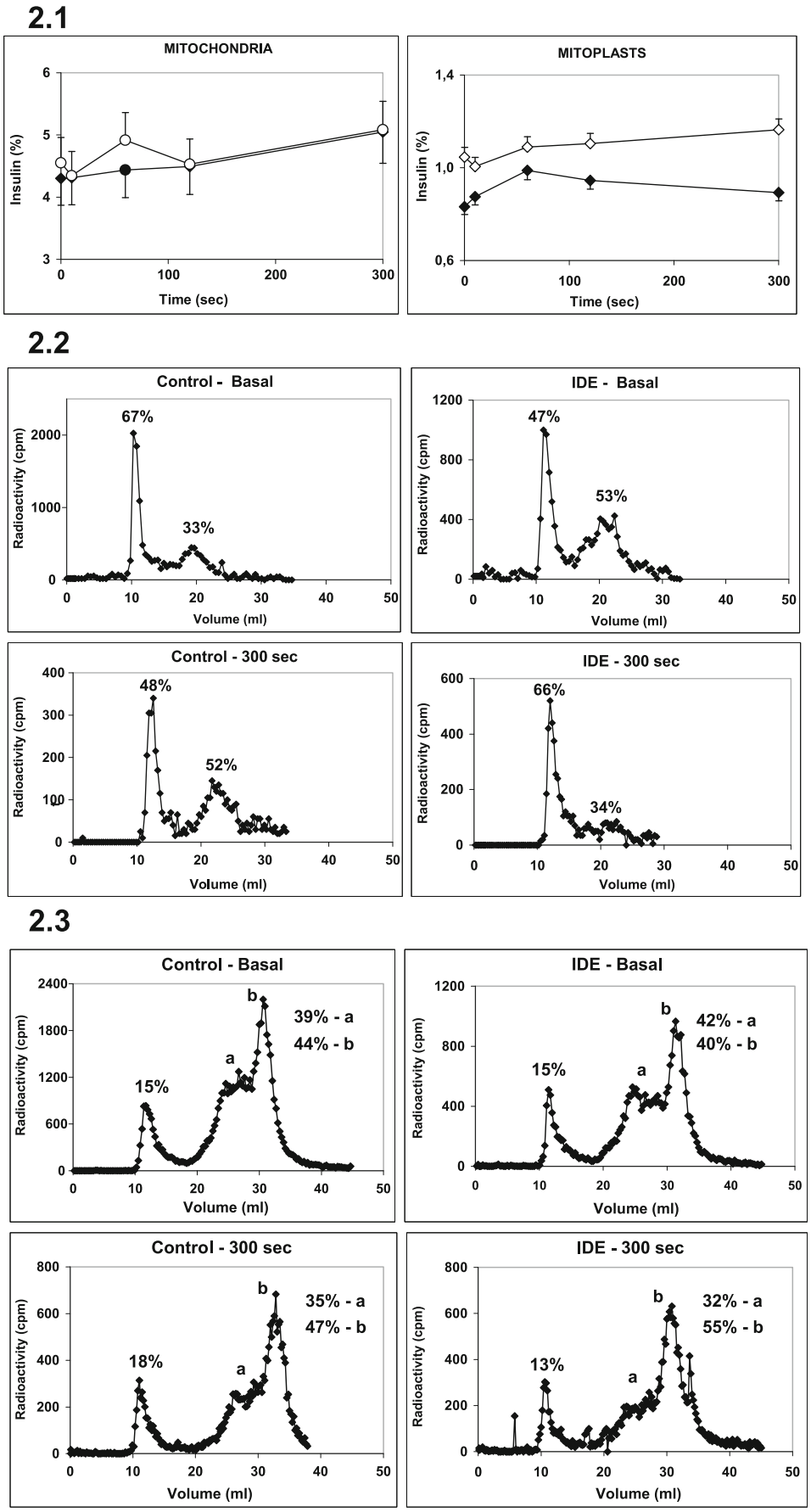


Fig. 2 Insulin accumulation and degradation in mitochondria. *2.1* Mitochondria: Control ●, IDE ○. There were not statistical differences between Control vs IDE. Mitoplasts: Control ♦, IDE ◊. IDE basal vs. Control basal: $p < 0.05$ (n: 4). IDE basal vs. IDE 300 s: $p < 0.05$ (n: 4). Control 300 s vs. IDE 300 s: $p < 0.01$ (n: 4). Increased uptake mean accumulation. *2.2* Mitoplasts; profiles of insulin chromatography. Mitoplasts were solubilized and subjected to chromatography in Sephadex G50 Superfine (25 ml column), eluted with 1 M acetic acid, 150 mM NaCl, 0.25 % BSA. Mitoplasts chromatography showed 3 peaks. The 1st was void volume, the 2nd was free insulin (1/20000 antibody excess: 70 %; n: 7); the 3rd was degraded insulin (antibody excess: 1 %, n: 6). Quantification of free insulin peaks; Control: *basal*: 33 %, *300 s*: 52 %; IDE: *basal*: 53 %, *300 s*: 34 %. *2.3* Mitochondrial insulin degradation; chromatographic profiles. The chromatography of mitochondrial incubation buffer showed 2 peaks: peak 1 was aggregated insulin, there was not peak 2 of free insulin and peak 3 had two peaks. Peak 3a was degraded insulin and peak 3b was iodinated tyrosine. Antibody excess 1/20000: peak 1: 7 % (n: 8); peak 3a: 3 % (n: 7); peak 3b: 1 % (n: 6). Radioactive areas are expressed as percentage of total area

participation of other enzyme(s) than IDE in mitochondrial insulin degradation.

