Implications of Antioxidant Enzyme Paraoxonase 2 in Insulin Synthesis and Secretion

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The diabetes epidemic nowadays appeared at the same time with the obesity epidemic their relationship being a causal one. The protection offered by paraoxonase 2 (PON2), a recently discovered member of the paraoxonase multigene family that has been proposed to have antioxidant properties, is still a matter of debate. We aimed to investigate if antioxidant enzyme PON2 is affected by glucose (glucotoxicity) and lipids (lipotoxicity) levels in β-pancreatic cells and if these, in turn, are related to functional changes in the synthesis and secretion of insulin. We determined the PON2 in β-pancreatic cell lines INS-1 (rat) and PANC-1 (human). Cells (2X10⁴ cells) were incubated with different concentrations of D-glucose (no glucose; hypo and hyperglycemia) in the presence or absence of either oleic acid (50μM), palmitic acid (1mM) or a mixture of them (oleate/palmitate:2/1). The cell lysates and media were separated by Tricine–SDS-PAGE and the insulin and PON2 were visualized by Western Blot and/or ELISA. With our model of in vitro islets, we showed that intracellular insulin accumulation was lower at higher glucose concentrations and this was associated with insulin secretion and PON2 changes in all cell lines studied. The effect was amplified by adding oleic acid in a combination with palmitic acid. Our findings suggest that PON2 is present in β-pancreatic cells and could protect against gluco and lipotoxicity being useful for prevention of type 2 diabetes obesity associated complications.

Key words: β-pancreatic cell lines, Paraoxonase2, Obesity, type 2 diabetes mellitus

Diabetes mellitus (DM) is a major health problem in the world. There were about 157 millions diabetic patients in 2000, and about 268 millions people are expected to be diabetic by the year 2025 in the world [1, 2]. Diabetes prevalence has increased constantly in Romania, over the past two decades due to lifestyle changes (increase overeating, overweight, and inactivity, alteration of dietary patterns) coming with the transition period in our country. The statistics shows that the prevalence has increased from 1.98% in 2003 to 3.5% 2011. In 2010 a number of 564,896 patients where diagnosed with diabetes, and in 2011 almost 590,000 [2].

Type 2 diabetes (DT2) is associated with a range of metabolic changes including impaired glycemic homeostasis, dyslipidemia (hypertriglyceridemia, low levels of high-density lipoprotein cholesterol (HDL-C), small dense LDL particles), hypertension and increased levels of markers reflecting oxidative, inflammatory, and thrombotic pathways. The major factor responsible for developing these abnormalities is considered to be the development of insulin resistance in association with abdominal obesity [3, 4].

Available data suggest that some of the cytokines arising from adipose tissue may be partly responsible for the metabolic, hemodynamic, and hemostatic abnormalities associated with insulin resistance [5-7]. In association with obesity and the metabolic syndrome, diabetes is considered a typical vascular disease, being an independent risk factor for atherosclerosis [8, 9]. The *epidemic* character of diabetes which precedes the *epidemy* of obesity is an important observation for the type 2 diabetes pathogeny.

Despite the numerous studies devoted to interrelation between type 2 diabetes/obesity, the pathogenic link is not yet clear. The main reason could be that diabetes is a *syndrome* including numerous phenotypes, each of them with their genetic background. For DT2 the recent GWA (Genome-Wide Association) studies have identified ~35 genes [10, 11], some of them related both with obesity and DT2. However, the majority of genes with known function are encoding the pancreatic β-cell molecules. The major defect consists in the incapacity of the pancreatic β-cell to produce *mature secretory vesicles.* In such vesicles the complex enzymatic mechanism of promolecules processing (proinsulin and proamylin) is not completely fulfilled, so that the proinsulin remains partially unsplitted. An immature secretory vesicle (SV) can not respond promptly and efficiently to the increase in blood glucose levels, explaining the progressive decompensation of blood glucose regulation [12-15].

The results of the research we carried out in the last 4 years on the basis of clinical, epidemiological, biochemical and hormonal data have proven that in the case of newly-diagnosed type 2 diabetes mellitus, and even in the early stages of prediabetes/metabolic syndrome (impaired fasting glucose-IFG and impaired glucose tolerance-IGT), serum levels of proinsulin and insulin, as well as the ratio between proinsulin/insulin are all increased. This disorder occurs early in the natural history of diabetes mellitus, and could be detected several years before the irreversible decompensation of glycemic regulation mechanisms [16-19].

