Modulation of Gene Expression Profiles by Hyperosmolarity and Insulin

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Key Words

Insulin resistance • Cell volume • Translation • Cyclins transport

Abstract

Cell hydration changes play a key role in the regulation of cell function and critically affect insulin sensitivity of carbohydrate- and protein metabolism. Here, the modulation of gene expression profiles by hyperosmolarity and insulin was examined in H4IIE rat hepatoma cells by cDNA/oligonucleotide array-, Northern- and Western blot analysis. Osmosensitive expression of the insulin-like growth factor binding protein Igfbp1, the multidrug resistance protein Mrp5 (Abcc5a) and cyclin D1 (Ccn1) was established at the mRNA and protein level. Despite a hyperosmotic increase of cyclin D1 mRNA induction by insulin, the cyclin D1 protein expression was decreased by hyperosmolarity, suggesting a hyperosmotic interference with cyclin D1 mRNA translation. Hyperosmolarity at the mRNA level blunted the insulin response of betaine homocysteine-S-methyl transferase, the multidrug resistance proteins Mdr1a (Abcb1a) and 2 (Abcb4), the Igfbp 2 and 5, cyclin

G1, dual specificity phosphatase Dusp1, signal transducers and activators of transcription Stat3 and 5, catalase and the bile salt export pump Bsep (Abcb11), whereas the insulin response was increased for Mrp5, cyclin D1 and the phosphoenolpyruvate carboxykinase. Insulin effects on the mRNA expression of the eukaryotic initiation factor 4E binding protein 4e-bp1, tubulin, gene 33, growth hormone receptor, keratin18, ornithine decarboxylase and heme oxygenase 1 were largely insensitive to hyperosmolarity. The data indicate that hyperosmolarity differentially modulates insulin sensitivity at the level of gene expression.

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Introduction

Moderate cell hydration changes as rapidly induced by hormones, substrates, second messengers, and oxidative stress play a critical role in the regulation of cell function [1-5]. In general, cell swelling supports anabolic metabolism and proliferation and protects cells from different kinds of stress, whereas cell shrinkage triggers

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catabolic pathways and promotes a proapoptotic state.

Studies with anisoosmotically exposed cells and tissues identified cell volume changes as modulators of gene expression. Hyperosmolarity in liver cells increased the expression of mRNAs of the phosphoenolpyruvate carboxykinase (Pck) [6-8], tyrosine amino transferase [7], serum and glucocorticoid-dependent serine/threonine kinase [9], Mi2 autoantigen [10] and the transporters of betaine, taurine or myo-inositol [11-14], but decreased betaine homocysteine-S-methyltransferase (BHMT) [15], argininosuccinate lyase [7], multidrug-resistance protein 2 (Mrp2) [16] and bile salt export pump (Bsep) [17] mRNA expression. The opposite responses were stimulated by hypoosmolarity.

A hyperosmotic dehydration contributes to insulin resistance even in healthy subjects [18-20]. Adequate rehydration improves the sensitivity of diabetic patients to low doses of insulin [21, 22]. More recent studies unravelled that hyperosmolarity impairs insulin activation of signaling components around the mammalian target of rapamycin [23, 24]. In perfused rat liver hyperosmolarity blunted K⁺ uptake, hepatocyte swelling and proteolysis inhibition in response to insulin [25].

Although insulin effects on gene expression have been extensively studied [26], little is known about the interference of hyperosmolarity with gene expression changes induced by insulin. In H4IIE rat hepatoma cells hyperosmolarity upregulated PEPCK mRNA expression but did not decrease the suppression of PEPCK mRNA expression by insulin [6]. Furthermore, hyperosmolarity delayed induction of the MAP-kinase phosphatase (Mkp1) expression by insulin [27].

The present gene expression profiling was performed in order to identify novel osmo-regulated genes and to address the hyperosmotic modulation of gene expression changes induced by insulin.

Materials and Methods

Materials

The polyclonal antibody raised against Mrp5 was a kind gift of Dr. Dietrich Keppler (Division of Tumor Biology, Deutsches Krebsforschungszentrum, Heidelberg, Germany) [28]. Rabbit polyclonal antibodies raised against IGFBP1 were from Abcam (Cambridge, UK) or Upstate Biotechnology (Lake Placid, USA). Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD). Insulin, sodium vanadate and Triton-X-100 were from Sigma (Taufkirchen, Germany). The polyclonal anti-Cyclin D1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal

anti-GAPDH antibody was from Biodesign International (Cologne, Germany). Anti-β actin antibody was from Abcam (Cambridge, United Kingdom). All other chemicals were from Merck (Darmstadt, Germany).

Cell culture and experimental treatment of the cells

H4IIE-C3 rat hepatoma cells were maintained on Cluster six dishes (Greiner, Frickenhausen, Germany) in DMEM-F12 / 5% CO₂ / 5 mM glucose at 37°C, pH 7.4, supplemented with 10% FCS. When cells had reached about 90% confluency, they were washed with phosphate-buffered saline and the culture was continued in 1.5 ml serum-free medium for an additional 24h. Hyperosmolarity (405 mosmol/l) and hypoosmolarity (205 mosmol/l) were adjusted by dilution with medium of elevated NaCl or without NaCl, respectively. In the normoosmotic control (305 mosmol/l), an identical volume of normoosmotic medium was added.

RNA preparation and cDNA synthesis

Total RNA from H4IIE cells was prepared with the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany). The amount and quality of the RNA was checked spectrometrically (absorption 220 nm–350 nm) and by capillary electrophoresis (Bioanalyzer, Agilent Technologies, Inc., Palo Alto, CA, U.S.A.). 100 µg of these RNA served as template for synthesis of the fluorescence labelled cDNA. The direct labeling with Cy3-dUTP (NEN, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, U.S.A.) was performed with Oligo-dT primers through the CyScribe-Kit (Amersham Pharmacia Biosciences, Freiburg, Germany). Unincorporated dye was removed with QIAquick PCR columns (Qiagen GmbH, Hilden, Germany).

Nylon Array hybridization

Nylon arrays (Atlas™ rat toxicology 1.2 arrays, Clontech Laboratories, Palo Alto, CA, U.S.A.) were used according to the manufacturers' recommendations. 5 µg total RNA was reverse transcribed in the presence of α-³²P-dATP using CDS Primer Mixes (Clontech). Unincorporated nucleotide was removed by two rounds of column chromatography (BioSpin 30, Bio-Rad, Hercules, CA, U.S.A.). The array membranes were prehybridized for 30min in ExpressHyb (Clontech) and 100 µg/ml sheared salmon testes DNA (Eppendorf, Waterbury, NY, U.S.A.). The probe was chemically denatured and membranes were hybridized at 68°C. The arrays were washed four times for 30 min each in 2X SSC, 1% SDS at 68°C, once with 0.1X SSC, 0.5% SDS for 30min at 68°C, then briefly rinsed in room temperature 2X SSC. After washing, the membranes were exposed to a phosphorimager screen and were scanned with an Fuji FLA 8000-Scanner (Raytest, Straubenhardt, Germany) with the following parameters: excitation 640 nm, filter B390, resolution 20 µm, scan speed 800 mm/s, photomultiplier voltage 100 %.

Oligonucleotide Array Production

Oligonucleotide probes were designed using OligoWiz [29] and synthesized from Operon (Cologne, Germany). The probes are 70-mers with an average T_m = 79°C and 5'-amino group with a C6 spacer. They were dissolved in spotting buffer

II (Quantifoil, Jena, Germany) and were printed as triplicates with a GMS417 (Affymetrix, UK Ltd., UK) at 55% relative humidity and room temperature on Epoxy-coated Slides (Eppendorf AG, Hamburg, Germany). The amino-modified probes were covalently bound to the epoxy surface in an acid-catalyzed reaction according to the protocol from Eppendorf. The quality of the printing process was proofed with a SYBR green II staining [30].

DNA Microarray hybridization and fluorescence determination

The fluorescence labeled cDNA was dissolved in 220 µl hybridization buffer (5xSSC, 4xDenhardtts solution, 200 mM Na₂HPO₄, 0.05% SDS, 50% Formamide, pH 8.0). The cDNA was denaturated 10min at 95°C and injected in an ASP hybridization station (Amersham Pharmacia Biosciences, Freiburg, Germany). The ASP used the following program: prehybridization 1h, 42°C with hybridization buffer; hybridization 12h, 42°C; washing 60s, 30°C with 2xSSC / 0.05 % SDS; two times washing 60s, 30°C with 2xSSC.

Hybridized oligonucleotide arrays were visualized with an Fuji FLA 8000-Scanner (Raytest, Straubenhardt, Germany) with the following parameters: excitation 532 nm, emission filter 570 nm, resolution 10 µm, scan speed 200 mm/s, photomultiplier voltage 100%.