Mitochondria incubated at 30 °C showed no changes of insulin accumulation in Control or IDE (Fig. 2.1); however mitoplasts showed a statistical insulin increment with IDE (Fig. 2.1). If we compared the temperature at 25 and 30 °C (Figs. 2 and 3) in Fig. 3.1 (25 °C) mitochondria and mitoplasts showed statistical differences in both cases with an increment in mitoplasts at 300 s. The differences of insulin accumulation were increased in mitoplasts at 25 °C. Mitoplasts chromatography at 30 °C showed 3 peaks; the first was in void volume position, the second was free insulin, and the third was a small peak of degraded insulin (Fig. 2.2, the percent was not calculated). Insulin characterization with antibody excess was free insulin: 70 % (n: 7) degraded insulin: 1 % (n: 6). Control had at 30 °C an increment of free insulin along the incubation time (Control: *basal*: 33 %, *300 s*: 52 %) while IDE showed the contrary (IDE: *basal*: 53 %, *300 s*: 34 %). Mitoplasts chromatography at 25 °C showed a fast first peak formation when IDE was added, followed by a decrease of this first peak with a big increment of free insulin in the second peak at 300 s (antibody excess, 79 %) (Fig. 3.2). Degraded insulin remains without changes at 25 °C.

When we studied the mitochondrial insulin degradation at 30 °C (Figs. 2 and 3), we observed 2 peaks; the peak 1 was in void volume position, there was no free insulin and the peak 3 showed 2 peaks of degraded insulin. Peak 3a was degraded insulin and peak 3b was iodinated tyrosine which appeared at the end of chromatography. To qualify the insulin distribution and degradation we controlled each peak with antibody excess; *peak 1*: 7 % (n: 8), *peak 3a*: 3 % (n: 7), *peak 3b*: 1 % (n: 6). Mitochondrial insulin degradation at 25 °C was always the same (~22 %, Fig. 3.3) and it was less than free insulin.

These results demonstrated the influence of degradation at 30 °C, suggesting that IDE in mitoplasts increased the speed of insulin accumulation and degradation, especially when degradation had been increased by the diet. The comparisons at both temperatures (25–30 °C) between the insulin accumulation in mitoplasts and its degradation led to the same conclusion.

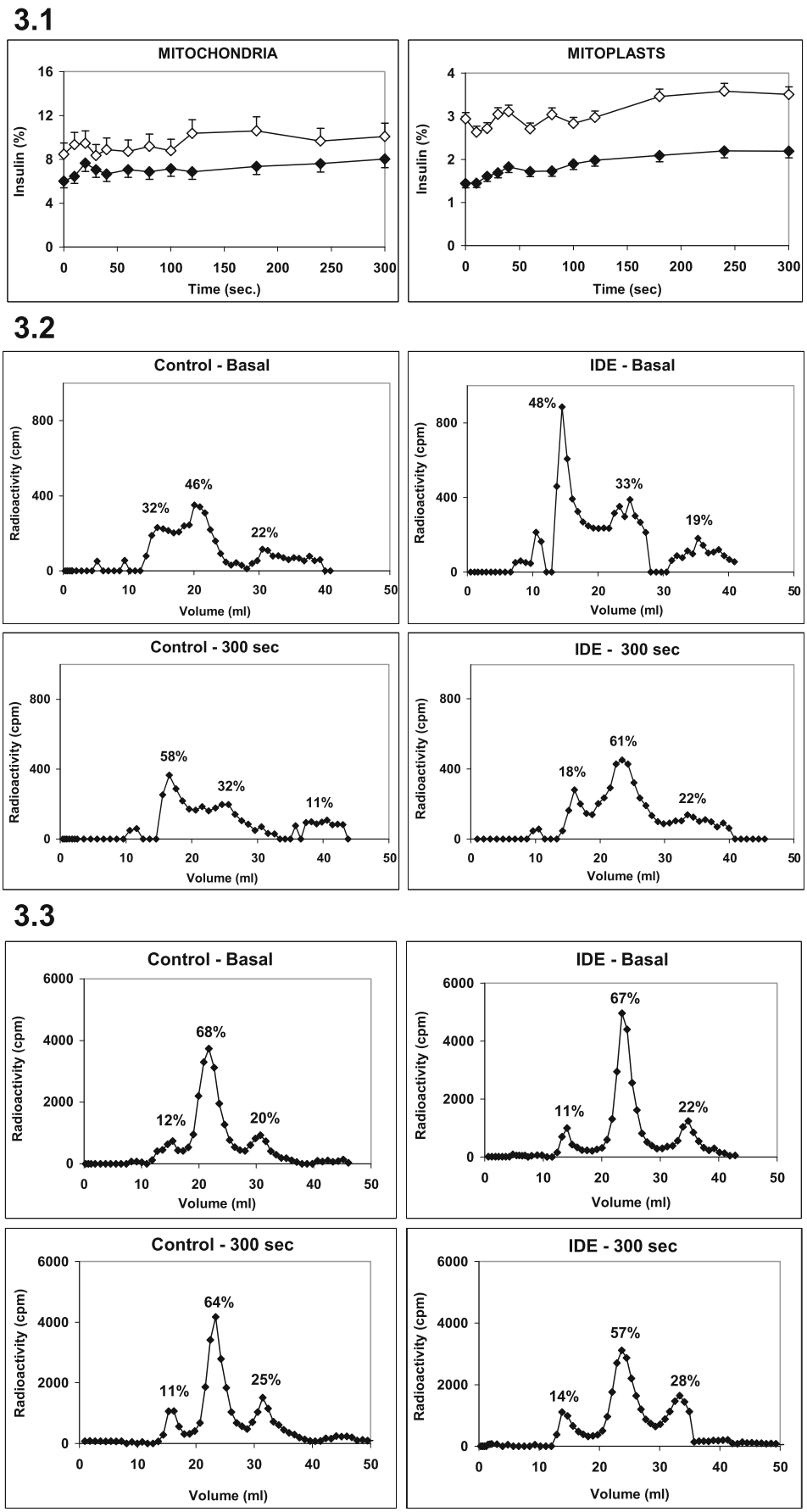
We analyzed the above results in Control and IDE with increasing insulin doses from 1 to 10000 ng/tube, with or without 0.1 mM NEM (Fig. 4). The experiment showed with 1 ng of insulin the same profile that Fig. 3.3 which changed with high insulin concentrations. The percentage of insulin in mitochondria and mitoplasts with 0.1 mM NEM doubled the insulin values of associated insulin without NEM (Fig. 4a vs. c; b vs. d), demonstrating that minimal inhibition of insulin degradation increased its accumulation in mitochondria and mitoplasts. High insulin concentrations with or without NEM (Fig. 4, a–c), did not modify its accumulation in mitochondria, but in mitoplats the insulin dose of 10000 ng was statistically lower than 1 or 10 ng, (Control or IDE; Fig. 4, b–d),