Beta-pancreatic cells are secretory cells specialized in the production of insulin in response to an increase in blood glucose levels. Although glucose is the main metabolite responsible for the regulation of insulin secretion in beta pancreatic cells [20], other metabolites that can stimulate insulin secretion have also been described, such as free fatty acids as well as incretin hormones like glucagon-like peptide-1 (GLP-1) [21].

In addition to stimulating insulin secretion, glucose also stimulates insulin synthesis by increasing transcription and translation [22]. Being secretory cells, beta pancreatic cells must ensure a high quantity of insulin, which is why they are susceptible to certain stressors. In order to adjust to this high demand, beta cells possess a very well-developed endoplasmic reticulum [23]. Therefore, there is a high demand for protein folding, and if the endoplasmic reticulum does not meet this demand, the stress inside the reticulum intervenes, which then triggers an unfolded protein response (UPR) [23, 24].

The UPR is also involved in establishing the protein folding capacity of the endoplasmic reticulum, requiring cellular effects that can reduce stress inside the endoplasmic reticulum, through a transient attenuation of translation, as well as an increase in chaperon protein expression and an increase in the degradation ratio of incorrectly folded proteins [24]. In mammalian cells, this process is mediated by three stress sensors inside the endoplasmic reticulum (PERK, IRE-1 and ATF4), which sense the increased levels of unfolded proteins in the reticulum and transmit cascade signals. The PERK pathway, by phosphorylating eIF2α, transiently attenuates translation and induces a change in the expression of several genes, by increasing the expression of the transcriptional factor ATF4. The IRE-1 pathway, through the ARNm XBP-1 splicing, causes an increase in XBP-1 translation, whereas ATF6 is activated through proteolytic regulations once it is transferred inside the Golgi apparatus. XBP-1 and ATF6 regulate the induction of most genes involved in UPR [24].

Chronic or persistent stress in beta pancreatic cells can induce apoptosis [25]. In conditions such as obesity, in the presence or even in the absence of diabetes mellitus, where insulin demand is high, beta pancreatic cells can be susceptible to programmed cellular death. These cells are also sensitive to oxidative stress, possibly due to the fact that beta pancreatic cells express low levels of antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, which work together to remove reactive oxygen species [26]. In these chronic conditions they can cause adverse effects such as decreasing the expression of insulin, increasing cellular demands as well as cellular death. Both endoplasmic reticulum stress and an increase in oxidative stress levels can lead to the accumulation of incorrectly folded proteins or aggregated proteins within cells [24]. It is very important for these cells to maintain an optimum level of insulin secretion. An excess in insulin secretion decreases blood glucose levels, leading to an onset of hypoglycemia, and consequently a decreased blood glucose level produces a decrease in insulin synthesis [27].

Paraoxonase 2 (PON2) is a recently discovered member of the paraoxonase multigene family that is ubiquitously expressed in cells and like its family member PON1, present in serum HDL, has been proposed to have antioxidant properties [28, 29].

The aim of these study was to investigate if antioxidant enzyme PON2 is affected by glucose and lipids (oleic versus palmitic acid) levels in β-pancreatic cells and if these, in turn, are related to functional changes in the synthesis and secretion of insulin.

Experimental part
Reagents, Cell Lines and Treatment
The pancreatic beta cell lines INS-1E (rat) and PANC-1 (human) were grown in RPMI 1640 medium, either EuroClone containing 11.1 mmoli/L D-glucose, 10% Fetal Bovine Serum (FBS), 10mmoli/L Hepes, 2mmoli/L L-glutamin, 1mmol/L piruvat de sodiu, 50μmoli/L β-mercaptoetanol, 100U/mL of penicilin and 100μg/mL of streptomycin (Sigma, St Louis, MO, USA) for INS-1E, or Life Tehnology 10% FBS, 100U/mL of penicilin and 100μg/mL of streptomycin (Sigma, St Louis, MO, USA) for PANC-1 respectively. Cells were kept in humidified incubators at 37°C containing 5% CO_2 and were passaged by trypsinization and subcultured every 2 to 3 days of culture.

