PROTECTIVE EFFECTS OF GLP-1 ANALOGUES AGAINST CELLULAR STRESS IN GLUCOSE UP-TAKING TISSUES UNDER GLUCO-LIPOTOXIC CONDITIONS

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Background and Objectives:

Increase in glucagon-like peptide-1 (GLP-1) activity has recently emerged as a useful therapeutic tool for the treatment of type 2 diabetes (T2D) by enhancing the glycemic control and also helping in maintaining or even decreasing body weight of most patients [1]. Lipotoxicity through increased free fatty acids(FFAs) levels is an important factor in the pathogenesis of type 2 diabetes resulting in defective beta cell proliferation and increased beta cell apoptosis. The action of GLP-1 and its mimetics on pancreatic β -cells as well as on nervous and digestive systems are relatively well established. The effect of this peptide and its analogues in other tissues including adipose, muscles and liver, however, are still poorly defined. On the other hand, activation of MAP-kinases (MAPKs) in diabetes is an established fact both in humans and animals [2. Therefore, we decided to investigate the impact of GLP-1 mimetics on the expression levels and activity of MAPKs family as well as on other downstream pathways using HepG2 liver cells.

Methods:

Using cell lines from liver (HepG2), adipose tissue (3T3-L1), Skeletal (L6) we analysed the effect of GLP-1 mimetic (Exendin-4) on MAPKs in the presence of stressing amounts of palmitic acid (PA) AND glucose. Differential protein expression was investigated using global proteomics approaches and label-free quantification with a focus on the MAPKs. Results were validated using western blotting and RT-PCR approaches.

Cell culture and treatment

HepG2 cells, 3T3-L1 and L6 (ATCC, Rockville, Baltimore, MD) at 80% confluency were pretreated with 100nM Exendin-4 for 2 hours followed by 400uM PA treatment for 1h, 4h, 24h with or without Exendin-4.

Nano LC-MS/MS Analysis

Peptide digest analysis was done using an Easy nano-LC (C18-A1 pre-column, and C18-A2 analytical column, 10 cm, 75um ID) coupled to an LTQ-Orbitrap Velos Mass Spectrometer. Peptides were eluted with a gradient of 5 to 35% acetonitrile with 0.1% formic acid over 80 min. Full MS spectra scan was performed at a resolution of 60,000. MS/MS spectra were acquired in a DDA mode. MaxQuant software was used for quantification of identified proteins.

Western blot analysis

Cells were lysed in RIPA buffer and 20 μ g of whole cell extracts was used for Western blots. GAPDH was used as an internal control to monitor protein loading. For densitometric analysis, the intensity of the bands was determined using Quantity One Software (BioRad, Hercules, CA).

Gene expression analysis

Total RNA extracted from cells AllPrep RNA/Protein extraction kits, (Qiagen) and converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and analyzed by quantitative real-time PCR using Rotor Gene Q-100 (Qiagen). Changes in gene expression of the selected genes between subjects were determined by the comparative $\Delta\Delta$ CT method using GAPDH as internal references.

Results:

In HepG2 cell line, we showed that:

- Phosphorylation levels of MAPKs (JNK, ERK1/2 and p38) were increased in the presence of Exendin-4 alone (Fig. 1).
- In the presence of PA, Exendin-4 has limited effect for short incubation periods but significantly attenuated MAPKs phosphorylation after 24h incubation (Fig. 1).
- Heat shock proteins (HSP60, HSP72, HSP90 and GRP78) and other chaperones were not affected by Exendin-4 regardless the used conditions.
- Comparable results were obtained using preadipocyte 3T3-L1 and pancreatic BTC-6 cell lines.
- Using proteomics approaches, a linked set of proteins were regulated by Exendin-4 (Table 1).
- New canonical signalling pathways involved in cellular proliferation and homeostasis such as EIF2, ILK, PKA and Rho were modulated by Exendin-4 (Fig. 2). Another set of upstream regulator proteins was regulated by Exendin-4 (Fig.3).

