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Research Article

Differential protein expression in pancreatic islets after treatment with an imidazoline compound

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Abstract. The effects of an imidazoline compound (BL11282) on protein expression in rat pancreatic islets were investigated with a proteomic approach. The compound increases insulin release selectively at high glucose concentrations and is therefore of interest in type 2 diabetes. Whole cell extracts from isolated drug-treated and native pancreatic rat islets were compared after separation by 2-D gel electrophoresis. Differentially expressed proteins were identified by mass spectrometry; 15 proteins were selectively up-regulated and 7 selectively down-regulated in drug-treated islets. Of special interest among the differentially expressed proteins are those involved in protein folding (Hsp60, protein disulfide isomerase, calreticulin), Ca^{2+} binding (calgizzarin, calcyclin and annexin I) and metabolism or signalling (pyruvate kinase, alpha enolase and protein kinase C inhibitor 1).

Keywords. 2-D gel electrophoresis, pancreatic islets, proteomics, type 2 diabetes, imidazolines, BL11282.

Introduction

Proteome analysis by comparison of patterns in 2-D gel electrophoresis (2-DE) of cells obtained from normal versus specific disease states has been used to trace disease marker proteins [1]. Although, for example, membrane proteins and scarce compounds constitute well-known problems in 2-DE analysis [2], useful information on particular proteins can be obtained using this technique. For example, this laboratory has defined patterns and markers in the development of colon and prostate cancer [1, 3] as well as between anatomical zones of normal prostate [4] and in an endothelial tissue [5]. Other laboratories have studied human, rat and mouse pancreatic islets and effects on the latter by glucose [6–9]. We have now used the 2-DE technique to analyze protein patterns between native cells of rat pancreatic islets and cells treated with an imidazoline compound, BL11282 (Formula 1), which possesses insulinotropic and anti-hyperglycaemic activity [10, 11].

Type 2 diabetes is characterized by a defective insulin response in the tissues relative to the insulin release from the pancreatic β cells. Compounds influencing pancreatic insulin release are therefore of particular

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interest in type 2 diabetes. In this regard, imidazolines have been shown to have an insulinotropic effect, and one such compound, BL11282, increases insulin release selectively at high glucose concentrations [11]. This imidazoline derivative is therefore of special interest since such a compound, if developable into a drug, may be effective without risk of insulin shock from hypersecretion in subjects with low or normal blood glucose.

The glucose-dependent insulinotropic activity of the present compound is not based on a K^{+}_{ATP} channel activity but is expected to be mediated through binding to a receptor or a regulatory protein [11]. Another imidazoline (RX871024) protects β cells against apoptosis [12], but such an effect has not yet been reported for BL11282. The aim of this study is to compare, using a proteomic approach, protein expression pattern of pancreatic islets that have lost their responsiveness to BL11282 (desensitized to BL11282) with control islets in which BL11282 stimulates insulin release. Interestingly, we detected a limited number of differentially expressed proteins, and identification of many of them revealed a few groups of selectively affected proteins. Although the compound itself is not yet a direct drug candidate, the results of this study indicate that treatment of pancreatic islets with this imidazoline type leads to changes in expression of islet proteins that can be detected by 2-DE.

Materials and methods

Sample collection. The study was approved by the Ethics Committee for Animal Research at the Karolinska Institutet. Pancreatic islets were isolated from 2- to 3-month-old male Wistar rats (200–250 g) by collagenase digestion [13]. Samples of 150 islets from normal rats were placed into Petri dishes and incubated in RPMI 1640 culture medium supplemented with 5.5 mM glucose, 10 % v/v heat-inactivated foetal calf serum, 2 mM glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin without (control islets) or with 50 μM BL11282 (conditions for desensitization to BL11282) at 37°C for 18–20 h. These conditions give desensitization without noticeable apoptosis [14] and maximal insulin response to the compound [11]. Each experiment required 2–4 rats, the pooled islets of which were divided for the treatment and the control. Following incubation the islets were transferred to Eppendorf tubes and washed three times in PBS buffer, followed by snap-freezing in liquid nitrogen. Samples were stored at –80°C.