Fig. 3 Insulin incubated at 25 °C: mitochondria and mitoplasts behavior and chromatographic profiles. *3.1* Insulin accumulation in mitochondria and mitoplasts. Control ♦, IDE ◊. In mitochondria IDE was higher than Control at 180 s ($p < 0.05$, n: 4). In mitoplasts IDE was higher than Control at all times ($p < 0.01$ to 0.001, n: 4). Comparisons of mitochondria and mitoplasts between both temperatures (25 and 30 °C) showed increased insulin accumulation at 25 °C. To show the differences, 3.2 and 3.3 have the same scale. *3.2* Mitoplasts; chromatography of insulin profiles. This experimental part had been published (Camberos et al. (Camberos et al. 2014)) and it is reproduced with permission of authors. Mitoplasts were solubilized and profiles of Control and IDE were determined through chromatography in Sephadex G50 Superfine (25 ml column) eluted with 1 M acetic acid, 150 mM NaCl, 0.25 % BSA. Similar amount of counts were placed in each column (the same scale was used for comparison): 3 peaks were observed, peak 1 was void volume (exclusion size of insulin aggregates), peak 2 was free insulin and peak 3 was degraded insulin. At 300 s, free insulin was accumulated by IDE in mitoplasts and identified with insulin antibody excess (1/20000). Peak 1: 52 % (n: 6); peak 2: 79 % (n: 14); peak 3: 4 % (n. 3). Added IDE in the basal incremented very fast the first peak and increased free insulin in mitoplasts at 300 s. Radioactive areas are expressed as % values of total area. *3.3* Mitochondrial insulin degradation; profiles of chromatography. Similar amount of counts were placed in each column (the same scale was used for comparison): 3 peaks were observed; peak 1 was insulin "void volume", peak 2 was free insulin, peak 3 was degraded insulin. Identification with insulin antibody excess (1/20000) was peak 1: 5 % (n: 8), peak 2: 82 % (n: 9), peak 3: 1 % (n: 5). Mitochondrial degradation at 25 °C was similar in Control or IDE. Radioactive areas are expressed as % values of total area

suggesting that high insulin concentrations can compete with radioactive insulin showing saturation of a putative [IDE-insulin] transport system.

We studied the chromatographic profile of insulin in mitoplats from 1 to 10000 ng of insulin with and without 0.1 mM NEM in Control and IDE (Fig. 4.1, mitoplasts). These results are not showed because they exhibited a similar profile of Fig. 2.2. In order to know how much insulin can be incorporated to mitoplasts exposed to high insulin concentrations, we quantified by RIA the free insulin peak in the chromatography without NEM. We assayed only the pork insulin added, because pork insulin was the standard and the antibody used made the endogenous rat insulin assayed insignificant. Free insulin peak was (μU/ml; 3 replicates): Control *1 ng insulin*: ND, *10000 ng insulin*: 96; IDE *1 ng insulin*: 27, *10000 ng insulin*: 71. These results demonstrated that high insulin concentration in hepatic mitochondria did not induce mitoplasts accumulation.

Chromatographic studies of mitochondrial degradation with 1 ng/insulin without NEM, in Control and IDE (Fig. 4.2) showed a similar chromatography profile compared with Fig. 2.3. Addition of insulin (10000 ng) decreased the peak in void volume and appears a small peak of free insulin followed by a big peak of degraded insulin (5 % TCA: 8.2 %). The only difference was that iodinated tyrosine disappeared with high insulin doses. The addition of 0.1 mM NEM did not change the amount of insulin degradation, but iodinated tyrosine was not present throughout the study (Fig. 4.3).


3.1
MITOCHONDRIA
Insulin (%)
Time (sec.)
MITOPLASTS
Insulin (%)
Time (sec)
3.2
Control - Basal
Radioactivity (cpm)
Volume (ml)
32%
46%
22%
IDE - Basal
48%
33%
19%
Control - 300 sec
58%
32%
11%
IDE - 300 sec
18%
61%
22%
3.3
Control - Basal
12%
68%
20%
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64%
25%
IDE - 300 sec
14%
57%
28%