Conclusions and Clinical Relevance

Identifying new pathways modulated by GLP-1 analogues provides insight into its beneficial effects beyond glycaemic control, such as MAPKs activity, energy homeostasis in glucose up-taking organs and body weight decrease.

References:

- [1]. Girard, J. (2008). *Diabetes Metab* 34(6): 550-559.
[2]. Khadir et al. (2016). *Am J Physiol Endocrinol Metab*, 308, E71-83.

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Acknowledgement:

Kuwait Foundation for the Advancement of Sciences (KFAS) for financial support.

Figure 1: Exendin-4 differentially modulates the activity of MAPKs in HepG2 Cells in presence of PA. HepG2 cells were pre-incubated with Exendin-4 (100nM) for 2h and then were treated for various durations with PA (400uM) in presence or absence of Exendin-4 (100nM). The levels of total proteins (JNK, ERK1/2 and p38) and their phosphorylated forms (p-JNK, p-ERK1/2 and p-p38) were analysed using Western blot and normalised based on total protein or GAPDH. The mRNA expression levels of JNK, ERK1 and p38 were measured under the same treatment conditions by qRT-PCR and normalized using GAPDH. The quantified data are presented as fold changes and obtained from 3 independent experiments. Quantified Data are expressed as the mean $\pm$ SEM. * denotes $p < 0.05$.

Table 1: List of proteins differentially expressed between HepG2 cells treated with PA + Exendin-4 vs. PA alone.