Protein extraction and sample preparation. Cell lyses and protein extractions were carried out essentially as described [15]. Samples were dissolved in 6× w/v solubilisation buffer containing 9 M urea, 65 mM dithiothreitol (DTT), 0.5 % octylphenyl-polyethylene glycol (Igepal CA 630), 1.5 % 3-[(3-cholamido propyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 5 % ampholyte 3–10, 35 mM Tris and complete mini proteinase inhibitor mix (Roche Diagnostics). Protein extracts were centrifuged for 15 min at 13 000 × g to remove cell debris, and supernatants were carefully transferred to new Eppendorf tubes. Protein concentrations were determined (after 50-fold dilution) by the Bradford assay [16].

2-D gel electrophoresis. Isoelectric focusing was performed using pre-casted immobilized pH-gradient (IPG) strips with a non-linear gradient from 3–10 (Bio-Rad). Samples (75 μg protein for analytical gels and 340–480 μg for preparative gels) were applied via active in-gel rehydration using rehydration buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 0.5 % Igepal CA 630, 0.5 % ampholyte 3–10 and 2.8 mg/mL DTT) to a final volume of 300 μL. Usually, a sample of 150 islets was sufficient for 1–2 analytical gels, while preparative gels were always obtained from pooled material. The isoelectric focusing was carried out in a PROTEAN IEF cell (Bio-Rad) at 20°C as follows: 12 h at 50 V (active rehydration), linear gradient to 500 V for 1 h, kept at 500 V for another 1 h, linear gradient to 2000 V for 2 h, linear gradient to 8000 V for 1 h and then held constant at 8000 V for 3.5 h. After isoelectric focusing, the strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS and a trace of bromphenol blue) with 2 % DTT, followed by 15 min with 2.5 % iodoacetamide in the same buffer. In the second dimension of SDS-PAGE, proteins were separated on 12 % gel (1.5 × 260 × 200 mm) in an Ettan™ DALTsix system (GE Healthcare) at constant current, 15 mA/gel for 1 h, followed by 40 mA/gel for an additional 6.5 h until the bromophenol dye front was 1 cm from the gel bottom. Analytical gels were stained with silver nitrate ("Long method A" in [17]), and preparative gels were stained with colloidal Coomassie G-250 or Sypro Ruby (Bio-Rad), followed by scanning at an 84.7 × 84.7 μm resolution with a flat-bed scanner GS-710 (Bio-Rad) and a fluorescent scanner Molecular Imager FX (Bio-Rad), respectively.

Image analysis and spot selection. Scanned gel images were analyzed with the PDQuest™ software (Bio-Rad, version 7.3.0) for spot quantification and matching using a Gaussian model for spot detection [18]. Background was subtracted and spot intensity quantified as ppm of the total integrated optical density. The individual quantities of resolved protein spots were normalized to the total intensity of valid spots. One match set containing 12 gel images was created, with all gel images in the match set merged into a master image (Fig. 1), a synthetic visualisation of the spots containing spot data of all gels [19]. Quantitative analysis tools within the PDQuest™ software were used to find spots that differed between treated and control islets. The scanned gel images in the match set were analysed as two groups, control (A) and treated (B), each group containing gel images from six islet isolates. The analysis tool "outside limits" was used, and ±1.5-fold change was set as the limit. A threshold value was thereby established by multiplying or dividing A by the factor 1.5. B was then compared to the threshold. The selected spots were manually examined, and only selections in which the spot of interest was of good quality and present in the entire match set were included. The histograms created from the analysis were also examined manually to verify that the trend was accurate.