Image analysis and statistics

The image analysis was computed with AIDA Array Analysis 3.21 (Raytest). The fluorescence intensities from each spot were integrated and the background intensities derived from weighted image regions were subtracted. The normalization was performed using a global approach:

$$N(clone) = \frac{I(clone) - B(clone)}{\frac{1}{N} \sum_i I(clone_i) - B(clone_i)}$$

$N(clone)$ normalized intensity

$I(clone)$ measured intensity

$B(clone)$ background fluorescence from I

Only the 50% upper values were used. In a next step the average normalized intensity for a specific condition was calculated from 4 arrays; clear artifacts were eliminated. Genes with a signal smaller 10 under all experimental conditions which implies a signal to noise ratio smaller 2.5 were declared as "not detectable".

The difference in the expression is:

$$Ratio = \log_{10} \frac{N_{(condition1)}}{N_{(condition0)}}$$

$N(condition)$ mean value of normalized intensity

This ratio was used to define five classes of genes. A ratio in the range ± 0.18 was declared as unregulated. A ratio from ± 0.18 to ± 0.30 indicates a moderate regulation. Genes with a ratio >0.30 were upregulated or with a ratio <-0.30 were downregulated.

Production of individual cDNA probes

cDNAs were synthesized from 1 µg total RNA through the 1st Strand cDNA kit for RT-PCR (AMV) from Roche Molecular Biochemicals (Mannheim, Germany) with Oligo-dT primers and gene-specific primers designed by using the Primer3 program (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>) and purchased from the MWG Biotech AG (Ebersberg, Germany).

The PCR reactions were performed using a PE Biosystems GeneAmp PCR System 2400 in 50 µl of the following reaction mixture: 1x reaction buffer, 2.5 mM MgCl₂, 0.2 mM dNTP mix, 0.2 pM each primer, 1.25 U Goldstar DNA-polymerase (Eurogentec, Seraing, Belgium) and 100ng cDNA. The following program was used: 5min 95°C; 40 cycles (40s 95°C; 50s 60°C; 1min 72°C); 7min 72°C; The PCR Products (161-486 bp) were separated and eluted from agarosegels after electrophoresis using the Qiaquick Gel extraction kit (Qiagen, Hilden, Germany). Sequence analysis confirmed the specificity of the PCR amplifications and was performed from the Biologisch-Medizinisches Forschungszentrum (Heinrich-Heine-University Düsseldorf, Germany). The taurine transporter (Slc6a6; taurine/ beta-alanine transporter) cDNA probe was prepared as described [12].

Northern-Blot Analysis

RNA samples (10 µg) were electrophoresed in 0.8% agarose / 3% formaldehyde and then RNA was blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) with 20xSSC (3 M NaCl, 0.3 M sodium citrate). After brief rinsing with water and cross-linking (Hoefer UV-crosslinker 500; Hoefer, San Francisco, CA, U.S.A.), the membranes were inspected under UV light with regard to RNA integrity and the location of the 28 S and 18 S rRNA spots. Blots were then subjected to a 3h prehybridisation at 43°C in 50% deionized formamide in sodium phosphate buffer (0.26 M, pH 7.2) containing 0.25 M NaCl, 1 mM EDTA, 100 µg/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with approx. 106 cpm/ml [α -³²P]dCTP-labelled random-primed cDNA probes (Rediprime labelling kit; Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). Then, membranes were washed three times in 2xSSC/0.1% SDS for 15min, twice in sodium phosphate buffer (25 mM, pH 7.2)/ EDTA (1mM)/0.1% SDS for 10min and twice in sodium phosphate buffer 10min at 53°C. Blots were exposed to Kodak AR X-Omat film (Eastman Kodak Company, Rochester; NY, U.S.A.) at -70°C with intensifying screens.

Concentration of IGFBP1 from H4IIE culture medium

IGFBP1 was concentrated from culture medium by Microcon® YM-10 centrifuge filter units (Millipore GmbH, Schwalbach, Germany) according to the manufacturers recommendation.

Gel Electrophoresis and Immunoblotting

Cells were homogenized in hypotonic buffer (0.1 mmol/L EDTA, 0.5 mmol/L sodium phosphate, pH 7.0) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany) using a Labsonic 2000 sonifier (1000 rpm/min). Crude membrane fractions were collected by centrifugation

Log ₁₀ x/y	down-regulated		non-regulated	up-regulated	
	< -0.3	-0.3 to -0.18	±0.18	+0.18 to +0.3	> +0.3
Conditions:					
N ₃₀₅ vs. N ₃₀₅	38	47	395	51	27
N ₄₀₅ vs. N ₃₀₅	52	53	394	40	19
N ₄₀₅ vs. N ₄₀₅	24	32	410	54	38
N ₄₀₅ vs. N ₃₀₅	33	51	409	33	32

Table 1. Global analysis of gene expression changes induced by hyperosmolarity and insulin: DNA arrays were hybridized with labeled cDNA representing gene expression levels (normalized intensity N by fluorescence or autoradiography, see “Methods”) in H4IIE cells after exposure to a) normoosmolarity (305 mosmol/l, “N₃₀₅”), b) hyperosmolarity (405 mosmol/l, “N₄₀₅”), c) insulin (I, 100 nmol/l) under normoosmotic conditions (“N₃₀₅ |”), d) insulin (100 nmol/l) under hyperosmotic conditions (“N₄₀₅ |”). As a criterion for the consideration of data points in this table a signal to noise cutoff ≥ 2.5 under at least one of the experimental conditions was taken, which led to the inclusion of 558 targets. Gene expression changes are expressed as logarithmic ratio. This ratio was used to define five classes of genes. A ratio in the range ± 0.18 was declared as unregulated. A ratio from ± 0.18 to ± 0.30 indicates a moderate regulation. Genes with a ratio >0.30 were upregulated or with a ratio <-0.30 were downregulated. i.e. + 0.3 represents a 2-fold increase and – 0.3 a 2-fold decrease. The number of up-, down- or non-regulated targets is given.

(20.000g, 4°C, 1h) and resuspended in hypotonic buffer. Alternatively, cells were immediately lysed at 4°C using 10 mmol/L Tris/HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF and 1 tablet / 50ml of protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Lysates were centrifuged at 20,000 g at 4°C for 10min. To perform SDS polyacrylamide gel electrophoresis and Western Blot analysis the supernatant was added to an identical volume of 2x gel loading buffer containing 200 mmol/L dithiothreitol (DTT, pH 6.8). After heating to 95°C for 5min, the proteins were subjected to SDS-PAGE (60 µg protein/lane, 10% gels). Following electrophoresis, gels were equilibrated with transfer buffer (39 mmol/L glycine, 48 mmol/L Tris/HCl, 0.03% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked overnight in 1-5% bovine serum albumin solubilized in 20 mmol/L Tris/HCl (pH 7.5) containing 150 mmol/L NaCl and 0.1% Tween 20 and then incubated for 3-4h with the respective primary antibody diluted 1:1,000. Following washing and incubation for 2h with horseradish peroxidase-coupled anti-rabbit-IgG antibody or horseradish peroxidase-coupled anti-mouse-IgG antibody (1:20,000, 4°C) the blots were washed again and developed using enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany).

Results

Experimental setting

To study osmosensitivity of gene expression H4IIE rat hepatoma cells were exposed for 8 h to normo- (305 mosmol/l) or hyperosmolarity (405 mosmol/l), adjusted by addition of 50 mmol/l NaCl. In some experiments,

insulin was added following a 1 h normo- or hyperosmotic pre-treatment and the incubation was continued for another 7h. RNA was isolated and probes were prepared for hybridization of commercially available cDNA-Arrays and the custom-made glass oligonucleotide array.

Global evaluation of gene expression changes induced by hyperosmolarity and insulin

At a signal to noise ratio ≥ 2.5 a target was considered as detectable. 558 targets were detectable at least under one experimental condition. As illustrated in Table 1, gene expression levels were compared as logarithmized ratios between the following conditions: normoosmolarity plus insulin vs. normoosmolarity, hyperosmolarity vs. normoosmolarity, hyperosmolarity plus insulin vs. hyperosmolarity, and hyperosmolarity plus insulin vs. normoosmolarity plus insulin.

Insulin changed the expression levels of 29% of the targets under normoosmotic conditions (upregulated: 14%, downregulated: 15% targets) and of 27% targets under hyperosmotic conditions (upregulated: 16%, downregulated: 10 %). In absence of insulin the expression of 29% of the 558 detected targets were sensitive to hyperosmolarity (upregulated: 11%, downregulated: 19%).

Hyperosmotic modulation of the insulin response

In case of $|\log_{10}(N_{305|}/N_{305}) - \log_{10}(N_{405|}/N_{405})| \geq 0.18$ the insulin response of a target was defined as modified by hyperosmolarity (Table 2A).