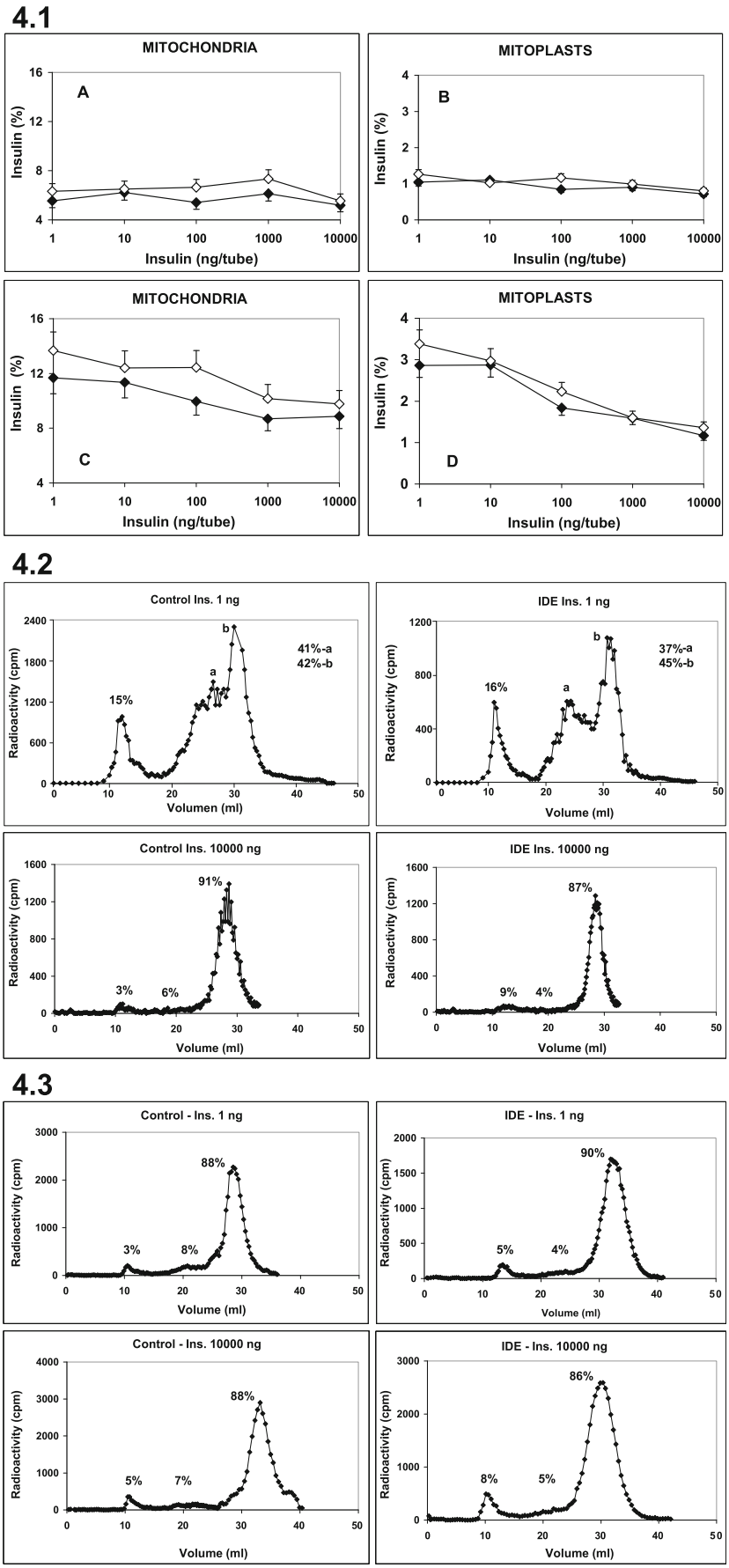


Fig. 4 Insulin accumulation and degradation in mitochondria and mitoplasts with increasing insulin doses. Mitochondrial degradation was studied in the incubation buffer and profile of degradation was determined through chromatography in Sephadex G50 Superfine (25 ml column) and eluted with 1 M acetic acid, 150 mM NaCl, 0.25 % BSA. To compare the effect of 0.1 mM NEM, mitochondria and mitoplasts have the same scale. *4.1* (**a**, **b**) - No NEM (incubation: ^{125}I-insulin ~50000 cpm during 60 s at 30 °C). **a** (mitochondria): Control ♦, IDE ◊. There were not statistical differences. **b** (mitoplats): Control ♦, IDE ◊. Control: 10 ng insulin vs. 10000 ng, $p < 0.01$ (n:5); IDE: 1 ng insulin vs. 10000 ng, $p < 0.05$ (n: 7). (**c, d**) 0.1 mM NEM (incubation: ^{125}I-insulin ~50000 cpm during 60 s at 30 °C). **c** (mitochondria): Control ♦, IDE ◊. There were not statistical differences. **d** (mitoplats): Control ♦, IDE ◊. Control: 1 ng insulin vs. 10000 ng, $p < 0.01$ (n: 4); IDE: 1 ng insulin vs. 10000 ng, $p < 0.02$ (n: 4). *4.2* Chromatography of mitochondrial insulin degradation (no NEM). There were 2 peaks with 1 ng of insulin: peak 1 was insulin aggregates, there was no free insulin and peak 3a and peak 3b were degraded insulin and iodinated tyrosine. With high insulin doses 3 peaks were observed: peak 1 was insulin aggregates; peak 2 showed small amount of free insulin and peak 3 was degraded insulin (5 % TCA: 8.2 %). Radioactive areas are expressed as percentage of total area. *4.3* Chromatography of mitochondrial insulin degradation (0.1 mM NEM). There were 3 peaks with 1 ng of insulin: peak 1 was insulin aggregates, peak 2 was free insulin and peak 3 was degraded insulin. All chromatography did not show iodinated tyrosine. Radioactive areas are expressed as percentage of total area

Insulin degradation in mitochondrial fractions at 37 °C Mitochondrial fractions were first studied in rats without diet (Fig. S2). The mitochondrial fractions had low levels of cytosolic contamination as observed in remaining LDH after isolation (mUI/mg protein, % cytosolic contamination): OM: 8.52 %, IMS: 1.43 %, IM: 0.29 %, Mx: 1.24 % (n: 3) and the same was observed with catalase as the expression of peroxisomal contamination (mU/mg protein, % peroxisomal contamination) OM: 0.35 %, IMS: 7.27 %, IM: 0.02 %, Mx: 0.17 % (n: 3). Then, IDE immunoblot concentration with antibody p15 (Fig. S2) was convincing because cytosolic and peroxisomal contamination was negligible. IDE at 110 kDa was present in IMS and Mx but a 76 kDa of immunoreactive carboxy-terminal band of degraded IDE was detected in all fractions. Insulin degradation in each fraction was (^{125}Insulin pg/mg protein, 30 min, 37 °C; n: 3) OM: 3.28 ± 0.25; IMS: 22.30 ± 1.18; IM: 7.18 ± 1.99; Mx: 27.97 ± 3.99.

