Aus dem Institut/der Klinik für Endokrinologie der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Insulin-like growth factor (IGF) binding protein-2, independently of IGF-1, induces GLUT-4 translocation and glucose uptake in 3T3-L1 adipocytes

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

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Zusammenfassung

Insulin-like growth factor bindendes Protein-2 (IGFBP-2) ist das vorherrschende IGF-bindende Protein, das während der Adipogenese produziert wird und bekanntlich die Insulin-stimulierte Glukoseaufnahme (GA) in Muskelfasern erhöht. Wir untersuchten die IGFBP-2-induzierten Veränderungen der basalen und Insulin-stimulierten GA in Adipozyten und die zugrunde liegenden Mechanismen. Wir bestimmten ferner die Rolle von Insulin und IGF-1-Rezeptoren bei der Mediation von IGFBP-2 und der Auswirkung von IGFBP-2 auf die IGF-1-induzierte GA. Vollständig differenzierte 3T3-L1-Adipozyten wurden mit IGFBP-2 in Gegenwart und Abwesenheit von Insulin und IGF-1 behandelt. Insulin, IGF-1 und IGFBP-2 induzierten eine dosisabhängige Zunahme der GA. IGFBP-2 erhöhte die Insulin-induzierte GA nach Langzeitinkubation. Die IGFBP-2-induzierte Wirkung auf die GA wurde weder durch Insulin- oder IGF-1-Rezeptorblockade noch durch Insulinrezeptor-Knockdown beeinflusst. IGFBP-2 erhöhte signifikant die Phosphorylierung von PI3K, Akt, AMPK, TBC1D1 und PKCζ/λ und induzierte die GLUT-4-Translokation. Darüber hinaus reduzierte die Hemmung von PI3K und AMPK die IGFBP-2-stimulierte GA signifikant. Zusammenfassend lässt sich sagen, dass IGFBP-2 die GA in 3T3-L1 Adipozyten durch Aktivierung von PI3K / Akt, AMPK / TBC1D1 und PI3K / PKCζ/λ/GLUT-4 Signalwege stimuliert. Die stimulierende Wirkung von IGFBP-2 auf die GA ist unabhängig von seiner Bindung an IGF-1 und wird möglicherweise nicht durch den Insulin- oder IGF-1-Rezeptor vermittelt. Diese Studie hebt eine mögliche Rolle von IGFBP-2 im Glukosestoffwechsel hervor.

Schlüsselwörter: IGFBP-2; Glukoseaufnahme; AMPK; GLUT-4.

Abstract

Insulin-like growth factor binding protein-2 (IGFBP-2) is the predominant IGF binding protein produced during adipogenesis and is known to increase the insulin-stimulated glucose uptake (GU) in myotubes. We investigated the IGFBP-2-induced changes in basal and insulin-stimulated GU in adipocytes and the underlying mechanisms. We further determined the role of insulin and IGF-1 receptors in mediating the IGFBP-2 and the impact of IGFBP-2 on the IGF-1-induced GU. Fully differentiated 3T3-L1 adipocytes were treated with IGFBP-2 in the presence and absence of insulin and IGF-1. Insulin, IGF-1 and IGFBP-2 induced a dose-dependent increase in GU. IGFBP-2 increased the insulin-induced GU after long-term incubation. The IGFBP-2-induced impact on GU was neither affected by insulin or IGF-1 receptor blockage nor by insulin receptor knockdown. IGFBP-2 significantly increased the phosphorylation of PI3K, Akt, AMPK, TBC1D1 and PKCζ/λ, and induced GLUT-4 translocation. Moreover, inhibition of PI3K and AMPK significantly reduced IGFBP-2-stimulated GU. In conclusion, IGFBP-2 stimulates GU in 3T3-L1 adipocytes through activation of PI3K/Akt, AMPK/TBC1D1 and PI3K/PKCζ/λ/GLUT-4 signaling. The stimulatory effect of IGFBP-2 on GU is independent of its binding to IGF-1 and is possibly not mediated through the insulin or IGF-1 receptor. This study highlights the potential role of IGFBP-2 in glucose metabolism.

Key words: IGFBP-2; Glucose uptake; AMPK; GLUT-4.