Table 2. Analysis of gene expression changes induced by hyperosmolarity and insulin: DNA arrays were hybridized with labeled cDNA representing gene expression levels in H4IIE cells after exposure to a) normoosmolarity (305 mosmol/l, “N₃₀₅”), b) hyperosmolarity (405 mosmol/l, “N₄₀₅”), c) insulin (I, 100 nmol/l) under normoosmotic conditions (“N_{305 I}”), d) insulin (100 nmol/l) under hyperosmotic conditions (“N_{405 I}”). Gene expression changes are expressed as logarithmic ratio. $\log_{10} (N_{305 I} / N_{305})$ indicates the insulin-induced response under normoosmotic conditions (column A), $\log_{10} (N_{405} / N_{305})$ indicates the hyperosmolarity-induced response in the absence of insulin (column B), $\log_{10} (N_{405 I} / N_{405})$ indicates the insulin-induced response under hyperosmotic conditions (column C) and $\log_{10} (N_{405 I} / N_{305 I})$ compares the gene expression levels in presence of insulin under hyper- and normoosmotic conditions (column D). At a ratio in the range ± 0.18 the gene was declared as unregulated (printed in black). A ratio from ± 0.18 to ± 0.30 indicates a moderate regulation (printed in black, bold). Genes with a ratio >0.30 (printed black, italic, bold) were upregulated or with a ratio < -0.30 were downregulated (printed black, italic, bold). The array type describes the origin of the data. N indicates ClonTech nylon arrays with cDNA probes hybridized with radioactive labelled targets, G indicates glass microarrays with oligonucleotide probes and fluorescence labelled first strand cDNA as target. In column E, A-C represents $\log_{10} (N_{305 I} / N_{305}) - \log_{10} (N_{405 I} / N_{405})$, which defines the degree of osmotic modulation of the insulin-induced response. (A) Hyperosmotic modulation of the insulin-induced response. In case of $\log_{10} |(N_{305 I} / N_{305}) - \log_{10} (N_{405 I} / N_{405})| \geq 0.18$ the insulin response of a target was defined as modified by hyperosmolarity. (B) Osmoregulated genes. This table summarizes a group of targets that was sensitive to hyperosmolarity [$|\log_{10} (N_{305} / N_{405})| \geq 0.18$] whereas the response to insulin was similar under normo- and hyperosmotic conditions [i.e. $\log_{10} (N_{305 I} / N_{305}) - \log_{10} (N_{405 I} / N_{405}) \leq 0.18$]. (C) Insulin-responsive genes not affected by hyperosmolarity. This table summarizes targets that responded to insulin [$\log_{10} (N_{305 I} / N_{305}) \geq 0.18$] but were largely insensitive to hyperosmolarity in either absence of insulin [$|\log_{10} (N_{405} / N_{305})| \leq 0.18$] or in its presence [$|\log_{10} (N_{305 I} / N_{305}) - \log_{10} (N_{405 I} / N_{405})| \leq 0.18$].

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					$\log_{10} (N_{305 I} / N_{305})$	$\log_{10} (N_{405} / N_{305})$	$\log_{10} (N_{405 I} / N_{405})$	$\log_{10} (N_{305 I} / N_{405 I})$	A - C
1	interleukin 5	Il5	NM_021834	N	0.64	1.02	-0.39	-0.01	1.03
2	insulin-like growth factor binding protein 5 Igfbp5		NM_012817	N	0.68	0.50	-0.02	-0.20	0.69
3	folistatin-like 1	Fstl1	NM_024369	N	0.27	0.45	-0.23	-0.05	0.50
4	adrenergic receptor kinase, beta 1	Adrbk1	NM_012776	N	0.41	0.17	-0.08	-0.32	0.49
5	interleukin 4	Il4	NM_201270	N	0.06	0.53	-0.42	0.04	0.49
6	mitogen activated protein kinase 14	Mapk14	NM_031020	N	0.52	0.40	0.04	-0.08	0.48
7	GNAS complex locus	Gnas	NM_019132	N	0.37	0.40	-0.10	-0.08	0.47
8	insulin-like growth factor binding protein 2 Igfbp2		NM_013122	G	0.71	0.23	0.24	-0.24	0.47
9	solute carrier family 22 (organic anion transporter), member 6	Slc22a6	NM_017224	G	0.57	0.00	0.12	-0.46	0.46
10	retinol binding protein 1, cellular	Rbp1	NM_012733	N	0.12	0.33	-0.33	-0.12	0.45
11	adenylate cyclase 6	Adcy6	NM_012821	N	0.25	-0.20	-0.20	-0.65	0.45
12	growth factor receptor bound protein 2	Grb2	NM_030846	N	0.23	0.30	-0.21	-0.15	0.44
13	protein kinase C, beta 1	Prkcb1	NM_012713	N	0.41	0.20	-0.03	-0.24	0.44
14	hydroxysteroid(17-beta)dehydrogenase 7 Hsd17b7		NM_017235	N	0.23	-0.30	-0.18	-0.70	0.41
15	cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	NM_012540	N	0.31	0.46	-0.09	0.06	0.40
16	c-src tyrosine kinase	Csk	NM_001030039	N	0.44	0.47	0.05	0.08	0.39
17	regucalcin	Rgn	NM_031546	N	0.03	0.30	-0.35	-0.09	0.39
18	surfactant associated protein C	Sftpc	NM_017342	N	0.37	0.30	-0.01	-0.08	0.38
19	phosphoenolpyruvate carboxykinase 1	Pck1	NM_198780	N	-0.39	0.18	-0.75	-0.18	0.36
20	damage-specific DNA binding protein 1	Ddb1	NM_171995	N	-0.16	-0.19	-0.51	-0.54	0.35

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A – C
21	RAB3D, member RAS oncogene family	Rab3d	NM_080580	N	0.18	0.16	-0.17	-0.18	0.34
22	adenylate kinase 3	Ak3	NM_013218	N	-0.54	-0.34	-0.88	-0.69	0.34
23	dual specificity phosphatase 1	Dusp1	NM_053769	G	0.74	0.40	0.40	0.07	0.33
24	Glucose-dependent insulinotropic peptide	Gip	NM_019630	N	0.06	0.23	-0.27	-0.10	0.33
25	phospholipase A2	Pla2g5	NM_017174	N	0.12	0.14	-0.20	-0.19	0.32
26	discoidin domain receptor family, member 1	Ddr1	NM_013137	N	0.25	0.26	-0.07	-0.06	0.32
27	calmodulin 3	Calm3	NM_012518	N	0.23	0.07	-0.09	-0.24	0.32
28	monoamine oxidase A	Maoa	XM_343764	G	0.08	0.28	-0.24	-0.04	0.31
29	acetyl-coenzyme A carboxylase alpha	Acaca	NM_022193	G	0.19	0.00	-0.12	-0.31	0.31
30	corticotropin releasing hormone receptor 1	Crrh1	NM_030999	N	0.54	0.13	0.23	-0.17	0.30
31	replication protein A2	Rpa2	NM_021582	N	0.42	0.47	0.12	0.17	0.30
32	solute carrier family 38, member 2	Slc38a2	NM_181090	G	0.28	0.52	-0.01	0.23	0.29
33	protein kinase, cAMP dependent regulatory, type II beta	Prkar2b	XM_343046	N	0.16	-0.46	-0.12	-0.74	0.28
34	matrix metalloproteinase 13	Mmp13	XM_343345	N	0.52	0.80	0.24	0.52	0.28
35	abl-interactor 2	Abi2	NM_173143	N	0.32	0.15	0.05	-0.13	0.27
36	signal transducer and activator of transcription 5A	Stat5a	NM_017064	N	0.60	-0.06	0.33	-0.33	0.27
37	calcium/calmodulin-dependent protein kinase I	Camk1	NM_134468	N	0.17	0.45	-0.10	0.19	0.26
38	tumor protein p53	Tp53	NM_030989	N	0.31	0.52	0.05	0.26	0.26
39	syndecan 1	Sdc1	NM_013026	N	0.68	0.35	0.42	0.09	0.26
40	insulin-like growth factor binding protein 1	Igfbp1	NM_013144	G / N	-0.18	0.47	-0.44	0.21	0.26
41	solute carrier family 22 (organic cation transporter), member 5; organic cation/carnitine transporter (OCTN2)	Slc22a5	NM_019269	G	-0.11	0.00	-0.36	-0.26	0.26
42	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	Slc7a1	NM_013111	G	0.59	0.15	0.33	-0.11	0.26
43	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	Slc7a7	NM_031341	G	-0.02	0.12	-0.27	-0.13	0.25
44	solute carrier family 22 (organic cation transporter), member 4	Slc22a4	NM_022270	G	0.40	0.01	0.16	-0.23	0.25
45	abcc5 ATP-binding cassette, sub-family C (CFTR/MRP), member 5	Abcc5a	NM_053924	G	0.02	1.04	-0.22	0.79	0.25
46	solute carrier family 38, member 5; amino acid transporter system N2, SN2	Slc38a5	NM_138854	G	0.20	-0.05	-0.05	-0.29	0.24
47	profilin 1	Pfn1	NM_022511	N	0.39	0.32	0.15	0.08	0.24
48	glucose-6-phosphatase, catalytic	G6pc	NM_013098	G	-0.33	0.24	-0.57	0.00	0.24
49	cell division cycle 42 homolog (S. cerevisiae)	Cdc42	NM_171994	N	0.15	0.23	-0.09	-0.01	0.24
50	zinc finger protein 354A	Zfp354a	NM_052798	N	0.22	0.35	-0.01	0.12	0.22
51	RAB5A, member RAS oncogene family	Rab5a	NM_022692	N	0.04	0.07	-0.18	-0.15	0.22
52	dual specificity phosphatase 5	Dusp5	NM_133578	G	0.21	0.16	-0.01	-0.06	0.22
53	epidermal growth factor receptor	Egfr	NM_031507	G	0.18	0.19	-0.04	-0.03	0.22
54	cyclin G1	Ccng1	NM_012923	N	0.27	0.39	0.06	0.17	0.22
55	high mobility group box 2	Hmgb2	XM_573903	N	0.08	-0.02	-0.13	-0.23	0.21