Before experiments cells were seeded at $2X10^4$ in 2mL corresponding medium per well in a six-well plate. After 24h, the medium was replaced with RPMI 1640 as above but with no glucose or 5mM, 11.1mM, 33.3mM and 100mM containing either BSA or BSA coupled to oleic acid (OA) or palmitic acid (PA) (final concetration 50μM for OA or 1mM for PA, respectivelly). Cells were analized 24 h after tratment. Cell viability by Trypan Blue exclusion was always ≥ 90%.

Glucose, oleic acid, palmitic acid, Bovine Serum Albumin (BSA) and Trypan Blue were purchased from Sigma Chemical, St Louis, MO, USA. Palmitat and oleat were precomplexed to FFA-free bovine serum albumin (BSA) at a 2:1 (fatty acid:BSA) molar ratio.

Western blot and ELISA measurements
After 24h different treatments medium was collected and cells were lysate with HEPES-CHAPS buffer containing Hepes 50mM, Chaps 2%, NaCl 200mM (pH 7.5) and a mixture of protease inhibitors (Sigma-Aldrich) for 1h, on ice. Cell lysates were centrifuged 10min at 10000g and the protein content was determined using the BCA method (Protein Assay kit, no. 23225, Thermo Fisher Scientific), following the protocol supplied by the manufacturer. Before SDS-PAGE and Western blotting the supernatant was boiled in the presence of 5mM DTT (reducing contitions). Supernatants were separated in Tricine SDS-PAGE gels and transferred to nitrocellulose membranes using a semi-dry blotter (Millipore). Equal loadind of protein (50μg) between lines was confirmed by Coomasie staining and subsequent β-actin immunoblots. Membranes were incubated in primary antibodies diluted in 5%BSA in Tris-buffered saline with 0.05% Tween (1:200) for 1h followed by the addition of goat anti-mouse (Santa Cruz, 1:10000 dilution) or donkey anti-rabbit antibody (Santa Cruz, 1:5000 dilution) 1h at room temperature and further immunodetection was performed by using an enhanced chemiluminescence (ECL) detection system (Amersham, UK) according to the manufacturer's instructions. The following primary antibodies were used: anti-insulin (H86 Sc-9168; 200μg/mL; Santa Cruz; 1:300 dilution) and antiPON2 (sc-373981 (D-12), 200μg/mL; Santa Cruz 1:200 dilution)

The human proinsulin and human/rat insulin enzyme-linked immunosorbent assay (ELISA) kits for PANC-1 cells were obtained from DRG Intruments GmbH, Germany while kits from Mercodia were used for proinsulin determination for INS-1E cells according to the manufacturer's instructions.

Results and discussions
Characterization of the beta-cells
It is well known that diabetes is one of the most severe and debilitating chronic diseases, and despite all efforts of finding efficient therapeutic methods for the management
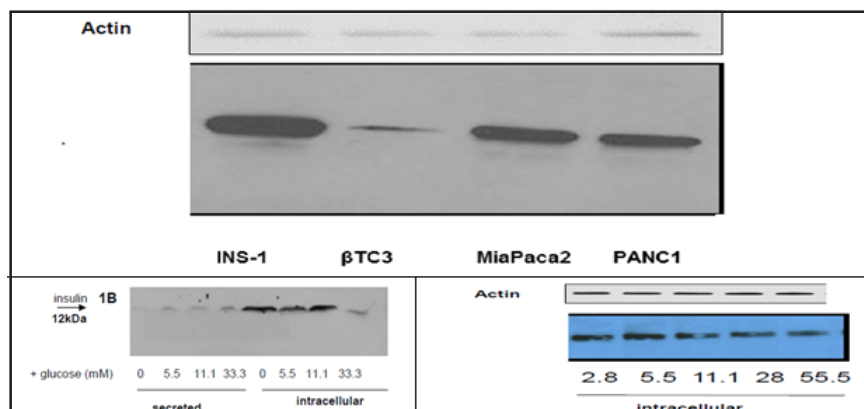
Fig. 1. Expression of PON2 in beta pancreatic cell-lines (1A); secreted and intracellular insulin in INS-1E cells incubated with different glucose concentrations (1B) and intracellular PON@ in PANC-1 cells incubated with different glucose concentration (1C)
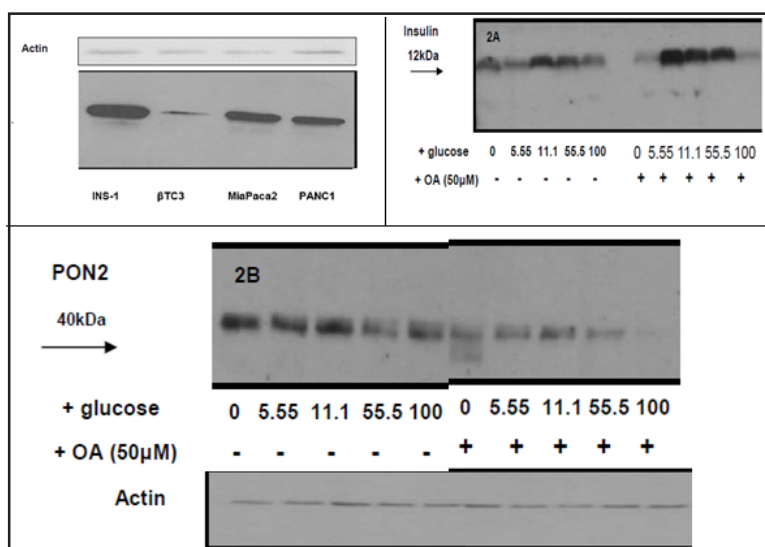