We used some inhibitors to further characterize IDE degradation activity (Table 1), and there was coincidence among different inhibitors. Particularly, high insulin concentrations induced the inhibition of 90 % of insulin degradation in IM and Mx, but did not reach similar values in OM and IMS, which would suggest the participation of other protease(s) than IDE in insulin degradation activity. The result with apyrase indicates that ATP proteases did not participate, in the study conditions, in insulin degradation.

In order to compare rats with and without supplementary diet, we studied the mitochondrial fractions IMS and Mx precipitated with 60 and 80 % ammonium sulfate, because these fractions were with the highest insulin degradation. The intention was to concentrate IDE and differentiate this enzyme from other proteases. In one study mitochondria were incubated with insulin to know if the hormone affected the distribution of mitochondrial IDE and its degradation activity. In the other study, degradation was assayed in each mitochondrial fraction with the addition of 2.5 mM ATP (see Methods).

The supplementary diet produced an increment of degradation in all fractions and conditions (Fig. 5.1). When we compared the fractions of rats without diet vs. diet in Control (no insulin-no ATP) there was a significant increment with diet. When we made the same comparisons after insulin incubation (Fig. 5.1-Insulin), we observed an increment in all fractions with diet, and this increment was significant at 80 % precipitation. When studied the response of diet to ATP (Fig. 5.1-ATP) the 60 or 80 % ammonium sulfate precipitation, depend on the fraction, showed divergent insulin degradation because 2.5 mM ATP inhibits degradation in Mx and stimulates it in IMS. Cytosol (**C**) was strongly inhibited with 2.5 mM ATP.

As can be deduced from Fig. 5.1, if we compared the Control (no insulin-no ATP) in rats without supplementary diet vs the insulin response in the same group (i.e., without diet), it showed little changes after insulin incubation. The same comparison (the group with no diet) with ATP showed inverse response in Mx and IMS (Mx was statistically decreased and IMS was statistically increased).

If we compare degradation in rats with supplementary diet, Control vs. incubation with insulin showed statistical increments in 60 % Mx. The response to ATP was the same as described before in rats without diet, but with the characteristic that 80 % IMS was the highest (Fig. 5.1-ATP).

IDE immunoblot was studied with the specific monoclonal IDE antibody 9B12, whether the rats were on a supplemented diet or not. The addition of insulin, under both dietary conditions, caused increments in the density of the bands corresponding to EDI. This effect was observed both in Mx (80 and 60 % fractions) and in IMS (60 % fraction-no diet and both fractions with supplemented diet). On the other hand, IDE in rats without diet at IMS 80 % precipitation fraction was not detected. Lane C corresponds to cytosol and was used as an IDE control (Fig. 5.2).

If we intended to compare "IDE concentration/IDE degradation" in Mx vs. Cytosol (rats without diet, 60 % precipitation), it was: (Mean ± SEM) Mx 4.16 ± 0.88 (n: 8); Cytosol 6.21 ± 0.32 (n: 7) $p < 0.03$, which would indicate a decrease of IDE activity in mitochondrial matrix in relation to cytosol. The same result was obtained in rats without diet, directed to study the relationship of IDE activity in mitoplasts vs. cytosol (60 % ammonium sulfate precipitation) which is not showed.

Table 1 Effect of different agents on mitochondrial fractions

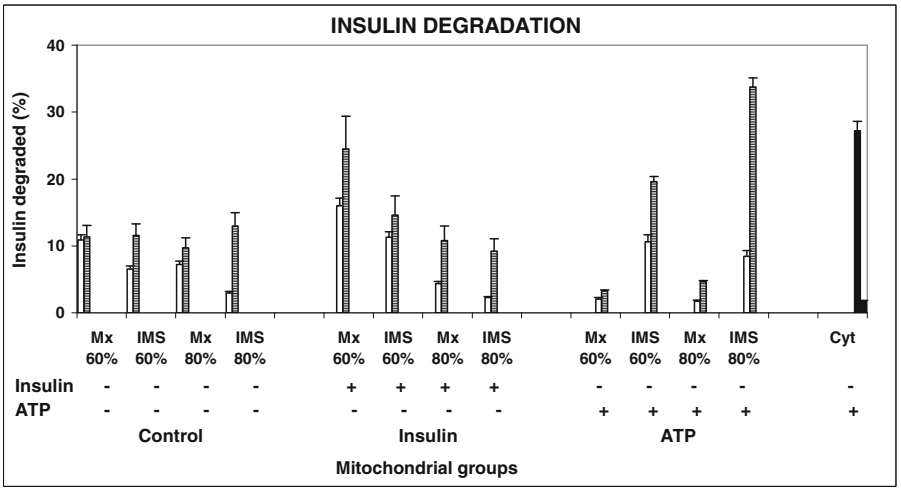
		% Inhibition of degradation			
Agent	Concentration	OM	IMS	IM	M
NEM	1 mM	98.64	95.63	96.03	97.05
1, 10-Phenatroline	1 mM	87.25	93.47	94.06	94.73
Bacitracin	1 mg/ml	77.85	65.15	90.57	87.07
PMSF	1 mM	6.43	3.00	5.32	1.02
Apyrase	25 mU/ml	0.00	0.00	7.87	0.69
Insulin	1 mM	66.98	63.91	94.54	94.10