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A - C
56	cyclin-dependent kinase 4	Cdk4	NM_053593	N	0.15	0.02	-0.07	-0.19	0.21
57	alpha 1 microglobulin/bikunin	Ambp	NM_012901	G	0.16	0.18	-0.06	-0.04	0.21
58	CDC-like kinase 3	Clk3	NM_134340	N	0.11	0.23	-0.09	0.03	0.20
59	metallothionein 1a	Mt1a	NM_138826	G / N	0.41	-0.37	0.21	-0.57	0.20
60	polymerase (DNA directed), gamma	Polg	NM_053528	N	0.36	-0.18	0.16	-0.38	0.20
61	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	Abcb1a	NM_133401	G	0.19	0.29	-0.01	0.09	0.20
62	mitogen activated protein kinase kinase 5	Map2k5	NM_001033987	N	0.09	0.09	-0.11	-0.10	0.20
63	RAB14, member RAS oncogene family	Rab14	NM_053589	N	0.07	0.12	-0.12	-0.06	0.19
64	Histone H2a	LOC498753	NM_001024282	N	0.18	0.04	0.00	-0.14	0.18
65	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin), member 1	Serpina1	NM_022519	N	-0.02	0.11	-0.20	-0.07	0.18
66	B-cell translocation gene 2, anti-proliferative	Btg2	NM_017259	N	0.20	0.07	0.02	-0.11	0.18
67	polyubiquitin	Ubb	NM_138895	G / N	0.01	0.11	-0.16	-0.07	0.18
68	ribosomal protein S29	Rps29	NM_012876	G	0.07	0.25	-0.10	0.08	0.18
69	serine (or cysteine) proteinase inhibitor, clade E, member 1	Serpine1	NM_012620	N	0.30	0.13	0.47	0.30	-0.18
70	solute carrier family 16 (monocarboxylic acid transporters), member 1	Slc16a1	NM_012716	N	0.13	-0.22	0.31	-0.04	-0.18
71	arginyl aminopeptidase (aminopeptidase B)	Rnpep	NM_031097	N	-0.11	-0.10	0.07	0.08	-0.18
72	carnitine palmitoyltransferase 1, liver	Cpt1a	NM_031559	N	-0.56	0.05	-0.39	0.22	-0.18
73	annexin A5	Anxa5	NM_013132	N	-0.10	0.00	0.08	0.18	-0.18
74	ornithine decarboxylase antizyme 1	Oaz1	NM_139081	G	-0.09	-0.09	0.09	0.09	-0.18
75	Cytochrome P450, subfamily IIC6	Cyp2c6	XM_574666	N	-0.26	-0.23	-0.08	-0.05	-0.18
76	glutamate-cysteine ligase, catalytic subunit	Gclc	NM_012815	N	-0.18	0.08	0.00	0.26	-0.18
77	heterogeneous nuclear ribonucleoprotein K	Hnrpk	NM_057141	N	-0.06	-0.16	0.12	0.02	-0.18
78	phosphatidylinositol 4-kinase, catalytic, beta polypeptide; PI4K92	Pik4cb	NM_031083	G	-0.13	-0.11	0.05	0.07	-0.18
79	isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	NM_031510	G	-0.52	-0.11	-0.33	0.08	-0.18
80	secreted phosphoprotein 1	Spp1	NM_012881	N	-0.16	0.13	0.02	0.31	-0.18
81	aspartyl-tRNA synthetase	Dars	NM_053799	G	-0.03	-0.40	0.15	-0.21	-0.19
82	inter-alpha trypsin inhibitor, heavy chain 3	Itih3	NM_017351	N	-0.11	-0.33	0.08	-0.14	-0.19
83	glutamate oxaloacetate transaminase 1	Got1	NM_012571	N	-0.23	-0.39	-0.04	-0.21	-0.19
84	UDP glycosyltransferase 1 family, polypeptide A7	Ugt1a7	NM_130407	N	-0.25	-0.12	-0.07	0.06	-0.19
85	heat shock transcription factor 1	Hsf1	XM_001061027	G	-0.02	0.30	0.17	0.49	-0.19
86	integrin beta 1	Itgb1	NM_017022	G / N	0.02	0.01	0.22	0.21	-0.19
87	sterol carrier protein 2	Scp2	NM_138508	N	-0.34	-0.12	-0.14	0.07	-0.19
88	phosphatidylinositol 4-kinase, catalytic, alpha polypeptide; PI4K230	Pik4ca	NM_022301	G	-0.09	-0.06	0.10	0.13	-0.19
89	cyclin D1	Ccnd1	NM_171992	N	0.05	0.15	0.24	0.35	-0.20
90	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	Slc25a5	NM_057102	G	0.06	-0.04	0.25	0.16	-0.20

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A – C
91	cytochrome P450, family 2, subfamily c, polypeptide 23	Cyp2c23	NM_031839	N	-0.34	-0.12	-0.14	0.08	-0.20
92	phosphatidylinositol synthase; CDP-diacylglycerol--inositol 3-phosphatidyltransferase	Cdipt	NM_138899	G	-0.10	-0.15	0.10	0.05	-0.20
93	dipeptidylpeptidase 4; bile canalicular domain-specific membrane glycoprotein	Dpp4	NM_012789	G	-0.25	-0.22	-0.04	-0.02	-0.20
94	carboxypeptidase D	Cpd	NM_012836	N	-0.26	0.08	-0.05	0.29	-0.21
95	glutamate oxaloacetate transaminase 2	Got2	NM_013177	N	-0.24	-0.23	-0.03	-0.03	-0.21
96	flavin containing monooxygenase 1	Fmo1	NM_012792	N	-0.23	-0.18	-0.02	0.03	-0.21
97	Bcl2-like 1	Bcl2l1	NM_001033670	N	0.20	0.22	0.40	0.43	-0.21
98	activating transcription factor 3	Atf3	NM_012912	G	1.12	-0.30	1.33	-0.08	-0.21
99	inositol 1,4,5-triphosphate receptor 1	Itpr1	NM_001007235	N	-0.34	-0.11	-0.13	0.11	-0.22
100	cytochrome P450, subfamily 21A, polypeptide 1	Cyp21a1	NM_057101	N	-0.26	-0.08	-0.04	0.13	-0.22
101	dopamine receptor 4	Drd4	NM_012944	N	0.25	-0.20	0.47	0.02	-0.22
102	fatty acid binding protein 1, liver	Fabp1	NM_012556	G / N	-0.19	-0.26	0.03	-0.04	-0.22
103	UDP glycosyltransferase 1 family polypeptide A4	Ugt1a4	NM_201424	N	-0.25	-0.10	-0.03	0.12	-0.22
104	catalase	Cat	NM_012520	G / N	-0.43	-0.10	-0.20	0.13	-0.22
105	fatty acid binding protein 5, epidermal	Fabp5	NM_145878	N	0.08	-0.37	0.31	-0.14	-0.23
106	DNA-damage inducible transcript 3; growth arrest and DNA-damage-inducible protein	Ddit3	NM_024134	G	0.49	-0.23	0.72	0.00	-0.23
107	alanyl (membrane) aminopeptidase	Anpep	NM_031012	N	-0.29	-0.29	-0.06	-0.06	-0.23
108	malic enzyme 1	Me1	NM_012600	N	-0.11	-0.25	0.13	-0.01	-0.24
109	ubiquinol cytochrome c reductase core protein 2	Uqcrc2	NM_001006970	N	-0.34	-0.27	-0.10	-0.03	-0.24
110	Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase	Mgat1	NM_030861	G	-0.15	-0.16	0.08	0.07	-0.24
111	glycogen phosphorylase liver type	Pygl	NM_022268	G	-0.09	-0.39	0.15	-0.15	-0.24
112	leukemia inhibitory factor	Lif	NM_022196	N	-0.07	-0.16	0.18	0.09	-0.25
113	Transferrin receptor	Tfrc	XM_340999	N	-0.09	0.43	0.16	0.68	-0.25
114	alpha-fetoprotein	Afp	NM_012493	N	-0.10	-0.09	0.16	0.17	-0.26
115	alkaline phosphatase, tissue-nonspecific	Alpl	NM_013059	N	0.12	-0.60	0.38	-0.34	-0.26
116	notch gene homolog 2 (Drosophila)	Notch2	NM_024358	N	-0.33	-0.17	-0.07	0.10	-0.27
117	solute carrier family 22 (organic anion transporter), member 7	Slc22a7	NM_053537	G	-0.26	-0.47	0.01	-0.20	-0.27
118	amiloride binding protein 1 (amine oxidase, copper-containing)	Abp1	NM_022935	N	-0.21	-0.23	0.06	0.03	-0.27
119	acyl-CoA synthetase long-chain family member 1	Acsl1	NM_012820	N	-0.52	-0.14	-0.25	0.13	-0.27
120	kangai 1	Kai1	NM_031797	N	-0.02	-0.36	0.25	-0.09	-0.27
121	thymidine kinase 1	Tk1	XM_001081763	G	0.00	-0.36	0.28	-0.09	-0.28
122	RAB8A, member RAS oncogene family	Rab8a	NM_053998	N	-0.17	-0.20	0.11	0.08	-0.28
123	solute carrier family 25, member 1	Slc25a1	NM_017307	G	-0.11	-0.30	0.17	-0.01	-0.28
124	actin, beta	Actb	NM_031144	G / N	-0.03	-0.20	0.26	0.08	-0.28
125	signal transducer and activator of transcription 3	Stat3	NM_012747	N	-0.28	0.00	0.01	0.30	-0.29
126	ATP-binding cassette, sub-family B (MDR/TAP), member 11	Abcb11	NM_031760	N	-0.28	-0.14	0.02	0.16	-0.30