Affidavit

I, Biruhalem Assefa Bayayibgn certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Insulin-like growth factor (IGF) binding protein-2, independently of IGF-1, induces GLUT-4 translocation and glucose uptake in 3T3-L1 adipocytes”.
I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date 01.11.2017 Signature

Detailed Declaration of Contribution

Biruhalem Assefa Bayayibgn had the following share in the following publication:

Assefa B**,** Mahmoud A, Pfeiffer AFH, Birkenfeld AL, Spranger J and Arafat AM. Insulin-like growth factor (IGF) binding protein-2, independently of IGF-1, induces GLUT-4 translocation and glucose uptake in 3T3-L1 adipocytes. *OXID MED CELL LONGEV.*, 2017

AMA conceived the study and acquired funding for the experiment. BA and AMM performed experiments and analysed the results. BA, AMM and AMA wrote the manuscript. AFHP, ALB and JS revised and commented on the manuscript. All authors read and approved the final manuscript.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Journal Data Filtered By: Selected JCR Year: 2016 Selected Editions: SCIE,SSCI Selected Categories: 'CELL BIOLOGY' Selected Category Scheme: WoS
Total:190 Journals

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS MOLECULAR CELL BIOLOGY	40,565	46.602	0.095730
2	CELL	217,952	30.410	0.593980
3	NATURE MEDICINE	70,491	29.886	0.178470
4	CANCER CELL	32,653	27.407	0.102790
5	Cell Stem Cell	21,524	23.394	0.092350
6	NATURE CELL BIOLOGY	38,128	20.060	0.103020
7	Cell Metabolism	25,575	18.164	0.099580
8	Science Translational Medicine	22,073	16.761	0.125580
9	CELL RESEARCH	11,885	15.606	0.038030
10	TRENDS IN CELL BIOLOGY	12,503	15.333	0.035140
11	Annual Review of Cell and Developmental Biology	9,131	14.917	0.020340
12	MOLECULAR CELL	57,961	14.714	0.184120
13	NATURE STRUCTURAL & MOLECULAR BIOLOGY	26,851	12.595	0.092800
14	TRENDS IN MOLECULAR MEDICINE	8,371	10.732	0.019430
15	CURRENT OPINION IN CELL BIOLOGY	13,266	9.937	0.029780
16	EMBO JOURNAL	66,603	9.792	0.097670
17	GENES & DEVELOPMENT	57,493	9.413	0.105120
18	DEVELOPMENTAL CELL	25,598	9.174	0.076130
19	CURRENT BIOLOGY	52,274	8.851	0.134650
20	Cold Spring Harbor Perspectives in Biology	11,216	8.769	0.056160
21	PLANT CELL	49,690	8.726	0.074760
22	Autophagy	12,494	8.593	0.032370
23	EMBO REPORTS	11,985	8.568	0.032750
24	Cell Systems	394	8.406	0.002250
25	CELL DEATH AND	17,711	8.339	0.034000

	DIFFERENTIATION			
26	Cell Reports	20,705	8.282	0.158370
27	JOURNAL OF CELL BIOLOGY	67,863	7.955	0.092150
28	ONCOGENE	65,039	7.519	0.079990
29	AGEING RESEARCH REVIEWS	4,198	7.452	0.011320
30	MATRIX BIOLOGY	4,439	7.400	0.007730
31	Stem Cell Reports	3,299	7.338	0.020450
32	CURRENT OPINION IN STRUCTURAL BIOLOGY	10,440	6.932	0.025760
33	Science Signaling	9,495	6.830	0.039960
34	CYTOKINE & GROWTH FACTOR REVIEWS	5,514	6.794	0.008440
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36	SEMINARS IN CELL & DEVELOPMENTAL BIOLOGY	7,898	6.614	0.023400
37	NEUROSIGNALS	653	6.143	0.000660
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39	Cell Death & Disease	11,593	5.965	0.042410
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41	CELLULAR AND MOLECULAR LIFE SCIENCES	21,448	5.788	0.042590
42	JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY	14,017	5.680	0.027240
43	STEM CELLS	20,822	5.599	0.038040
44	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR AND CELL BIOLOGY OF LIPIDS	8,359	5.547	0.018450
45	FASEB JOURNAL	42,242	5.498	0.057590
46	Protein & Cell	1,898	5.374	0.007310
47	Pigment Cell & Melanoma Research	3,903	5.170	0.007980
48	Oncotarget	30,241	5.168	0.078660
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50	MOLECULAR	7,764	4.974	0.016440