Spot picking and in-gel digestion. Spots on Coomassie-stained gels were excised manually, and those on Sypro Ruby-stained gels were excised with a spot-cutter robot (Bio-Rad, Hercules, CA, USA). Proteins were in-gel digested with trypsin using a MassPREP robotic protein handling system (Waters) as described [3]. Peptide extracts from equivalent spots on 1–4 gels were pooled and concentrated under a stream of nitrogen to 5–10 μL.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using a Q-TOF Ultima mass spectrometer employing data-dependent acquisition over a mass range of 300–2000 m/z (Waters) and a Waters Atlantis C_{18} LC-column (3 μm, 100 Å, 75 μm ID × 15 cm) after desalting with a LC Packings Nano-precolumn cartridge (300 μm ID × 1 mm; 5 % acetonitrile/0.1 % formic acid; 20 μL/min). The peptides were eluted with a linear gradient of 14–50 % acetonitrile in 0.1 % formic acid for 20 min at 200 nL/min and introduced into the mass spectrometer using a Pico Tip sprayer. Data analysis was performed using ProteinLynx Global SERVER 2.1 (PLGS 2.1, Waters) software and MassLynx peptide sequence software version 4.0 (Waters). Data sets were analyzed using the NCBI BLAST and Mascot search engines against Swissprot. The genomic database of *Rattus norvegicus* was chosen for both search methods and with no constrictions regarding molecular weight or pI. The Mascot search was set for carbamidomethylation of cysteine residues and to allow for oxidation of methionine residues. The sequences found by Mascot were manually con-

firmed. We found examples where Mascot missed peptides by selecting the wrong precursor ion; therefore all the LC profiles were also examined manually. Mascot compares the mass values of observed product ions with mass values calculated for theoretical product ions from peptide sequences present in a specified genomic database. The Mascot score for an MS/MS match is based on the absolute probability that the observed match between the experimental data and the database sequence is a random event. The threshold for significance was chosen as $p<0.05$. For identification, we required either a significant match in the Mascot search or a peptide acquired from a high-quality spectrum with a minimum length of ten amino acid residues, combined in both cases with a BLAST search showing only one relevant protein match with a low Expect value. The Expect value reflects, like the Mascot score, the probability that the sequence match is a random event and should consequently be low. Expect values were typically in the order of 10^{-4} to 10^{-10} (with the next match 3–5 orders of magnitude higher and in most cases above 1).

Results

Protein extracts from six imidazoline (BL11282)-treated and six non-treated isolations of pancreatic islets were separated by 2-DE. Each set of treated/non-treated isolations was processed in parallel through protein extraction, 2-DE and staining to avoid differences from sample handling. The resulting 12 gel images were analyzed and evaluated as one match set with the PDQuest ™ software. On the average, 600 matched spots were detected on each gel, and 53 were found to differ consistently in expression level between the two groups: 30 were overexpressed (more than 1.5-fold) in treated islets, and 23 were underexpressed (more than 1.5-fold). Additional differences were noticed in individual gels but varied within the set and were not included. In total, 43 spots were successfully matched to and excised from preparative gels stained with Coomassie or Sypro Ruby. Of these, we could identify 22 proteins in 22 spots (Fig. 1) by LC-MS/MS (Table 1). Many of the differentially expressed protein spots were among those faintly stained with Sypro Ruby. Sypro Ruby is considered to have sensitivity in the range of silver staining, and we are therefore close to the detection limit of the instrumentation used. Even so, we identified half of the excised spots. Of the 22 proteins identified, 15 were up-regulated and 7 down-regulated (Table 1). A few of the proteins identified had multiple or unexpected gel positions versus theoretical Mw/pI values (Table 1), as known from other reports using this technique, suggesting the presence of modifications or complexes.

Three proteins involved in protein folding, Hsp 60 (Fig. 1, spots 6 and 7), calreticulin (spot 5) and protein disulfide isomerise (PDI; spots 2 and 3) were found to be up-regulated in drug-treated islets. These three proteins prevent protein aggregation as a result from misfolding and may have important roles in depository diseases. Furthermore, we found up-regulation of four Ca^{2+}-binding proteins in drug-treated cells: calcyclin (Fig. 1, spot 14), annexin 1 (spot 11), calreticulin (spot 5) and calgizzarin (spot 4). Significantly, Ca^{2+}-binding proteins are known to be involved in insulin secretion. A third group of interest contains important housekeeping enzymes or inhibitors of enzymes, such as pyruvate kinase (Fig. 1, spot 9), alpha enolase (spot 8), RhoA (spot 19) and protein kinase C inhibitor 1 (spot 21). Other differentially expressed proteins not mentioned above are involved in protein synthesis/degradation, energy homeostasis and proliferation/cell growth.