Fig. 2. Expression of secreted and intracellular insulin with different glucose concentrations (2A); intracellular insulin (B) and PON2 (2C) after glucose and 50μm OA (2C) treatments in INS-1E cells

of this affliction, insulin still remains the only efficient treatment, as pancreatic islet transplants and generation of beta pancreatic cells from stem cells have encountered several difficulties in obtaining either donors or an efficient coupling of glucose with insulin generating cells. Keeping in mind that a loss in beta pancreatic cells represents an essential factor in the onset of diabetes mellitus, it is essential to have an understanding of beta cell physiology, mainly the synthesis and secretion of insulin, as well as the way in which these processes can be affected during the progression of this disease. Moreover, the development of new therapeutic resources for the management of diabetes mellitus, such as pancreatic islet transplant, maintenance and replacement of beta pancreatic cells or stem cell therapy require a profound knowledge of how the presence of different nutritional substances and signals can regulate insulin secretion as well as beta cellular mass. About 40-60% of patients with diabetes develop diabetic neuropathy, a severe chronic complication for which new methods in proteomics for initial assessing were develop in recent years [30]. The aim of these study was to investigate if glucose and lipid excess (gluco and lipotoxicity) influences the insulin and PON2 levels.

The presence of PON2 in selected cell lines is shown in figure 1A. Results after glucose stimulation with 5.5, 11.1 and 33.3mM for INS-1E cells are shown in figure 1B while after glucose 2.8, 5.5, 11.1, 28 and 55.5mM are shown in figure 1C. We observed decreased levels of insulin at higher glucose concentrations in both cell lines. When OA (50μM) was added the intracellular insulin decreased even more (fig. 2A and 2B) and these was accompanied by PON2 decreasing (fig. 2C).