	CANCER RESEARCH			
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53	Wiley Interdisciplinary Reviews-RNA	1,680	4.838	0.009060
54	PLANT AND CELL PHYSIOLOGY	14,978	4.760	0.019460
55	Disease Models & Mechanisms	3,915	4.691	0.014960
56	Oxidative Medicine and Cellular Longevity	5,741	4.593	0.013820
57	IMMUNOLOGY AND CELL BIOLOGY	4,865	4.557	0.011210
58	CELLULAR MICROBIOLOGY	8,693	4.554	0.017740
59	BIOCHIMICA ET BIOPHYSICA ACTA-MOLECULAR CELL RESEARCH	13,829	4.521	0.036580
60	JOURNAL OF CELLULAR AND MOLECULAR MEDICINE	10,258	4.499	0.017860
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62	MOLECULAR AND CELLULAR BIOLOGY	59,340	4.398	0.057640
63	Stem Cell Research & Therapy	3,159	4.211	0.009660
64	NITRIC OXIDE-BIOLOGY AND CHEMISTRY	3,422	4.181	0.005350
65	TRAFFIC	6,150	4.133	0.014410
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68	JOURNAL OF CELLULAR PHYSIOLOGY	17,632	4.080	0.023450
69	JOURNAL OF LEUKOCYTE BIOLOGY	17,441	4.018	0.023770
70	Journal of Tissue Engineering and Regenerative Medicine	3,191	3.989	0.007160

71	Stem Cell Research	2,401	3.963	0.008060
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78	CELLULAR ONCOLOGY	1,099	3.786	0.001930
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82	CELL CALCIUM	4,918	3.707	0.007930
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97	CYTOTHERAPY	4,952	3.203	0.008780
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106	Stem Cell Reviews and Reports	1,705	2.967	0.005120
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114	Journal of Cell Communication and Signaling	693	2.705	0.001500
115	Current Stem Cell Research & Therapy	1,000	2.684	0.002030
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117	INFLAMMATION RESEARCH	3,570	2.659	0.005340
118	PROSTAGLANDINS LEUKOTRIENES	3,920	2.653	0.004230

	AND ESSENTIAL FATTY ACIDS			
119	BIOLOGY OF THE CELL	2,410	2.649	0.002680
120	PROSTAGLANDINS & OTHER LIPID MEDIATORS	2,053	2.640	0.002760
121	JOURNAL OF BIOENERGETICS AND BIOMEMBRANES	2,702	2.576	0.002630
122	DIFFERENTIATION	2,768	2.567	0.003170
123	HISTOCHEMISTRY AND CELL BIOLOGY	4,097	2.553	0.006030
124	Cellular and Molecular Bioengineering	660	2.535	0.002350
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130	Nucleus	988	2.387	0.006130
131	CYTOPATHOLOGY	1,112	2.380	0.001650
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133	EUROPEAN CYTOKINE NETWORK	1,188	2.364	0.000920
134	JOURNAL OF MOLECULAR HISTOLOGY	1,242	2.362	0.002060
135	CELL BIOLOGY AND TOXICOLOGY	1,369	2.333	0.001250
136	MOLECULAR REPRODUCTION AND DEVELOPMENT	5,298	2.316	0.004290
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138	DNA AND CELL BIOLOGY	2,908	2.236	0.004130
139	CELL BIOCHEMISTRY AND FUNCTION	2,214	2.186	0.003140

140	Cytoskeleton	1,082	2.173	0.004790
141	CELL COMMUNICATION AND ADHESION	449	2.147	0.000810
142	DEVELOPMENT GROWTH & DIFFERENTIATION	2,045	2.145	0.003610
143	MOLECULAR MEMBRANE BIOLOGY	1,142	2.095	0.001230
144	JOURNAL OF MUSCLE RESEARCH AND CELL MOTILITY	1,326	2.052	0.001730
145	HISTOLOGY AND HISTOPATHOLOGY	4,340	2.025	0.005230
146	GENES TO CELLS	3,849	1.993	0.006130
147	Human Cell	387	1.930	0.000510
148	CELL STRUCTURE AND FUNCTION	978	1.900	0.000750
149	Biochemistry and Cell Biology	2,539	1.895	0.002920
150	CYTOTECHNOLOGY	1,976	1.857	0.002330
151	CONNECTIVE TISSUE RESEARCH	1,970	1.832	0.002540
152	CELL BIOLOGY INTERNATIONAL	3,657	1.831	0.004650
153	GROWTH HORMONE & IGF RESEARCH	1,215	1.828	0.001510
154	Neural Regeneration Research	1,886	1.769	0.005270
155	PATHOBIOLOGY	912	1.703	0.001150
156	Biopreservation and Biobanking	504	1.698	0.001580
157	JOURNAL OF MEMBRANE BIOLOGY	4,062	1.696	0.004010
158	Acta Naturae	528	1.667	0.001340
159	GROWTH FACTORS	1,459	1.644	0.001770
160	Cell Journal	472	1.636	0.001350
161	JOURNAL OF RECEPTORS AND SIGNAL TRANSDUCTION	829	1.614	0.001530
162	TISSUE ANTIGENS	3,455	1.596	0.003860
163	DEVELOPMENT GENES AND EVOLUTION	1,556	1.422	0.001630
164	MOLECULAR AND CELLULAR PROBES	1,614	1.403	0.001560