Results are mean of three experiments in duplicate

Insulin degradation by an ATP-dependent protease The peak of IMS chromatography (80 % precipitation) showed in Sephadex G15 many lines in SDS-PAGE (Fig. 6, right). Then, we selected the proteins for its ATP-dependent insulin degradation and in a new chromatography in the same conditions we found a single band at molecular weights 85 kDa (Fig. 6, middle). In a new chromatography we selected proteins with increased ATP insulin degradation, as before from the same material. They were lyophilized and subject to chromatography in the same conditions. In the SDS-PAGE (Fig. 6, left), the protein concentration was scarce showing only one line at 56 kDa. This 56 kDa protease showed (% of insulin

Fig 5 Insulin degradation in mitochondria of rats without (□) or with diet (■). *5.1* Insulin degradation of fractions IMS and Mx (Control, Insulin and ATP). Statistical comparison of 60 % and 80 % sulfate precipitation of mitochondrial fractions. Incubation conditions: 60 min at 37 °C. Only are informed the fractions with statistical differences. *5.2* IDE immunoblot (Mx and IMS fractions). IDE immunoblot was performed using a specific monoclonal IDE antibody 9B12. Mitochondrias from rats, without or with diet, were incubated with or without insulin, matrix (Mx) and intermembrane (IMS) fractions were isolated and then precipitation fractions (60 and 80 %) were obtained from Mx and IMS. Each lane was loaded with 10 μg/proteins. **C** (cytosol) is shown as "IDE Control"

5.1



5. 2

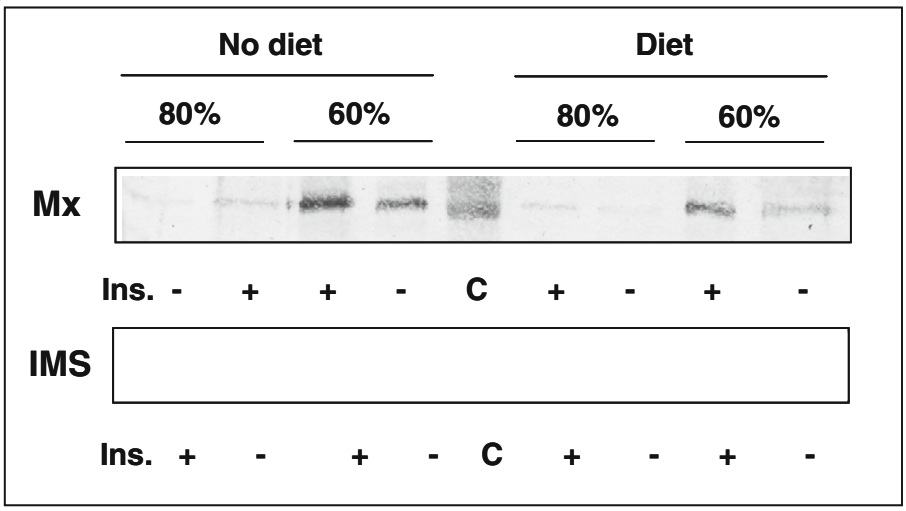


Fig. 5.1 - Statistical analysis

Rats with diet no supplemented vs. supplemented
(Only are informed the fractions with statistical differences)

Control, 60% IMS: 6.55±1.09 (n: 11) vs. 11.56±0.92 (n: 10) p<0.001
80% IMS: 2.98±0.43 (n: 11) vs. 12.99±4.10 (n: 9) p<0.008
Insulin, 80% Mx: 4.39±1.20 (n: 6) vs. 10.80±3.80 (n: 4) p<0.05
80% IMS: 2.30±0.47 (n: 11) vs. 9.22±2.45 (n: 9) p<0.003
ATP, 60% IMS: 10.61±1.46 (n: 6) vs. 19.61±2.34 (n: 10) p<0.008
80% IMS: 8.46±0.10 (n: 11) vs. 33.76 ±4.08 (n: 9) p<0.0001

Rats with diet no supplemented.
Control vs. Insulin or ATP

Insulin, 60% IMS: 6.55±1.09 (n: 11) vs. 11.31±1.78 (n: 7) p<0.01
ATP 60% Mx (*decreased*): 10.91±2.08 (n: 8) vs. 2.10± 0.36 (n: 8) p<0.0005
80% Mx: 7.24±1.94 (n: 7) vs. 1.73±0.64 (n: 6) p<0.01
60% IMS (*increased*): 6.55±1.09 (n: 11) vs. 10.61±1.46 p<0.02
80% IMS: 2.98±46 (n: 11) vs. 8.46±0.10 (n: 11) p<0.0001

Rats with supplemented diet.
Controls vs. insulin or ATP

Insulin, 60% Mx: 11.09±5.22 (n: 4) vs. 24.50±4.12 (n: 6) p<0.04.
ATP 60% Mx (*decreased*): 11.09±5.22 (n: 4) vs. 3.30±0.74 (n: 8) p<0.03; 80% Mx: 9.73±2.44 (n: 6) vs. 4.66±1.49 (n: 8) p<0.04 **-** 60% IMS (*increased*): 11.56±0.92 (n: 10) vs. 19.61±2.34 (n: 10) p<0.003; 80% IMS: 12.99±4.10 (n: 9) vs. 33.76±4.08 (n: 9) p<0.001.