ID	Symbol	Protein description	Peptides	PEP	Location	Type(s)	Expr Fold Change (PA+E : PA)	p-value
IPI00010290	FABP1	fatty acid binding protein 1	4	2.7E-15	Cytoplasm	transporter	1.52	0.046
IPI00012011	CFL1	cofilin 1	11	1.4E-252	Nucleus	other	1.11	0.036
IPI00012069	NQO1	NAD(P)H quinone dehydrogenase 1	8	1.8E-125	Cytoplasm	enzyme	1.47	0.004
IPI00013485	RPS2	ribosomal protein S2	8	2.8E-146	Cytoplasm	other	1.48	0.043
IPI00020008	NEDD8	neural precursor cell expressed, developmentally down-regulated 8	2	4.8E-61	Nucleus	enzyme	1.67	0.004
IPI00026546	PAFAH1B2	platelet activating factor acetylhydrolase 1b catalytic subunit 2	3	8.3E-123	Cytoplasm	enzyme	-1.92	0.001
IPI00027448	ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex subunit G	4	1.0E-18	Cytoplasm	enzyme	1.30	0.024
IPI00029631	ERH	enhancer of rudimentary homolog (Drosophila)	2	9.2E-52	Nucleus	other	1.40	0.007
IPI00031420	UGDH	UDP-glucose 6-dehydrogenase	24	0	Nucleus	enzyme	1.17	0.004
IPI00147874	NANS	N-acetylneuraminate synthase	7	4.2E-76	Cytoplasm	enzyme	1.36	0.015
IPI00179589	MTFN	Myotrophin	3	1.6E-71			1.76	0.043
IPI00216298	TXN	thioredoxin	4	1.4E-28	Cytoplasm	enzyme	2.10	0.012
IPI00216691	PFN1	profilin 1	9	1.5E-265	Cytoplasm	other	1.21	0.005
IPI00218310	TPM3	tropomyosin 3	14	0	Cytoplasm	other	1.56	0.028
IPI00218914	ALDH1A1	aldehyde dehydrogenase 1 family member A1	25	0	Cytoplasm	enzyme	1.13	0.028
IPI00220362	HSP61	heat shock protein family E (Hsp10) member 1	8	2.8E-94	Cytoplasm	enzyme	1.27	0.001
IPI00220827	TM5B10	thymosin beta 10	1	3.7E-80	Cytoplasm	other	2.80	0.005
IPI00220834	XRC5	X-ray repair cross complementing 5	18	4.6E-191	Nucleus	enzyme	-1.33	0.010
IPI00222122	SUB1	SUB1 homolog, transcriptional regulator	5	3.1E-109	Nucleus	transcription regulator	2.02	0.023
IPI00257508	DPYSL2	dihydropyrimidinease like 2	11	3.1E-205	Cytoplasm	enzyme	1.54	0.018
IPI002891467	SLC25A6	solute carrier family 25 member 6	10	2.7E-26	Cytoplasm	transporter	1.60	0.014
IPI00297779	CCT2	chaperonin containing TCP1 subunit 2	20	0	Cytoplasm	kinase	-1.20	0.033
IPI00300086	QPRT	quinolinate phosphoribosyltransferase	7	1.8E-52	Cytoplasm	enzyme	-1.52	0.033
IPI00304692	RBMX	RNA binding motif protein, X-linked	8	4.2E-68	Nucleus	other	1.39	0.005
IPI00383581	GANAB	glucosidase II alpha subunit	11	3.3E-27	Cytoplasm	enzyme	1.37	0.017
IPI00479186	PKM	pyruvate kinase, muscle	27	0	Cytoplasm	kinase	1.10	0.049
IPI00479997	STMN1	stathmin 1	6	3.5E-62	Cytoplasm	other	1.66	0.001
IPI00550882	PYCR1	pyroline-5-carboxylate reductase 1	8	3.4E-202	Cytoplasm	enzyme	-1.75	0.046
IPI00553185	CCT3	chaperonin containing TCP1 subunit 3	16	1.5E-126	Cytoplasm	other	1.13	0.049
IPI00604620	NCL	nucleolin	22	0	Nucleus	other	1.18	0.032
IPI00642816	SRP9	signal recognition particle 9	3	7.4E-13	Cytoplasm	other	1.59	0.042
IPI00719135	GPC3	glypican 3	9	8.5E-98	Plasma Membrane	other	1.32	0.036
IPI00844578	DHX9	DEH-H-box helicase 9	22	4.6E-91	Nucleus	enzyme	1.52	0.034
IPI00853068	HBA1	Hemoglobin subunit alpha	7	1.1E-123			-1.96	0.008
IPI00900293	FLNB	filamin B	31	9.9E-290	Cytoplasm	other	1.21	0.043
IPI00916768	CALM	calmodulin 1 (phosphorylase kinase, delta)	9	0			2.56	0.002

Figure 2: Proteins and major canonical pathways affected by Exendin-4 treatment. (A) Volcano plot featuring proteome data with the mean ratio in LFQ intensities between HepG2 cells treated with PA+Exendin-4 and PA vs. PA on the X-axis, versus transformed statistical significance on the y-axis. Significantly regulated proteins are located above the dashed line. (B) Proteins differentially expressed were selected and analysed using IPA to identify most altered canonical pathways caused by Exendin-4 treatment. Bars represent $-\log(p\text{ value})$ for a given pathway and reflecting its significance in the effect of Exendin-4 under each condition. The dashed line at 1.3 shows the p -value threshold at 0.05. Legends stand for: PE/P; ratio PA + Exendin-4 to PA, E/C; ratio Exendin-4 to Control.

Figure 3: Functional annotation of upstream molecules and regulators affected by Exendin-4. (A) Activation Z-score of upstream regulators obtained using IPA analysis and the protein expression data set of HepG2 cells treated under various indicated conditions. $-2 \leq Z\text{-score} \leq 2$ is considered significant. (B) Relationship network of the upstream regulators constructed by STRING and showing a direct connection between most of those regulators affected by Exendin-4 treatment. Legends stand for: PE/P; ratio PA + Exendin-4 to PA, E/C; ratio Exendin-4 to Control.