165	FOLIA HISTOCHEMICA ET CYTOBIOLOGICA	984	1.389	0.001220
166	ACTA HISTOCHEMICA	1,765	1.360	0.003110
167	CYTOGENETIC AND GENOME RESEARCH	2,735	1.354	0.003470
168	CELL AND TISSUE BANKING	846	1.331	0.001490
169	CELL BIOCHEMISTRY AND BIOPHYSICS	3,323	1.320	0.007360
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171	CELLULAR & MOLECULAR BIOLOGY LETTERS	953	1.260	0.001110
172	TISSUE & CELL	1,835	1.232	0.001340
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174	Analytical Cellular Pathology	333	1.078	0.000570
175	ZYGOTE	984	1.053	0.001260
176	IET Systems Biology	405	1.048	0.000830
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178	IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY-PLANT	1,787	1.024	0.001400
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182	ACTA HISTOCHEMICA ET CYTOCHEMICA	375	0.879	0.000620
183	CELLS TISSUES ORGANS	2,111	0.776	0.002630
184	Molecular & Cellular Biomechanics	146	0.500	0.000210
185	Analytical and Quantitative Cytopathology and Histopathology	568	0.454	0.000460
186	Animal Cells and	128	0.376	0.000270

	Systems			
187	BIOLOGICHESKIE MEMBRANY	115	0.130	0.000080
188	JOURNAL OF HISTOTECHNOLOGY	106	0.111	0.000040
189	POSTEPY BIOLOGII KOMORKI	36	0.064	0.000020
190	HLA	208	Not Available	0.000000

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Research Article

Insulin-like growth factor (IGF) binding protein-2, independently of IGF-1, induces GLUT-4 translocation and glucose uptake in 3T3-L1 adipocytes

Short title: IGFBP-2 induces GLUT-4 translocation and glucose uptake.

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Abstract

Insulin-like growth factor binding protein-2 (IGFBP-2) is the predominant IGF binding protein produced during adipogenesis and is known to increase the insulin-stimulated glucose uptake (GU) in myotubes. We investigated the IGFBP-2-induced changes in basal and insulin-stimulated GU in adipocytes and the underlying mechanisms. We further determined the role of insulin and IGF-1 receptors in mediating the IGFBP-2 and the impact of IGFBP-2 on the IGF-1-induced GU. Fully differentiated 3T3-L1 adipocytes were treated with IGFBP-2 in the presence and absence of insulin and IGF-1. Insulin, IGF-1 and IGFBP-2 induced a dose-dependent increase in GU. IGFBP-2 increased the insulin-induced GU after long-term incubation. The IGFBP-2-induced impact on GU was neither affected by insulin or IGF-1 receptor blockage nor by insulin receptor knockdown. IGFBP-2 significantly increased the phosphorylation of PI3K, Akt, AMPK, TBC1D1 and PKCζ/λ, and induced GLUT-4 translocation. Moreover, inhibition of PI3K and AMPK significantly reduced IGFBP-2-stimulated GU. In conclusion, IGFBP-2 stimulates GU in 3T3-L1 adipocytes through activation of PI3K/Akt, AMPK/TBC1D1 and PI3K/PKCζ/λ/GLUT-4 signaling. The stimulatory effect of IGFBP-2 on GU is independent of its binding to IGF-1 and is possibly not mediated through the insulin or IGF-1 receptor. This study highlights the potential role of IGFBP-2 in glucose metabolism.

Key words: IGFBP-2; Glucose uptake; AMPK; GLUT-4.

1. Introduction

Insulin-like growth factor-1 (IGF-1) bears structural homology with pro-insulin [1], and plays a key role in the proliferation and differentiation of adipocytes [2]. *In vitro*, it is known to exert mitogenic effects at nanomolar concentrations [3] and to induce insulin-like metabolic effects in both muscle and adipose tissue [4]. The production and secretion of IGF-1 is affected by age, nutritional status and other hormones [5]. Because of the ability of insulin to induce hepatic growth hormone (GH) receptor gene expression [6] and protein abundance [7], the GH-induced synthesis and release of IGF-1 is highly dependent on the hepatic insulin sensitivity. This interplay among GH, insulin and IGF-1 is of key importance for metabolic and growth regulation [8].