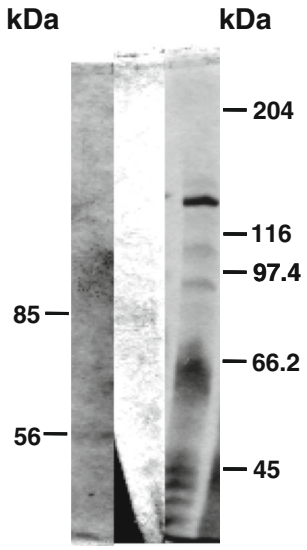


Fig. 6 SDS-PAGE of Sephadex G-15 chromatography. On the right hand side, MW standards are showed. On the left hand side, the bars indicate the MW of isolated proteins (see Methods). Right - Chromatography of IMS, 80 % precipitation. Molecular weight of proteins (from the top to the bottom): 146, 135, 125, 110, 96, 85 kDa. There were other bands with lower MW. Middle - Purification chromatography of protease ATP-dependent. There was only one band at 85 kDa. Left - Repurification chromatography of proteae ATP-dependent. There were scarce proteins. Only one line was detected at 56 kDa MW

degradation); no ATP: no detectable; ATP 2.5 mM: 2.49 %. We used ^{125}I-insulin for this experiment. With ^{125}I-insulin decay we have an insulin mass of 21.43 μU and in 60 min, for 1 μg of insulin (325.63 pg of insulin degradation). We had for 0.054 μg of proteins and degradation was (Mean ± SEM, pg/h) 325.63 ± 25.24 pg (n: 4) and protease degradation. (ng/min/mg protein): 5.43 ± 0.02 (n: 4).

Discussion

We showed that insulin was translated to mitochondria by IDE and it conform an IDE plus insulin complex. The distribution of IDE and insulin was not homogeneous in mitochondria because in confocal microscopy studies insulin was more externally distributed than IDE and both appears associated in some parts into mitochondria (merge).

To reach high insulin degradation we started to feed rats with hard boiled eggs and apples in order to obtain conditions for high and constant mitochondrial insulin degradation. Our interest was only to reach this objective, and we obtained this result with a prolonged supplementary diet. It was based on publications by several authors showing that egg supplementation demonstrated the quality dependence on protein in the case of growing rats, and the contribution of apples with minerals, vitamins, pectin, polyphenols, and other nutrients (Hao et al. 2007; Shen et al. 2008; Forbes et al. 1958; Juskiewicz et al. 2011; Miura et al. 2007). Lombardo et al and Pighin et al showed mitochondrial adaptation to changes in the diet of rats. These authors demonstrated that high sucrose diet induced insulin resistance and decreased the activity of pyruvate dehydrogenase complex (Lombardo and Chicco 2006; Pighin et al. 2003). Hein et al showed that fish oil n-3 polyunsaturated fatty acids modified many cytosolic and mitochondrial enzymes in insulin resistance rats induced by sucrose diet (Hein et al. 2010). Shen et al (Shen et al. 2008) demonstrated that a combination of cofactor and nutrients stimulates mitochondrial mRNA genes involved in lipid metabolism, increasing complex I and II in the electron transport chain of GK rats. This rat strain showed a dysfunctional IDE allele that explained most of biochemical alterations (Fakhrai-Rad et al. 2000).

The differences in free insulin and insulin degradation that showed between 30 and 25 °C (Figs. 2 and 3) could be interpreted as an increase in insulin turn-over (transfer and degradation) in a more active mitochondrial metabolism. In these conditions, [IDE-Insulin] complex as proposed in a previous paper (Camberos et al. 2014) showed a fast insulin accumulation in mitoplasts followed with mitochondrial degradation, more yet with the supplementary diet. Otherwise, the results with high insulin concentrations demonstrated that mitoplasts accumulation was not dependent on insulin concentration. Conversely, we found an apparent decrease of insulin accumulation in mitoplasts with increased insulin concentrations. High cold insulin competed with radioactive insulin on a putative mitoplasts transport system, and this competition was enhanced with 0.1 mM NEM. Simultaneously, the capacity of liver mitochondria to degrade high amounts of insulin could be observed. The chromatographic profile of degraded insulin in liver mitochondria was similar to one found before with isolated liver cells (Cresto et al. 1989).

The quantification of free insulin by RIA demonstrated a limited insulin accumulation in hepatic mitoplasts when exposed to high amounts of insulin, suggesting that insulin transfer and accumulation were dependent on other factors. Minimal inhibition of degradation with 0.1 mM NEM (an alkene agent that is reactive toward thiol groups) which is not sufficient to impede IDE activity, duplicated the insulin concentration in mitochondria and mitoplasts. Then, insulin transfer and accumulation in mitoplasts appeared to be limited by putative transport system and insulin degradation.

It was shown that IDE facilitates the transport of steroids hormones receptors to nucleus (Udrisar et al. 2005; Kupfer et al. 1993, 1994). Similar behavior was observed after castration during replacement therapy, where gold labeled IDE showed nucleus and mitochondrial accumulation (Fig. S3). Our results suggested two possibilities: either that IDE accumulated the hormone in mitoplasts or that insulin increased IDE accumulation in mitochondrial matrix. Udrisar et al (Udrisar et al. 2005) demonstrated that after androgen receptors were transferred to the nucleus, the enzyme lost its insulin degradation activity, and we found a fall of insulin degradation

in mitochondrial matrix when it was compared with the cytosolic enzyme, suggesting that matrix IDE had some regulation that decreased its insulin degradation activity.

The capacity of liver mitochondria to degrade high amounts of insulin allowed us to suppose that other proteases than IDE could participate, whether associated to IDE or not, in insulin degradation activity. The study of mitochondrial fractions with specific carboxy-terminal IDE antibody (P.15) showed IDE at 110 kDa in IMS and Mx. However, a 76 kDa of immunoreactive band of degraded IDE was detected in all fractions, with a similar profile of degraded IDE found by Hamel et al in peroxisomes (Hamel et al. 2001). Contamination with cytosol or other organelles was minimal as shown by LDH and peroxidase assays. Insulin degradation in these fractions was inhibited by known IDE inhibitors, but in OM and IMS it was not completely inhibited by insulin excess. The absence of inhibition with insulin excess in both fractions could suggest that insulin degradation in these fractions could not only be dependent on IDE. Moreover, the partial inhibitory effect with Bacitracin or NEM on insulin degradation (Fig. S1-A and B) supported the search for other proteases than IDE. The results with apyrase were not conclusive because ATP-dependent enzymes decreased their activities with apyrase when they were stimulated by ATP, and we did not know the levels of ATP in the isolated mitochondrial fractions.