Discussion

In the present study, the effects of an imidazoline compound (BL11282) were investigated using a proteomic approach. Whole-cell extracts from isolated pancreatic rat islets were separated on 2-DE, and 22 differentially expressed proteins were identified by mass spectrometry. The differential results between normal pancreatic islets and those treated with the imidazoline compound are interesting. The gel pattern demonstrates that 53 spots out of ~600 matched spots, *i.e.* ~10 % of valid spots, differ between the treated and untreated islets, showing that treatment with the insulin-releasing drug has considerable cellular effects; clearly, it binds to cellular components. It is difficult to find a proper control for this proteomics study, as there are no directly comparable proteomics data available for pancreatic islets treated with sulfonylurea or GLP-1, insulinotropic compounds whose mechanisms of action on pancreatic islets have been intensively investigated. Mechanisms of insulinotropic activity require further studies, but the already differing proteins now identified include several known regulatory proteins.

Among the differentially expressed proteins, we find at least three that are involved in protecting the cell from misfolded proteins: Hsp60, calreticulin and PDI, which are all up-regulated in BL11282-treated islets. This finding may be of interest, because type 2 diabetes is a risk factor for developing Alzheimer's disease: the incidence of Alzheimer's is more than twofold increased in patients with type 2 diabetes [20, 21]. Misfolded proteins have a propensity to aggregate. Hsp 60 may be a critical chaperone preventing this activity. It has been shown that another heat shock protein, Hsp70, prevents protein aggregation in Alzheimer β-amyloid fibrils [22, 23]. Calreticulin has many functions and is found in several cell types; important functions include the binding of Ca^{2+} and a chap-

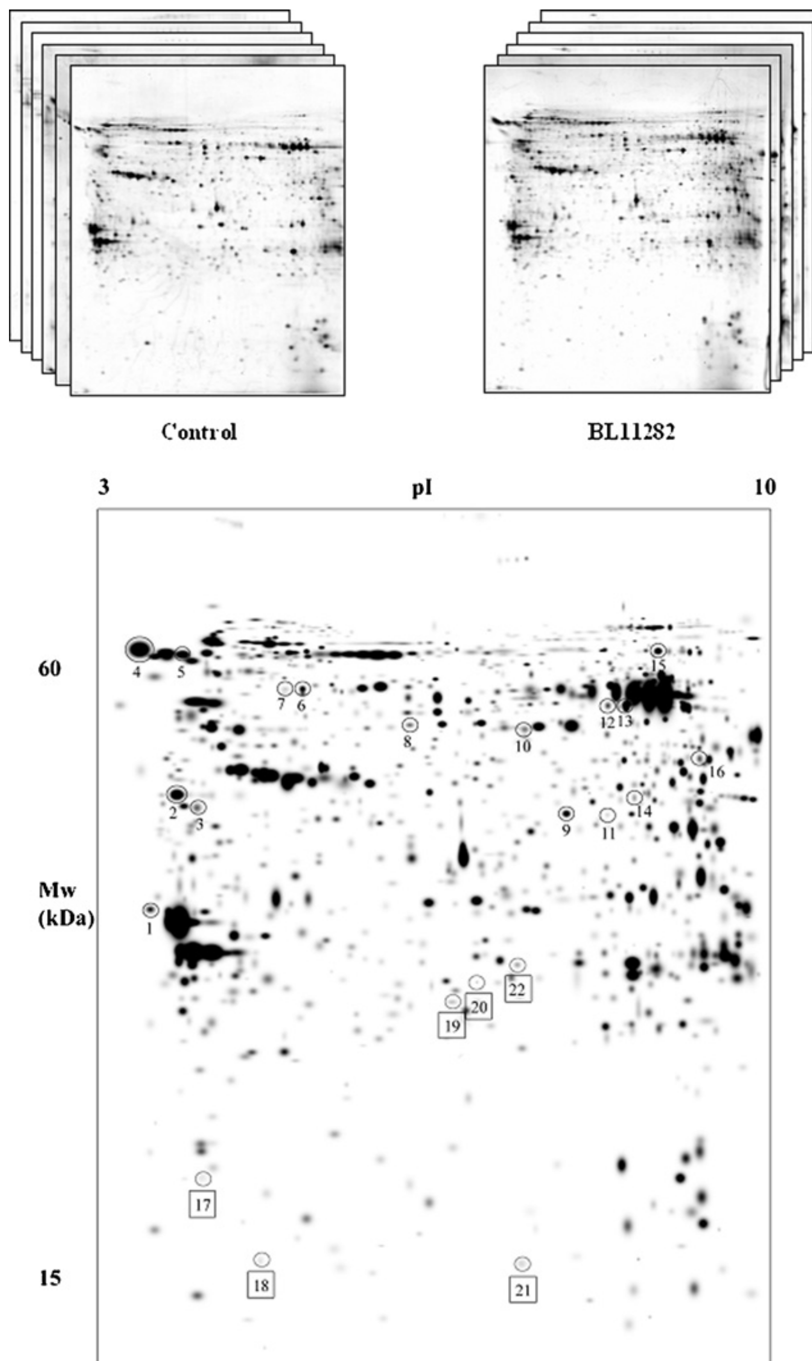
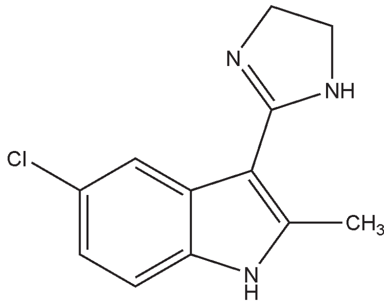