The bioavailability of IGFs is regulated by a family of seven structurally conserved binding proteins (IGFBPs) [9-11]. These IGFBPs bind IGF-1 and IGF-2 but not insulin [12]. The IGF-1 independent role of IGFBPs in growth and metabolism has also been reported at least *in vitro* [13, 14]. IGFBP-2 is the predominant binding protein produced during adipogenesis of white preadipocytes [15]. Both inhibitory and stimulatory effects of IGFBP-2 on the cellular actions of IGF-1 and IGF-2 have been reported [16, 17]. IGFBP-2 is reported to be a key regulator of metabolic diseases, such as diabetes and obesity. Low IGFBP-2 has been shown to be associated with higher fasting glucose levels and reduced insulin sensitivity suggesting it as a biomarker for identification of insulin-resistant individuals [18]. Moreover, IGFBP-2 gene expression was down-regulated in visceral white adipose tissue of mice and its circulating levels were reduced in obese ob/ob, db/db and high fat-fed mice [19]. Low levels of circulating IGFBP-2 have also been reported in obese adults [20] and children [21].

Wheatcroft and colleagues demonstrated that IGFBP-2–overexpression conferring protection against age-associated decline in insulin sensitivity in mice [22]. Moreover, the leptin-induced overexpression of IGFBP2 has been shown to reverse diabetes in insulin-resistant obese mice and hyperinsulinemic clamp studies showed a 3-fold improvement in hepatic insulin sensitivity following IGFBP-2 treatment of ob/ob mice [23]. However, only few information exists to date regarding the mechanisms underlying the positive IGFBP-2-induced impact on glucose metabolism. Indeed, IGFBP-2 has been shown to increase the insulin-stimulated glucose uptake in myotubes [24] but nothing is known about its impact on glucose uptake in adipocytes with respect to the insulin or IGF-1 induced effects. We, therefore, aimed to investigate the IGFBP-2-induced changes in both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes and the underlying mechanisms. We further investigated the role of insulin and IGF-1 receptors in mediating the IGFBP-2 and even the impact of IGFBP-2 on the IGF-1 induced improvement in glucose uptake.

2. Materials and methods

2.1. Reagents, hormones and antibodies

IGF-1 and IGF-1 Long R3 (IGF-1 LR3) were purchased from BioVision Inc. (Milpitas, CA, USA). IGFBP-2, Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Biochrom AG (Berlin, Germany). Insulin, dexamethasone, LY294002 and picropodophyllin (PPP) were supplied by Sigma Aldrich (Darmstadt, Germany). 3-isobutyl-1-methylxanthine (IBMX), S961, wortmannin and Compound C were purchased from Biomol GmbH (Hamburg, Germany), Phoenix Biotech (Beijing, China), Merck Chemicals (Darmstadt, Germany) and BIOZOL Diagnostica Vertrieb (Eching, Germany), respectively. RevertAid First Strand cDNA Synthesis Kit, SYBR Green master mix, Bicinchoninic Acid (BCA) protein assay kit and ECL reagent were supplied by Thermo Fisher Scientific (Dreieich, Germany). DNA primers were purchased from Eurogentec Deutschland GmbH (Köln, Germany). All other chemicals were supplied by Sigma Aldrich (Darmstadt, Germany).

2.2. Cell culture

The murine fibroblast cell line 3T3-L1 (ATCC, Manassas, VA, USA) was cultured in DMEM supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 4 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin until confluence. The cells were incubated to differentiate into adipocytes following the method of Woody et al [25] with slight modifications. Briefly, 2 days post-confluence, cells were treated with 0.5 mM IBMX, 1 µM dexamethasone and 1µM insulin supplemented DMEM for 2 days. The cells were then maintained in 1µM insulin supplemented growth medium for 3 days and in growth medium for 4 days prior to experiments.

2.3. Transfection of insulin receptor (INSR) siRNAs

Differentiated 3T3-L1 adipocytes were transfected with control or INSR specific siRNA (validated siRNA from Dharmacon) using LipofectamineRNAiMAX (Invitrogen) for 72 h. The efficiency of transfection was assessed by using qPCR and western blot.

2.4. Measurement of glucose uptake

Glucose uptake was assayed using the method described by Yamamoto et al [26]. Briefly, differentiated 3T3-L1 adipocytes were serum starved for 4 h followed by incubation in D-glucose free

DMEM for 1 h. The adipocytes were washed with PBS (pH 7.4) and then incubated for 30 min in Krebs-Ringer bicarbonate buffer (KRBP) with different concentrations of insulin, IGF-1, IGF-1 LR3 and/or IGFBP-2. IGF1 LR3 is an analogue of IGF-1 in which the glutamic acid at carbon 3 (Glu3) is replaced by arginine and contains 13 extra amino acids to the N-terminus. It has a very low affinity towards IGFBPs as compared to IGF-1 [27]. The rationale for using IGF1-LR3 was to investigate whether IGFBP-2 is able to impact the IGF-1 induced increase in glucose uptake regardless of its binding to IGF-1 itself. Had IGFBP-2 exerted additive effect on the IGF-1 induced glucose uptake, it would be imperative to scrutinize the observed effect as due to binding or other means. In some experiments, the adipocytes were incubated with 100 nM S961 (INSR blocker) for 2 h, 60 nM PPP (IGF-1 receptor blocker) for 4 h, 100 µM LY294002 (PI3K inhibitor) for 1 h, 200 nM wortmannin (PI3K inhibitor) for 30 min or 200 µM Compound C (AMPK inhibitor) for 20 min before the treatment. The adipocytes were treated with [^{3}H] 2-Deoxy-D-glucose (0.5 µCi/ml in HEPES) for 10 min at room temperature (RT) and then washed with PBS. Thereafter, the cells were lysed in 50 mM NaOH/1% Triton X-100 for scintillation counting using a liquid scintillation counter (PerkinElmer Wallac GmbH, Freiburg, Germany). Each experiment was performed with three technical replicates and total number of experiments was three.