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A - C
127	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	Ywhah	NM_013052	N	0.09	-0.15	0.39	0.15	-0.30
128	leukemia inhibitory factor receptor	Lifr	NM_031048	N	-0.35	-0.80	-0.04	-0.49	-0.31
129	cytochrome c, testis	Cyct	NM_012840	N	-0.52	-0.08	-0.21	0.23	-0.31
130	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat	NM_017300	G	-0.49	-0.20	-0.18	0.12	-0.31
131	nuclear receptor subfamily 1, group H, member 2; oxysterol receptors LXRbeta	Nr1h2	NM_031626	G	-0.37	-0.09	-0.06	0.22	-0.32
132	protein disulfide isomerase related protein (calcium-binding protein, intestinal-related); endoplasmic reticulum stress protein 72	Erp70	NM_053849	G	-0.28	-0.06	0.04	0.25	-0.32
133	protein kinase inhibitor, alpha	Pkia	NM_053772	N	0.13	-0.33	0.45	-0.01	-0.32
134	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	Atp5a1	NM_023093	N	-0.34	-0.20	-0.02	0.13	-0.33
135	cathepsin C	Ctsc	NM_017097	G	-0.40	-0.11	-0.08	0.22	-0.33
136	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	Atp1b1	NM_013113	N	-0.26	0.20	0.07	0.53	-0.33

For example, hyperosmolarity did not affect catalase (Cat) mRNA expression but blunted the insulin-induced decrease of *Cat* mRNA expression (Table 2A, no. 104; Figure 1). As reported [15, 31], hyperosmolarity and insulin decreased the betaine homocysteine-S-methyltransferase (*Bhmt*) mRNA expression in H4IIE cells (Table 2, no. 145; Figure 1). Under hyperosmotic conditions, however, *Bhmt* mRNA expression was not further decreased by insulin (Table 2A, no. 145; Figure 1). Similar observations were made for the multidrug resistance protein (*Mdr2*, *Abcb4*) mRNA (Table 2A, no. 154). In cases of insulin-like growth factor-binding protein (Igfbp)2 and 5, cyclin G1 (*Ccng1*) and the P-glycoprotein *Mdr1a* (*Abcb1a*), both, hyperosmolarity and insulin increased the respective mRNA levels, however, an additional mRNA induction by insulin was not observed under hyperosmotic conditions (Table 2A, no. 8, 2, 54, 61). Confirming an earlier report [27], the mRNA of the dual specificity phosphatase (*Dusp1*) was induced by insulin and hyperosmolarity, whereas insulin did not further increase *Dusp1* mRNA expression under hyperosmotic conditions (Table 2A, no. 23). Also the insulin response of the signal transducers and activators of transcription (*Stat*) 3 and 5 mRNA expression was osmosensitive (Table 2A, no. 125, 36). Insulin suppressed *Stat3*- but increased *Stat5* mRNA levels. Hyperosmolarity, by itself was essentially without effect (confirming [32] in the case of *Stat3*) but blunted the insulin response of

both genes. In line with an earlier report [17], hyperosmolarity slightly decreased the ATP-dependent bile salt export pump (*Bsep*, *Abcb11*) mRNA expression. As shown in Table 2A (no. 126), insulin decreased *Bsep* mRNA expression, which was blunted under hyperosmotic conditions.

Examples of a hyperosmotic sensitization to mRNA expression changes by insulin include the *Mrp5* (*Abcc5a*), *Igfbp1* and cyclin D1 (*Ccnd1*). *Mrp5* mRNA expression was increased by hyperosmolarity but remained largely unchanged by insulin. Under hyperosmotic conditions, however, insulin decreased *Mrp5* mRNA expression (Table 2A, no. 45; Figure 1). As reported [33], the *Igfbp1* mRNA levels were decreased by insulin in H4IIE cells (Table 2A, no. 40; Figure 1). Hyperosmolarity potentially upregulated *Igfbp1* mRNA expression and increased the potency of insulin to suppress *Igfbp1* mRNA expression (Table 2A, no. 40; Figures 1, 2A). Similar observations were made for phosphoenolpyruvate carboxykinase (*Pck1*) mRNA expression (Table 2A, no. 19), confirming an earlier report [6]. *Ccnd1* mRNA expression only weakly responded to hyperosmolarity, however, the induction of *Ccnd1* mRNA by insulin was increased under hyperosmotic conditions (Table 2A, no. 89; Figure 1).

Osmoregulated genes

A group of targets listed in Table 2B was sensitive to hyperosmolarity [$|\log_{10}(N_{305}/N_{405})| \geq 0.18$]

Table 2B

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A 305 ↓ 305 I	B 305 ↓ 405	C 405 ↓ 405 I	D 305 I ↓ 405 I	E A - C
1	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	XM_343065	N	-0.03	0.52	0.10	0.65	-0.13
2	K-kininogen, differential splicing leads to HMW Kngk	Kng_v1	NM_012741	N	0.22	0.46	0.07	0.30	0.16
3	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	Slc6a9	NM_053818	G / N	0.00	0.45	0.04	0.50	-0.05
4	nuclear factor I/X	Nfix	XM_213849	N	0.18	0.38	0.01	0.21	0.17
5	presenilin 1	Psen1	NM_019163	N	0.00	0.36	-0.01	0.36	0.00
6	solute carrier family 21, member 1	Slc21a1	NM_017111	G	0.10	0.34	-0.05	0.19	0.15
7	protein kinase C alpha	Prkca	XM_343975	G	0.18	0.31	0.22	0.36	-0.04
8	p21 (CDKN1A)-activated kinase 2	Pak2	NM_053306	N	0.56	0.30	0.63	0.37	-0.07
9	tripartite motif protein 28	Trim28	XM_344861	N	0.14	0.28	0.03	0.17	0.11
10	cadherin 6	Cdh6	NM_012927	N	-0.05	0.27	-0.19	0.13	0.14
11	syndecan 2	Sdc2	NM_013082	N	-0.25	0.26	-0.36	0.15	0.12
12	v-crK sarcoma virus CT10 oncogene homolog A (avian)	Crk	NM_019302	N	-0.07	0.25	0.00	0.31	-0.06
13	insulin receptor	Insr	NM_017071	N	0.07	0.24	-0.04	0.14	0.11
14	cyclin dependent kinase 2	Cdk2	NM_199501	N	0.33	0.23	0.26	0.16	0.07
15	matrix metalloproteinase 14 (membrane-inserted)	Mmp14	NM_031056	N	0.24	0.22	0.31	0.30	-0.08
16	protein kinase C delta	Prkcd	NM_133307	G / N	0.15	0.22	0.08	0.16	0.06
17	prothymosin alpha	Ptma	NM_021740	N	0.35	0.21	0.27	0.14	0.08
18	DnaJ-like protein	Dnaja1	NM_022934	N	0.12	0.21	0.16	0.25	-0.04
19	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	Rela	NM_199267	N	-0.04	0.21	0.01	0.26	-0.05
20	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	Atp1a1	NM_012504	G	-0.02	0.21	0.04	0.27	-0.06
21	pyruvate carboxylase	Pc	NM_012744	G	-0.03	0.20	-0.10	0.14	0.07
22	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	Slc6a12	NM_017335	G	-0.11	0.20	-0.05	0.26	-0.06
23	E2F transcription factor 5	E2f5	XM_574892	N	0.12	0.20	0.04	0.12	0.08
24	solute carrier family 29 (nucleoside transporters), member 1; NBMPR-sensitive; es-type nucleoside transporter	Slc29a1	NM_031684	G	0.05	0.20	0.13	0.28	-0.08
25	nuclear receptor subfamily 2, group F, member 6	Nr2f6	NM_139113	N	0.19	0.20	0.11	0.11	0.09
26	protein tyrosine phosphatase 4a2	Ptp4a2	NM_053475	G	0.30	0.19	0.28	0.18	0.01
27	intercellular adhesion molecule 1	Icam1	NM_012967	N	-0.03	0.19	-0.04	0.19	0.00
28	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	Ywhab	NM_019377	N	0.34	0.19	0.36	0.21	-0.02
29	CD24 antigen	Cd24	NM_012752	N	-0.19	0.18	-0.05	0.32	-0.14
30	phosphatase and tensin homolog	Pten	NM_031606	G / N	-0.22	0.18	-0.12	0.27	-0.09
31	phospholipase C, gamma 1	Plcg1	NM_013187	N	0.08	0.18	-0.06	0.04	0.14
32	apolipoprotein A-I	Apoa1	NM_012738	N	0.06	-0.18	0.04	-0.20	0.02
33	heat shock 90kDa protein 1, alpha	HSPCA	NM_001017963	N	0.09	-0.18	0.22	-0.05	-0.13
34	aquaporin 8	Aqp8	NM_019158	G	-0.26	-0.18	-0.15	-0.08	-0.11
35	apurinic/aprimidinic endonuclease 1	Apex1	NM_024148	N	-0.19	-0.18	-0.14	-0.14	-0.04