Figure 1. Six BL11282-treated and six native islet isolates separated on 2-DE were merged into one match set with a synthetic master image containing the spot data of all gels in the match set. The numbers assigned to proteins down-regulated after treatment with the drug are shown in squares; up-regulated proteins are indicated by numbers without squares. Both groups are identified with the same numbers in Table 1.

erone role in the folding of MHC class I molecules [24, 25]. PDI, in addition to inhibiting protein aggregation, may accelerate posttranslational processing of proteins necessary to facilitate insulin uptake [26]. Furthermore, PDI may have a beneficial effect on the secretion of insulin, since overexpression of PDI stabilizes secretory proteins [27]. Misfolded proteins are translocated from the endoplasmatic reticulum, polyubiquinated and transported to cytosolic proteasomes for degradation [23], but we found that proteasome subunits a and b are down-regulated.

In addition to calreticulin, mentioned above, we found up-regulation of three other Ca^{2+}-binding proteins (calcyclin, calgizzarin and annexin I). Ca^{2+}-binding proteins are important mediators of the stimulus-secretion response in β cells following a rise in the concentration of free cytoplasmic Ca^{2+} [28, 29]. They are commonly divided into two groups: the bigger group, EF-hand proteins, includes the S100 proteins;



Formula 1

proteins of the other group, the annexins, bind Ca^{2+} and phospholipid [28]. Calcyclin and calgizzarin belong to the S100 family of Ca^{2+}-binding proteins, a family consisting of 20 small acidic proteins with Ca^{2+}-binding motifs and tissue-specific expression, which implies distinct functions in specific tissues or cell types. Members of this family can interact with effector proteins to regulate enzymatic activity, cell growth and calcium homeostasis [30]. Calcyclin enhances Ca^{2+}-stimulated insulin release in permeabilized pancreatic β cells [28]. Annexin I is located in insulin-containing granules in the β cell. The levels of annexin I and its phosphorylation are significantly increased at elevated glucose concentrations, a phenomenon synchronized with insulin release. Consequently, it has been suggested that annexin I is actively involved in the secretion of insulin [31].

The insulinotropic effect of the imidazoline compound BL11282 is dependent on protein kinase C (PKC), and inhibition of this kinase blocks the effect of the compound [10]. Histidine-triad nucleotide-

Table 1. Differential protein expression in BL11282-treated versus untreated pancreatic islets.