2.5. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from 3T3-L1 adipocytes using TRizol reagent and was treated with DNase I. RNA was quantified at 260 nm using a Nanodrop (Peqlab Biotechnologie, Erlangen, Germany) and samples with A260/A280 ratios < 1.8 were discarded. One µg RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Dreieich, Germany). cDNA was amplified using SYBR Green master mix (Thermo Fisher Scientific, Dreieich, Germany) with the primers set outlined in Table 1 and the following conditions; initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 60 sec at annealing temperature of respective primer, and 60 sec at 72°C for extension. Melting curve analysis was used to assess the quality of PCR products and the cycle threshold (CT) values were analyzed using the $2^{-\Delta\Delta Ct}$ method. Data were normalized to 36B4 and presented as % of control.

2.6. Preparation of a plasma membrane fraction for glucose transporter (GLUT)-4 translocation assay

The amount of GLUT-4 in the cell membranes was determined using subcellular fractionation [28] followed by western blotting analysis. Adipocytes were washed 3 times with ice cold HEPES-EDTA-sucrose (HES) buffer (pH 7.4) containing proteinase inhibitors. The cell suspension was homogenized by passing through 22-gauge needle 10 times on ice. The homogenate was centrifuged at 16000 g for 30 min at 4°C and pellet was suspended in HES buffer followed by centrifugation at 16000 g for 30 min at 4°C. The pellet was resuspended in HES buffer, layered on the top of sucrose cushion (38.5% sucrose, 20 mM HEPES and 1 mM EDTA, pH 7) in 1:1 volume ratio and centrifuged at 100000 g for 1 h at 4°C. The plasma membrane fraction (middle layer) was carefully collected and centrifuged at 40000 g for 20 min at 4°C. The pellet was used to determine amount of GLUT-4 using Western blotting.

2.7. Western blot analysis

Treated 3T3-L1 adipocytes were lysed in RIPA buffer supplemented with inhibitors for proteinases and phosphatases. For the GLUT-4 translocation experiments, the samples were lysed in a specific buffer (10 mM Tris-HCl [pH 7.2], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) supplemented with proteinase and phosphatase inhibitors. The protein content in the samples was measured by BCA assay kit. Proteins (30-50 µg) were denatured and resolved in 10% SDS/PAGE and transferred to nitrocellulose membranes. For GLUT-4, 8% SDS/PAGE was used. Blots were blocked for 1 h and probed with 1:1000 diluted primary antibodies for phosphoinositide 3-kinase (PI3K) p85, phospho-(Tyr) PI3K p85, protein kinase B (Akt), phospho-Akt (Ser473), AMP-activated protein kinase alpha (AMPKα), phospho-AMPKα (Thr172), atypical protein kinase (PKCζ), phospho-PKCζ/λ (Thr410/403), TBC1D1 (tre-2/USP6, BUB2, cdc16 domain family member 1), phospho-TBC1D1 (Ser237) and GAPDH over night at 4°C, and with GLUT-4 antibody and Na^+/K^+ ATPase for 1 h at RT. The blots were washed and incubated with 1:2000 diluted corresponding horseradish peroxidase (HRP)-labeled secondary antibodies. Details of the used antibodies are listed in Table 2. After washing, the membranes were developed with ECL reagents,

visualized and densitometry analysis using Image Lab™ Software (Bio-Rad Laboratories GmbH, Munich, Germany) was used to quantify protein signal.

2.8. Statistical analysis

Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using GraphPad Prism 5 (La Jolla, CA, USA). The results were presented as means ± standard error of the mean (SEM) with values of $P<0.05$ were considered significant.