Insulin degradation was increased with the diet, and this degradation was selectively modified by insulin or ATP in mitochondrial fractions. Immunoblot with specific IDE antibody 9B12 showed that IDE was increased by insulin treatment in both fractions (IMS and Mx), but Mx was the place with maximal accumulation. These results suggested that insulin induced an endogenous [IDE-Insulin] redistribution into mitochondria, because IDE was not added. The long IDE mitochondrial presequence did not argue against this redistribution because, as shown, there was an allosteric difficulty in [IDE-Insulin] mitochondrial transfer (Camberos et al. 2014), and it could be overcome by insulin excess.

The increment of insulin degradation in mitochondrial IMS with 2.5 mM ATP should not be ascribed to IDE because ATP at this concentration had an inhibitory effect on IDE insulin degradation (Camberos et al. 2001; Camberos and Cresto 2007), and this ATP concentration had also an inhibitory effect of IDE on hepatic cytosol (Fig. 5.1) as shown by other (Udrisar and Wanderley 1992). In a previous paper, we observed at 25 °C that 0.5 mM ATP directed and incremented the insulin in OM + IMS (Camberos et al. 2014). These results suggested that, at physiological ATP concentrations, insulin could be not only directed to mitoplasts by IDE, but also by IDE + ATP to IMS. ATP and polyphosphates stimulates the degradation activity of IDE on small substrates, which was not observed with large substrates like insulin (Camberos et al. 2001; Camberos and Cresto 2007; Song et al. 2004). This dual IDE behavior with insulin depending on ATP concentrations was demonstrated in cross-linking studies with purified IDE because bound insulin was incremented or inhibited between 0.5 to 10 mM ATP concentrations (Camberos and Cresto 2007).

We found an ATP-dependent protease participating in insulin degradation in IMS. A similar protease was explained by Sitte et al (Sitte et al. 1998) in IMS and described as oligomer with molecular weight up to 200 kDa. This ATP-dependent protease showed, with an ATP binding-site antibody, molecular weights of 85 and 58 kDa (Sitte et al. 1998), similar to our finding. Desautels and Goldberg (Desautels and Goldberg 1982) found in rats that 1 mg of mitochondrial proteins (supernatant of sonicated mitochondria without ATP) could degrade ~81 ng/min of insulin, and the protease described represents 7 % of this mitochondrial insulin degradation. Otherwise, we did not know how much insulin this protease could degrade in its oligomeric form.

The increment of ATP produced a fall of insulin degradation by IDE due to Vmax decrease without changes in Km (Camberos et al. 2001). McCord et al (McCord et al. 2013) showed that glycine to alanine mutation in aa. 366 and 369 decreased IDE degradation of peptide substrate by lowering Vmax without Km decrease; moreover, the amino acid sequence 331–369 from IDE had been proposed as a putative site for ATP binding (Camberos and Cresto 2007). Otherwise, it has been demonstrated that the enzyme can hydrolyze ATP with an apparent Km ~60 μM and a Vmax 500 μM, which suggests IDE can be saturated at normal cellular ATP concentrations, and this ATP hydrolysis could modify IDE conformation (Camberos and Cresto 2007; Im et al. 2007; McCord et al. 2013). It was worth considering that ATP synthesis is partially dependent on insulin, and changes in ATP concentration could regulate insulin degradation (Camberos et al. 2001).

In a recent paper, (da Cruz and Seabra 2014) in a computational model of IDE, it was shown that the association of IDE, ATP and amyloid-ß42 inhibits IDE degradation by keeping the enzyme in a close state. In this model, a supplementary energy was required to open the enzyme and release the degraded substrate. Purified IDE + ATP have a tendency to keep IDE in an open state, but the interaction with large substrates like ß-amyloid keeps IDE in a closed state. This result suggests that a closed state was physicochemical more stable when IDE and ATP were associated to large substrates, explaining the contradictory results of ATP on large substrates.

For the above experimental results, we might suggest that ATP binding to IDE in the glycine-rich zone can induce the enzyme to adopt a conformation with insulin bound without degradation, facilitating mitochondrial transfer, matrix accumulation, and its release due to ATP increments in matrix. The association of these parameters {[IDE-Insulin] + ATP} suggests that ATP could not only contribute to insulin transfer from OM + IMS to matrix (Camberos et al. 2014), but also

as an insulin transfer from cytoplasm to mitochondrial IMS for insulin degradation.

Conclusions

Insulin degradation forms part of the mitochondrial activity, and it was not associated with any mitochondrial function. The supplementary diet increased mitochondrial insulin degradation and the activity of ATP-dependent protease in IMS. We showed that liver mitochondria participated actively in insulin degradation, and a decrease in insulin degradation improved its mitoplast accumulation. Mitochondrial incubation with insulin increased IDE in matrix. High insulin levels did not induce insulin accumulation in liver cells mitoplasts, and saturated a possible insulin transport system. ATP, an expression of cell energy, appears to be a regulator of insulin degradation.

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Authors' contributions All of the authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. MCC and JCC conducted the experiments. JCC wrote the manuscript. AP, GP, LCM performed the insulin degradation studies. MIW and DPU participated in the antibody production and paper discussion.

Compliance with ethical standards

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Competing interests All authors state that they have not been paid for the work and they have not any economic interest on the present work.

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