	Spot	Fold change	Protein	Accession number	Calculated Mw/pI	LC MS/MS sequences
A	1	1.84	Glycoprotein GC1QBP	O35796	23596/4.39	204–216
	2	2.75	Protein disulfide isomerase	P04785	54983/4.77	455–463, 427–438
	3	2.47	Protein disulfide isomerase	P04785	54983/4.77	319–328, 378–387
	4	2.27	Calgizzarin (S100 calcium-binding protein A11)	Q6B345	11065/5.60	32–47
	5	2.61	Calreticulin	P18418	46348/4.33	112–120, 74–80
	6	2.13	Hsp60 Heat shock protein 60	P63039	57926/5.35	345–352, 406–417
	7	1.68	Hsp60 Heat shock protein 60	P63039	57926/5.35	143–156, 293–101
	8	1.77	Alpha enolase	P04764	46997/6.16	71–79, 412–419, 406–412
	9	1.61	pyruvate kinase M1/M2	P11980	57687/6.69	106–114, 141–150
	10	1.62	Glycine amidinotransferase	P50442	44183/6.15	370–381, 190–194
	11	1.83	Annexin A1	P07150	38698/7.13	113–123, 166–176
	12	1.66	Glutamate dehydrogenase1	P10860	55948/6.71	536–545, 163–171, 192–200, 172–183, 152–162
	13	1.46	Glutamate dehydrogenase 1	P10860	55948/6.71	536–545, 192–200, 172–183, 152–162
	14	1.72	Calcyclin	P05964	10035/5.30	2–17
	15	2.38	Transketolase	P50137	67644/7.22	472–493, 187–204
	15	2.38	Heterogeneous nuclear ribonucleoprotein Q (hnRNP-Q)	Q7TP47	59711/8.86	82–94
	16	1.72	3-ketoacyl-CoA thiolase	P13437	41871/8.09	26–38, 159–171
	16	1.72	Isocitrate dehydrogenase	P56574	50967/8.88	51–67, 250–260, 362–374
B	17	0.65	Keratin type 2, Cytoskeletal keratin 8	Q10758	53854/5.82	252–263, 264–272, 285–294, 316–324, 372–380
	18	0.61	Retinol binding protein	P02696	15703/5.11	69–80
	19	0.66	Transforming protein RhoA	P61589	21443/5.83	85–98
	20	0.58	Proteasome subunit beta type 3	P40112	22965/6.15	71–77
	20	0.58	40S Ribosomal protein SA	P38983	32693/4.80	120–127, 89–101
	21	0.50	Protein kinase C inhibitor 1	P62959	13660/6.65	7–20, 30–36
	22	0.62	Proteasome subunit alpha type 6	P60901	27399/6.35	31–43

Identification of proteins differentially up-regulated (**A**) or down-regulated (**B**). For spot locations see Fig. 1. Molecular weight (Mw) and isoelectric point (pI) values given in the Table are as calculated for the unprocessed precursor. Numbers in the column LC MS/MS sequences show start and end positions (in the unprocessed protein) of the sequences identified.

binding protein 1, also known as PKC inhibitor 1, was found to be down-regulated in the imidazoline-treated islets. Further resemblance with protein patterns in PKC-activated islets is likely but cannot yet be evaluated. Pyruvate kinase and α-enolase were found to be up-regulated, and RhoA was down-regulated. Pyruvate kinase and α-enolase are key enzymes in glycolysis; depression of glycolysis in the heart of diabetic rats has been reported [32]. In a mouse model of diabetic cardiomyopathy, RhoA, a small ras-related protein, was up-regulated [33].

In conclusion, the insulin-releasing imidazoline compound BL11282 has profound effects on pancreatic islet function. The exact mechanisms of action require further studies, but our results demonstrate that antidiabetic compounds such as BL11282 not only potentiate glucose-induced insulin release but also induce specific changes in protein expression profiles compatible with systemic functions important in type 2 diabetes. This is interesting and may suggest that imidazoline compounds work not only as insulin secretagogues but also interfere with other signalling events in the pancreatic islet cells.

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