3. Results

3.1. Effect of IGFBP-2 on basal as well as insulin and IGF-1 induced increase in glucose uptake in 3T3-L1 adipocytes

To study the effect of insulin, IGF-1, IGF-1 LR3 and IGFBP-2 on glucose uptake in 3T3-L1 adipocytes, the cells were incubated with different concentrations of all tested agents for 30 min and [^{3}H] 2-Deoxy-D-glucose uptake was assayed. Insulin and IGF-1 were able to exert statistically significant effects on glucose uptake. As represented in Figure 1A, different concentrations of insulin (10, 20, 50 and 100 nM) were able to exert a significant ($P<0.001$) increase in glucose uptake. IGF-1 as well produced a significant increase in glucose uptake at either 10 nM ($P<0.05$) or higher concentrations ($P<0.001$) as depicted in Figure 1B.

Treatment of the cells with the lengthened analogue of IGF-1, IGF-1 LR3, induced significant increase in glucose uptake first at higher concentrations (20, 50 and 100 nM) (Fig. 1C). Similarly, IGFBP-2 was able to significantly ($P<0.01$) increase glucose uptake in adipocytes first at concentration of 100 nM as compared to control cells (Fig. 1D).

Next, we determined both the short and long-term impact of IGFBP-2 on insulin, IGF-1 and IGF-1 LR3 induced glucose uptake in adipocytes.

Short-term incubation of the cells with 1:1 stoichiometric ratio of IGFBP-2 and either insulin, IGF-1, or IGF-1 LR3 for 30 min resulted in no additive increase in glucose uptake when compared to insulin, IGF-1 or IGF-1 LR3 alone (Fig. 1E).

Long-term incubation (24 h) of the cells with IGFBP-2 significantly ($P<0.05$) increased basal glucose uptake and exerted an additive effect ($P<0.01$) on insulin-stimulated glucose uptake. However, adipocytes treated with IGFBP-2 for 24 h showed non-significant changes in either IGF-1 or IGF-1 LR3 induced glucose uptake (Fig. 1F).

3.2. The IGFBP-2 induced impact on glucose uptake is not mediated through the activation of insulin or IGF-1 receptor

To investigate whether the stimulatory effect of IGFBP-2 on glucose uptake is mediated through its binding to insulin or IGF-1 receptors, we incubated 3T3-L1 adipocytes with either insulin receptor blocker (S961) or IGF-1 receptor blocker (PPP).

3T3-L1 adipocytes incubated for 2 h with S961 showed a significant ($P<0.05$) decrease in basal glucose uptake when compared with the control cells (Fig. 2A). The insulin receptor blocker S961 significantly reduced insulin ($P<0.001$), IGF-1 ($P<0.001$) and IGF-1 LR3 ($P<0.01$) stimulated glucose uptake, whereas no impact ($P>0.05$) of such treatment on IGFBP-2 stimulated glucose uptake was seen.

When compared with S961, the IGF-1 receptor blocker PPP was not able to induce any significant ($P>0.05$) effect on glucose uptake in adipocytes neither under basal conditions nor following stimulation with IGF-1, IGF-1 LR3 or IGFBP-2 (Fig. 2B).

In 3T3-L1 adipocytes transfected with control or INSR specific siRNA (Fig. 2C and 2D), insulin (Fig. 2E) and IGF-1 stimulated glucose uptake (Fig. 2F) was significantly ($P<0.05$) reduced, whereas INSR knockdown potentiated the effect of IGFBP-2 on glucose uptake when compared with the control cells ($P<0.01$) (Fig. 2G).

3.3. IGFBP-2 stimulates glucose uptake in a PI3K-dependent manner

Adipocytes treated with insulin and IGF-1 for 30 min exhibited significant ($P<0.001$) increase in PI3K phosphorylation when compared with the control cells. Similarly, IGFBP-2 induced a significant increase in PI3K phosphorylation in 3T3-L1 adipocytes treated for either 30 min ($P<0.01$) or 24 hr ($P<0.001$) (Fig. 3A).

The effect of PI3K inhibitors (LY294002 and wortmannin) on glucose uptake was investigated to further determine the role of PI3K in mediating the IGFBP-2 stimulated glucose uptake in 3T3-L1 adipocytes. Treatment of the adipocytes with either LY294002 (Fig. 3B) or wortmannin (Fig. 3C) induced a significant decline in basal as well as insulin-, IGF-1- and IGFBP-2- stimulated glucose uptake ($P<0.001$).

3.4. IGFBP-2 induces Akt- and AMPK-phosphorylation and the subsequent increase in GLUT-4 translocation in a PI3K-dependent manner

We further investigated the impact of IGFBP-2 on Akt and AMPK phosphorylation as well as on GLUT-4 translocation. As expected, insulin and IGF-1 significantly ($P<0.001$) up-regulated Akt phosphorylation in treated 3T3-L1 adipocytes. Similarly, IGFBP-2 induced a noticeable increase in Akt phosphorylation in 3T3-L1 adipocytes treated for either 30 min ($P<0.05$) or 24 h ($P<0.01$) (Figure 4A).