The process of insulin exocytosis involves the presence of several proteins. The increase in blood glucose triggers quickly, in less than a minute, mature secretory vesicle exocytosis, already crowded near the cell membrane, forming vesicles group ready to be exocitate. These are mature secretory vesicles (VS), meaning they were held in all the processing necessary that those vesicles can be promptly and effectively exocitate [31]. When the message is to increase insulin synthesis begin the complex process of transcribing approximately 70 molecules that are contributions to the formation of new secretory vesicles (about 70 different genes, 70 different molecules mRNA/tRNA and will be produced about 70 polypeptide, preproinsulin molecule being only one of them). Except the main 3 secretory molecules, insulin, C-peptide and amylin, the other molecules will form secretory vehicle will be introduced, along with numerous enzymes, chaperon, zinc transporters, ion channels (for calcium and other ions) proton pump, and other molecules that compose the secretory cell membrane. All these molecules will be found and will be assemble in the form of vesicles inside the ER membrane provisional immature. All processes mentioned are taken not more then 10-20 min. Mini nascent vesicles are then retrieved from the Golgi apparatus, their function depending on the achievement of specific physicochemical environment (pH of 5-5.5 acid; high concentration of Zn^{2+}, Ca^{2+}), allowing unfolding post-translational processing of the two molecules secretory proinsulin and proamilina. First, by a split between two successive acidic amino acids (Arg 31 and Arg 32) mediated proconvertaza 3/1 (resulting in a 32-33 split proinsulin to insulin semisplitata called) and then between other two acidic amino acids (Lys 64 and Arg 65), mediated proconvertaza 2 (resulting in a split and at the other end of the molecule, producing split proinsulin 65.66). Since these two splits are not synchronous, the secretory
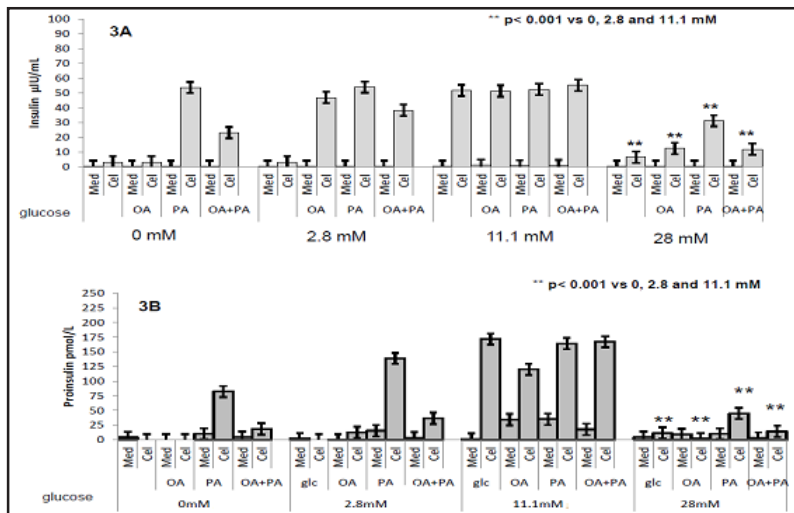
vesicle (and therefore systemic circulation) can be determined 2 molecules split proinsulin incomplete. Proinsulin concentrations of intact or partially split are elevated in type 2 diabetes, expressing â cell dysfunction of variable intensity. Normally, splitting proinsulin must be complete, freeing mature insulin (51 amino acids) and C-peptide (31 amino acids). Following these splits at the ends of the molecule of insulin or C-peptide remains one acidic amino acid (Arg or Lys) which will be quickly removed by carboxypeptidase H (or E). A vesicle containing about 95% mature insulin and C-peptide and proinsulin less than 5% intact or partially cleaved. Increasing for instance of proinsulin in mature secretory vesicle means an immaturity of their molecular consequence of a defective b-cell different from a diabetic phenotype to another [31, 32].

Both β-pancreatic cell lines selected are very sensitive to glucose concentrations [33] and we decided to go further for hypoglycemia with 2.8mM and for hyperglycemia with 28mM and we did 3 experiments in duplicate with the same concentrations of OA and PA. The ELISA results are presented in figure 3A and 3B. We observed decreased insulin and proinsulin levels after saturated and unsaturated acids treatments and the difference was higher when PA and OA were associated with high glucose as well.

Despite the fact that high blood glucose level and reduction of advanced glycation end-products (AGE) are the major contributors to the development of vascular complications in diabetes mellitus [34] and most studies were performed on animal models [35, 36] an increasing body of evidence links profiles of fatty acids into the relation between dietary lipids and the development of diabetes and cardiovascular diseases [37, 38].

Conclusions

In conclusion, by using cell lines β-pancreatic as an experimental model for the study of diabetes, we showed that the intracellular insulin was lower when concentrations of glucose was higher and this was associated with changes to both PON2 and insulin secretion. The effect was amplified by the addition of oleic acid in combination with palmitic acid and high concentration of glucose. Given that oxidative stress is thought to be a major initiator of atherosclerotic lesion development, it is likely PON2 activity modulation may be a useful approach for preventing atherosclerotic vascular diseases. Further studies are needed to discover the mechanisms underlying such effects.

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