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A - C
36	proliferating cell nuclear antigen, cyclin	Pcna	NM_022381	G / N	0.27	-0.19	0.40	-0.05	-0.13
37	UDP glycosyltransferase 2 family, polypeptide B4	Ugt2b4	NM_001004271	N	-0.25	-0.19	-0.30	-0.23	0.04
38	thymosin, beta 10	Tmsb10	NM_021261	N	0.14	-0.19	0.10	-0.22	0.04
39	apolipoprotein B	Apob	NM_019287	N	-0.16	-0.19	-0.14	-0.17	-0.02
40	acetyl-Coenzyme A acyltransferase 1	Acaa1	NM_012489	N	-0.39	-0.19	-0.25	-0.06	-0.13
41	retinoid X receptor gamma	Rxrg	NM_031765	N	0.14	-0.20	0.04	-0.30	0.11
42	Transferrin	Tf	NM_001013110	N	-0.07	-0.20	0.08	-0.05	-0.15
43	glutamate decarboxylase 1	Gad1	NM_017007	N	-0.12	-0.21	-0.07	-0.15	-0.05
44	solute carrier family 5 (sodium-dependent vitamin transporter), member 6	Slc5a6	NM_130746	G	0.07	-0.21	0.18	-0.10	-0.11
45	keratin complex 2, basic, gene 8	Krt2-8	NM_199370	N	0.29	-0.21	0.24	-0.25	0.04
46	myosin Ib	Myo1b	NM_053986	G / N	-0.03	-0.21	0.09	-0.09	-0.12
47	actin, alpha 1, skeletal muscle	Acta1	NM_019212	N	0.48	-0.21	0.54	-0.15	-0.07
48	mevalonate kinase	Mvk	NM_031063	G	-0.15	-0.21	-0.03	-0.10	-0.12
49	adenine phosphoribosyl transferase (predicted)	Aprt_pre dicted	NM_001013061	N	-0.14	-0.21	-0.08	-0.15	-0.06
50	cathepsin H	Ctsh	NM_012939	G	-0.12	-0.22	0.05	-0.05	-0.17
51	UDP glycosyltransferase 1 family polypeptide A2	Ugt1a2	NM_201423	G	-0.15	-0.22	0.02	-0.05	-0.17
52	copper chaperone for superoxide dismutase	Ccs	NM_053425	G	-0.12	-0.23	-0.03	-0.14	-0.09
53	ornithine decarboxylase 1	Odc1	NM_012615	G / N	0.20	-0.24	0.36	-0.07	-0.16
54	aldehyde dehydrogenase 2	Aldh2	NM_032416	N	0.04	-0.24	0.12	-0.16	-0.08
55	caspase 9	Casp9	NM_031632	N	-0.10	-0.24	0.06	-0.08	-0.17
56	annexin A4	Anxa4	NM_024155	N	0.01	-0.25	0.04	-0.22	-0.02
57	phospholipase A2, group IB	Pla2g1b	NM_031585	N	-0.06	-0.25	0.03	-0.16	-0.09
58	hepatocyte nuclear factor 4, alpha	Hnf4a	NM_022180	N	-0.46	-0.27	-0.45	-0.26	-0.01
59	Janus kinase 1	Jak1	XM_342872	N	-0.12	-0.29	0.00	-0.16	-0.13
60	biliverdin reductase A	Blvra	NM_053850	G / N	-0.04	-0.30	-0.17	-0.42	0.13
61	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hmgcr	NM_013134	G	0.03	-0.30	0.18	-0.15	-0.15
62	topoisomerase (DNA) 2 alpha	Top2a	NM_022183	N	-0.26	-0.30	-0.20	-0.24	-0.06
63	apolipoprotein C-III	Apoc3	NM_012501	N	-0.06	-0.30	0.02	-0.22	-0.08
64	MAD homolog 1 (Drosophila)	Smad1	NM_013130	N	0.23	-0.30	0.11	-0.43	0.13
65	choline transporter	Slc6a8	NM_017348	G	-0.14	-0.31	-0.01	-0.17	-0.13
66	alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	NM_017270	N	-0.10	-0.31	-0.27	-0.48	0.17
67	glutamine synthetase 1	Glul	NM_017073	G	-0.30	-0.31	-0.20	-0.22	-0.10
68	keratin complex 1, acidic, gene 18	Krt1-18	NM_053976	N	0.33	-0.32	0.32	-0.33	0.02
69	NADH-cytochrome b5 reductase; diaphorase 1	Dia1	NM_138877	G / N	-0.16	-0.33	-0.12	-0.28	-0.05
70	alpha-2-HS-glycoprotein	Ahsg	NM_012898	N	0.06	-0.33	0.00	-0.40	0.06
71	dopa decarboxylase	Ddc	NM_012545	N	-0.17	-0.34	-0.17	-0.33	-0.01

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A – C
72	branched chain keto acid dehydrogenase kinase	Bckdk	NM_019244	N	-0.21	-0.35	-0.05	-0.19	-0.16
73	early growth response 1	Egr1	NM_012551	N	0.02	-0.36	0.06	-0.32	-0.04
74	actin, gamma, cytoplasmic	Actg	XM_213540	G	0.16	-0.36	0.29	-0.23	-0.13
75	fibrinogen, gamma polypeptide	Fgg	NM_012559	N	-0.23	-0.36	-0.27	-0.40	0.04
76	abcc2 ATP-binding cassette, sub-family C (CFTR/MRP), member 2; cmoat (Oligo 3)	Abcc2	NM_012833	G / N	-0.13	-0.36	-0.10	-0.32	-0.04
77	nuclear receptor binding factor 1	Nrbf1	NM_017209	G	-0.13	-0.37	-0.12	-0.37	0.00
78	uncoupling protein 2	Ucp2	NM_019354	N	0.27	-0.38	0.27	-0.38	0.00
79	v-raf oncogene homolog 1 (murine sarcoma 3611 virus)	Araf1	NM_001033663	N	-0.17	-0.41	-0.03	-0.27	-0.14
80	ATP-binding cassette, sub-family D (ALD), member 3	Abcd3	NM_012804	N	-0.51	-0.41	-0.38	-0.29	-0.12
81	aldehyde dehydrogenase family 1, subfamily A4	Aldh1a4	NM_017272	N	-0.34	-0.41	-0.29	-0.36	-0.05
82	arginase 1	Arg1	NM_017134	G	-0.50	-0.44	-0.39	-0.33	-0.11
83	acetyl-Coenzyme A dehydrogenase, long-chain	Acadl	NM_012819	N	-0.21	-0.44	-0.10	-0.32	-0.12
84	sulfotransferase family 1B, member 1	Sult1b1	NM_022513	N	-0.34	-0.48	-0.29	-0.43	-0.05
85	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	Echs1	NM_078623	N	-0.23	-0.55	-0.11	-0.43	-0.12
86	glial fibrillary acidic protein	Gfap	NM_017009	N	0.28	-0.61	0.30	-0.59	-0.02

whereas the response to insulin was similar under normo- and hyperosmotic conditions [i.e. $\log_{10}(N_{305\text{ I}} / N_{305}) - \log_{10}(N_{405\text{ I}} / N_{405}) \leq 0.18$]. Northern blot detection of taurine transporter (Taut, Slc6a6) mRNA expression served as a control. As expected [12], *Taut* was upregulated by hyperosmolarity. Insulin further increased the response of *Taut* to hyperosmolarity but was largely without effect by itself (Figure 1).

The glycine transporter Glyt (Slc6a9, Table 2B, no. 3, Figure 1) and the multispecific organic anion transporter Oatp1 (Slc21a1, Table 2B, no. 6) mRNAs were upregulated by hyperosmolarity but insensitive to insulin under normo- and hyperosmotic conditions. Mrp2 (Abcc2) mRNA expression was downregulated by hyperosmolarity ([16]; Table 2B; no. 76) but was largely insensitive to insulin under normo- and hyperosmotic conditions (Table 2B, no.76). Different to findings on Madin-Darby canine kidney cells [34] also the transcription factor “early growth response” Egr-1 mRNA expression was downregulated by hyperosmolarity (Table 2B, no. 73).

The mRNAs of keratin 18 (Krt1-18) and, in line with earlier reports [35-38], ornithin decarboxylase (Odc1) and actin (Acta1) were downregulated by hyperosmolarity but upregulated by insulin (Table 2B, no. 68, 53 and 47).

Hepatocyte nuclear factor (Hnf4) α , arginase1 (Arg1) and, in line with [32], γ fibrinogen (Fgg) mRNA expression each were additively downregulated by hyperosmolarity and insulin (Table 2B, no. 58, 82, 75).

Insulin-responsive genes not affected by hyperosmolarity

These targets responded to insulin [$|\log_{10}(N_{305\text{ I}} / N_{305})| \geq 0.18$] but were largely insensitive to hyperosmolarity in either absence [$|\log_{10}(N_{405} / N_{305})| \leq 0.18$] or in presence of insulin [$|\log_{10}(N_{305\text{ I}} / N_{305}) - \log_{10}(N_{405\text{ I}} / N_{405})| \leq 0.18$].

One example that largely fits these criteria is the eukaryotic initiation factor 4E-binding protein (4ebp1) whose mRNA expression only slightly decreased in response to hyperosmolarity but was upregulated by insulin to a similar degree under normo- and hyperosmotic conditions (Table 2C, no.7; Figure 1). In agreement with findings on cultured hepatocytes [39] also tubulin (Tuba1) mRNA abundance was increased by insulin but was largely insensitive to hyperosmolarity (Table 2C, no. 19). Also the reported increase of gene 33 mRNA expression by insulin [40] was found (Table 2C, no. 2) and was preserved under hyperosmotic conditions. Interestingly, an insulin-induced downregulation of growth hormone

Table 2C

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A - C
1	mitogen-activated protein kinase kinase 8	Map3k8	NM_053847	G	0.41	-0.10	0.25	-0.26	0.16
2	similar to Mitogen-inducible gene 6 protein homolog (Mig-6) (Gene 33 polypeptide)	gene 33	NM_001014071	G	0.39	-0.14	0.43	-0.09	-0.04
3	thymine-DNA glycosylase	Tdg	NM_053729	N	0.38	-0.07	0.53	0.08	-0.16
4	solute carrier family 5 (inositol transporters), member 3	Slc5a3	NM_053715	G	0.34	-0.08	0.31	-0.11	0.03
5	vascular endothelial growth factor A	Vegfa	NM_031836	N	0.32	0.00	0.44	0.11	-0.12
6	ATP-binding cassette, sub-family B (MDR/TAP), member 9	Abcb9	NM_022238	G	0.31	0.03	0.22	-0.06	0.09
7	eukaryotic translation initiation factor 4E binding protein	Eif4ebp1	NM_053857	G	0.31	-0.11	0.26	-0.16	0.04
8	chloride channel, nucleotide-sensitive, 1A Clns1a	NM_031719		N	0.30	0.09	0.17	-0.03	0.13
9	dynein, cytoplasmic, light chain 1	Dncl1	NM_053319	N	0.30	-0.06	0.31	-0.05	-0.01
10	cortactin isoform B and C	Cttnb	NM_021868	G	0.29	-0.03	0.18	-0.14	0.11
11	Jun D proto-oncogene	Jund	NM_138875	N	0.27	0.06	0.14	-0.07	0.13
12	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Slc3a2	NM_019283	G	0.27	-0.02	0.41	0.12	-0.14
13	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	Atrx	XM_217570	N	0.27	0.00	0.12	-0.15	0.15
14	nuclear receptor binding factor 2	Nrbf2	NM_022186	G	0.27	-0.05	0.20	-0.12	0.07
15	H-ras proto-oncogene; transforming protein p21	Hras	XM_001061671	N	0.27	0.10	0.16	-0.01	0.10
16	cold shock domain protein A	Csda	NM_031979	N	0.26	-0.03	0.17	-0.12	0.09
17	solute carrier family 19 (folate carrier), member 1	Slc19a1	NM_017299 NM_001035232	G	0.25	0.02	0.28	0.06	-0.03
18	transcription factor 1	Tcf1	NM_012669	G	0.25	-0.08	0.15	-0.18	0.10
19	tubulin, alpha 1	Tuba1	NM_022298	N	0.25	-0.10	0.26	-0.09	-0.01
20	macrophage migration inhibitory factor	Mif	NM_031051	N	0.24	0.03	0.09	-0.12	0.15
21	heme oxygenase (decycling) 1	Hmox1	NM_012580	G	0.22	-0.03	0.26	0.01	-0.04
22	protein phosphatase 2a, catalytic subunit, beta isoform	Ppp2cb	NM_017040	N	0.21	0.13	0.11	0.03	0.10
23	amiloride-sensitive cation channel 5, intestinal	Accn5	NM_022227	G	0.21	0.09	0.17	0.05	0.04
24	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	Ywhag	NM_019376	N	0.20	-0.01	0.22	0.00	-0.01
25	endothelin receptor type A	Ednra	NM_012550	G	0.20	-0.03	0.04	-0.20	0.16
26	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Atp2a2	NM_017290	N	0.20	0.01	0.22	0.03	-0.02
27	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2	NM_032056	G	0.19	-0.12	0.10	-0.21	0.09
28	protein tyrosine phosphatase, non-receptor type 1	Ptpn1	NM_012637	G / N	0.19	0.08	0.09	-0.02	0.10
29	hydroxysteroid (17-beta) dehydrogenase 4	Hsd17b4	NM_024392	N	-0.18	-0.02	-0.04	0.12	-0.14
30	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51) (predicted)	Itgav	XM_230950	N	-0.18	-0.02	-0.04	0.12	-0.13
31	monoamine oxidase B	Maob	NM_013198	G	-0.19	-0.10	-0.09	0.00	-0.10
32	UDP glycosyltransferase 1 family, polypeptide A1	Ugt1a1	NM_012683	N	-0.19	-0.06	-0.17	-0.04	-0.02
33	macrophage stimulating 1 (hepatocyte growth factor-like)	Mst1	NM_024352	G	-0.19	-0.08	-0.03	0.07	-0.15

Index	Name	NCBI Gene Symbol	NCBI Accession	Array Type	A	B	C	D	E
					305 ↓ 305 I	305 ↓ 405	405 ↓ 405 I	305 I ↓ 405 I	A - C
34	mesenchyme homeo box 2	Meox2	NM_017149	N	-0.19	-0.02	-0.06	0.11	-0.13
35	adenosine A1 receptor	Adora1	NM_017155	N	-0.21	0.04	-0.13	0.12	-0.08
36	alcohol dehydrogenase 1	Adh1	NM_019286	N	-0.21	0.03	-0.21	0.02	0.00
37	microsomal glutathione S-transferase 1	Mgst1	NM_134349	N	-0.21	-0.03	-0.05	0.13	-0.16
38	ferredoxin reductase	Fdxr	NM_024153	N	-0.21	-0.01	-0.26	-0.06	0.05
39	interferon regulatory factor 2 (predicted)	Irf2_pre- dicted	XM_214350	N	-0.24	-0.04	-0.39	-0.19	0.15
40	growth hormone receptor	Ghr	NM_017094	G / N	-0.26	-0.12	-0.42	-0.29	0.17
41	acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	NM_017340	N	-0.26	-0.12	-0.12	0.02	-0.14
42	Cytochrom c oxidase subunit VIII-H (heart/muscle)	Cox8h	NM_012786	N	-0.26	-0.01	-0.09	0.16	-0.17
43	solute carrier family 27 (fatty acid transporter), member	Slc27a1	NM_053580	G	-0.27	0.10	-0.28	0.10	0.00
44	aldolase C, fructose-biphosphate	Aldoc	NM_012497	N	-0.28	-0.05	-0.23	-0.01	-0.05
45	progesterone receptor membrane component 1	Pgrmc1	NM_021766	N	-0.28	-0.09	-0.17	0.01	-0.10
46	retinoblastoma-like 2	Rbl2	NM_031094	N	-0.32	0.13	-0.37	0.09	0.05
47	RAB13, member RAS oncogene family	Rab13	NM_031092	N	-0.38	-0.04	-0.23	0.10	-0.15

Fig. 1. Northern blot analysis of mRNA expression of selected genes: H4IIE cells were exposed to normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium for 8h. Where indicated insulin (100 nmol/l) was added following a 1h normo- or hyperosmotic pre-treatment and the incubation was continued for another 7h. RNA was isolated and subjected to Northern blot analysis as described in the Materials and Methods. Representatives of a least three independent experiments are shown.

receptor (Ghr) mRNA was found (Table 2C, no. 40), which fits an earlier study demonstrating the decrease of Ghr protein expression upon insulin treatment of H4IIE cells [41]. The recently in renal adenocarcinoma cells reported insulin-induced heme oxygenase (Hmox1) mRNA expression [42] was also found in H4IIE cells under normo- and hyperosmotic conditions. Similar to that found in rat hepatocytes [43] hyperosmolarity only slightly decreased Hmox1 mRNA expression in the H4IIE cells (Table 2C, no. 21).

Fig. 2. Osmosensitivity of Igfbp1, Mrp5 (Abcc5a) und cyclin D1 (Ccnd1) expression: H4IIE cells were exposed to normo-, hypo- and hyperosmotic (305, 285-205 and 330-405 mosmol/l) media for the indicated time periods. mRNA and protein levels were analyzed by Northern- and Western blot, respectively. Representatives of at least 3 independent experiments are shown. (A) Igfbp1. At the end of the incubations media were collected for extraction of secreted Igfbp1. Cell lysates were analyzed for the expression of *Igfbp1* mRNA and Igfbp1 protein. (B) Mrp5 (Abcc5a). Plasma membrane preparations were analyzed for Mrp5 expression by Western blot. (C) Cyclin D1 (Ccnd1). Where indicated insulin (100 nmol/l) was added following a 1 h normo- or hyperosmotic pre-treatment and the incubation was continued for another 1.5, 3, and 7 h, respectively. Cell lysates were analyzed for the expression of cyclin D1 protein.

The protein level

The osmosensitivity of *Igfbp1* mRNA and Igfbp1 expression was studied in H4IIE cells by Northern and Western blot analysis, respectively. As shown in Figure 2A and consistent with the data shown in Table 2A (no. 40) and Figure 1, *Igfbp* expression increased by hyperosmolarity (330-405 mosmol/l). Hypoosmolarity (285-205 mosmol/l) decreased *Igfbp1* expression (Figure 2A). Similar observations were made at the levels of cellular Igfbp1 as well as Igfbp1 protein secreted into the culture medium (Figure 2A).

The osmosensitivity of Mrp5 (Abcc5a) was examined in H4IIE cells exposed to hypo- normo- or hyperosmotic medium for 6 or 24 h. Figure 2B shows that Mrp5 expression was hardly detectable under hypo- and normoosmotic conditions but was strongly induced by hyperosmolarity, in line with the induction of *Mrp5* mRNA expression by hyperosmolarity (Figure 1, Table 2A, no. 45).

The osmosensitivity of cyclin D1 (Ccnd1) expression was studied in H4IIE cells exposed to normo- or hyperosmotic medium in the presence or absence of insulin (Figure 2C). Insulin in H4IIE cells increased Ccnd protein expression, which was consistent with the increase of *Ccnd1* mRNA expression found by Northern blot analysis (Figure 1). Hyperosmolarity suppressed basal and insulin-induced Ccnd1 expression (Figure 2C) but increased *Ccnd1* mRNA expression in the presence of insulin (Table 2A, no. 89, Figure 1), suggesting that hyperosmolarity affects Ccnd protein expression at the posttranscriptional level.

Discussion

This study by DNA array, Northern- and Western blot analysis identified the genes encoding insulin-like growth factor-binding protein (Igfbp1), the multidrug

resistance protein Mrp5 (*Abcc5a*) and Cyclin D (*Ccnd1*) to be osmoregulated at both, the mRNA and the protein level. Further, as revealed by DNA array- and Northern blot analysis, at the mRNA level hyperosmolarity blunted the response of catalase (*Cat*) and betaine-S-homocystein methyltransferase (*Bhmt*) to insulin but increased the insulin response of *Mrp5* (*Abcc5a*), cyclin D1 (*Ccnd*) and was essentially without effect on insulin-induced changes of ornithine decarboxylase (*Odc*), eukaryotic initiation factor 4E-binding protein (*4ebp1*) and keratin 18 (*Krt1-18*). The reliability of the array analysis was further corroborated by confirming insulin- and/or hyperosmolarity-induced gene expression patterns in perfused rat liver, cultured hepatocytes or H4IIE cells that already have been reported. Examples for this include *Bhmt* [15], phosphoenolpyruvate carboxykinase (*Pck1*) [6-8], *Igfbp1* [33], actin (*Acta1*) [36], tubulin [39], growth hormone receptor (*Ghr*) [41], multidrug resistance protein (*Mrp*)2 [16], bile salt export pump (*Bsep*) [17], the dual specificity phosphatase (*Dusp1*) [27], signal transducer and activator of transcription (*Stat*)3 and γ fibrinogen (*Fgg*) [32].

The hyperosmotic upregulation of the glycine transporter *Glyt* (*Slc6a9*) mRNA reported here (Table 2B, no. 3; Figure 1) fits well into the adaptive response to hyperosmolarity, which involves also an increased expression of the taurine transporter (*Taut*, *Slc6a6*) [12] (this paper, Figure 1), and the betaine/ γ -aminobutyrate transporter (*Slc6a12*) (Table 2B, no. 22) mRNAs and a decreased expression of the *Bhmt* mRNA [15] (this paper, Table 2A, no. 145; Figure 1), leading altogether to a cellular accumulation of compatible organic osmolytes. On the other hand, Mrp5 (*Abcc5a*) physiologically acts as an export pump for cyclic nucleotides (cAMP, cGMP) [28] and glutathione conjugates [44, 45]. Its hyperosmotic upregulation (this paper, Table 2A, no. 45; Figures 1 and 2B) may compensate for an impaired excretion of organic anions via Mrp2 (*Abcc2*), whose mRNA expression was decreased by hyperosmolarity in H4IIE cells (this paper, Table 2B, no. 76) and cultured rat hepatocytes [16]. There is indirect evidence that hyperosmolarity in H4IIE cells increases cAMP production [46]. Further, hyperosmolarity in H4IIE cells stimulates nitric oxide production (F. Schliess, D. Häussinger, unpublished data), which may increase cGMP levels. It remains to be proven, whether upregulation of Mrp5 represents an overflow mechanism contributing to the clearance of intracellular cyclic nucleotides under dehydrating conditions.

Some findings of this study further corroborate the

concept that dehydration drives cells into a catabolic, antiproliferative and proapoptotic state. One example is the hyperosmotic upregulation of IGFBP1 expression (Table 2A, no. 40; Figures 1 and 2). A major function of IGFBPs is to bind insulin-like growth factor (IGF)1 and thereby to inhibit IGF1 receptor binding [47]. It seems well conceivable that hyperosmotic upregulation of IGFBP1 expression antagonizes the known mitogenic, antiapoptotic and metabolic effects of IGF1 and thus confers IGF resistance. Further, the pronounced downregulation of growth hormone receptor (*Ghr*) mRNA in presence of insulin plus hyperosmolarity (Table 2C, no. 40) may decrease growth hormone-induced signal transduction. Although hyperosmolarity increased cyclin D1 mRNA expression by insulin (Table 2A, no. 89; Figure 1), the insulin-induced *Ccnd1* protein expression was suppressed by hyperosmolarity (Figure 2C). Similarly, hyperosmolarity suppressed the induction of *Dusp1* protein expression by insulin despite a high expression of *Dusp1* mRNA [27, 46] (this paper, Table 2A, no. 23), suggesting that hyperosmolarity prevents translation of both, cyclin D1 and *Dusp1* mRNA. Bearing in mind that mTOR signaling is involved in the translation of both mRNAs [27, 48], the hyperosmotic interference with insulin signaling via mTOR recently shown in H4IIE cells [27] could well explain the hyperosmotic suppression of cyclin D1 (this paper) and *Dusp1* [27, 46] expression by insulin.

Simultaneously, hyperosmolarity may trigger compensatory responses which at least in part could antagonize cellular resistance to insulin and other growth factors. For example, the mRNAs encoding α and β subunits of the Na^+/K^+ -ATPase (Table 2B, no. 20; Table 2A, no. 136) are upregulated under hyperosmotic conditions. Provided that this is reflected also at the protein level this may help to maintain insulin-induced K^+ uptake, which is essential for assembling of the entire metabolic insulin response [49]. Likewise, upregulation of the retinoic X receptor mRNA (Table 2A, no. 149) by hyperosmolarity in presence of insulin and of the insulin receptor (*IR*) β mRNA by hyperosmolarity (Table 2B, no. 13) may reflect an attempt to counteract a hyperosmotic impairment of insulin signaling.

In summary, gene expression profiling identified novel osmoregulated genes with impact on cell volume regulation, hepatobiliary transport, and proliferation. Insulin-induced gene expression changes were differentially affected by hyperosmolarity, indicating that hyperosmotic insulin resistance at the level of gene expression is not a general phenomenon.

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