IGF-1 significantly ($P<0.001$) increased, whereas insulin failed to induce ($P>0.05$) AMPK phosphorylation in 3T3-L1 adipocytes (Fig. 4B). Similarly, treatment of adipocytes with IGFBP-2 for either 30 min or 24 h induced a significant ($P<0.001$) increase in AMPK phosphorylation.

To further confirm the involvement of AMPK phosphorylation in IGFBP-2 stimulated glucose uptake, adipocytes were treated with IGFBP-2 with or without previous incubation with the AMPK inhibitor Compound C. Treatment of the 3T3-L1 adipocytes with IGFBP-2 significantly ($P<0.01$) increased glucose uptake, an effect that was significantly ($P<0.001$) abolished by Compound C (Fig. 3C).

Insulin and IGF-1 stimulation increased TBC1D1 phosphorylation significantly ($P<0.05$) when compared with the control adipocytes (Fig. 4D). Similarly, treatment of the 3T3-L1 adipocytes with IGFBP-2 for either 30 min or 24 h induced a significant ($P<0.05$) increase in TBC1D1 phosphorylation (Fig. 4D).

GLUT-4 translocation was assessed by subcellular fractionation followed by western blotting. Treatment of the adipocytes with insulin significantly ($P<0.01$) stimulated GLUT-4 translocation from

the cytoplasm to plasma membrane. IGF-1 was also able to significantly ($P<0.05$) stimulate GLUT-4 translocation. Similarly, IGFBP-2 induced a significant ($P<0.05$) increase in GLUT-4 translocation in treated 3T3-L1 adipocytes (Fig. 4E).

3.5. IGFBP-2 stimulates PKCζ/λ Thr410/403 phosphorylation in 3T3-L1 adipocytes

A significant increase in the phosphorylated levels of the PKCζ/λ isoform was seen after stimulation with rather insulin ($P<0.05$) or IGF-1 ($P<0.01$) (Fig. 5). Similarly, treatment of the cells with IGFBP-2 induced a significant increase in PKCζ/λ phosphorylation after either 30 min ($P<0.01$) or 24 h ($P<0.001$) (Fig. 5).

4. Discussion

Previous studies have indicated the role of IGFBP-2 in adipogenesis and lipogenesis, but its effects on basal glucose uptake and the underlying mechanistic pathways have not yet been addressed. We, herein, provide the first evidence for insulin and IGF-1 independent positive impact of IGFBP-2 on glucose uptake in adipocytes. We further show that the effect of IGFBP-2 on glucose uptake is mediated through the activation of PI3K/Akt- and AMPK-pathways. Finally, we show that IGF-1 receptor is neither involved in the IGF-1 induced nor in the IGFBP-2 induced increase in glucose uptake.

Insulin and IGF-1 exerted significant dose-dependent effects on glucose uptake in 3T3-L1 adipocytes. These findings are in agreement with the reports from different previous studies [29-33]. Multiple *in vivo* studies reported the role of IGF-1 in enhancing insulin sensitivity and glucose metabolism. A low serum level of IGF-1 has been associated with insulin resistance and treatment with recombinant IGF-1 has been shown to improve insulin sensitivity and glucose metabolism [34, 35]. A study by Arafat et al [36] revealed that long term treatment of GH deficient patients with low dose GH results in improved insulin sensitivity and enhanced glucose metabolism. This improvement in insulin sensitivity is believed to be mediated by IGF-1, which is secreted as a result of GH stimulation. In another clinical study, IGF-1 combined with IGFBP-3 has been shown to improve insulin sensitivity

and to reduce complications associated with insulin resistance in HIV/AIDS patients on anti-retroviral therapy [37]. Blocking the insulin receptor with S961 or knocking down the INSR using siRNA significantly reduced basal and insulin-stimulated glucose uptake. The mechanisms behind the effect of S961 on basal glucose uptake in 3T3-L1 are not known so far. However, our results were concordant with previously reported impact of S961 on insulin-stimulated glucose uptake in 3T3-L1 adipocytes [38, 39]. Despite the fact that insulin and IGF-1 have different affinities to INSR and IGF-1R, they are able to stimulate both receptors [40]. However, blocking the IGF-1 receptor using PPP [41] in our present study did not affect the impact of IGF-1 on glucose uptake, whereas blocking or even knocking down the INSR did, pointing to the role of INSR in mediating these IGF-1 effects. In the study of Girnita et al. [41], PPP efficiently blocked IGF-1R activity, and reduced phosphorylation of Akt and extracellular signal regulated kinase 1 and 2 (Erk1/2) in cultured IGF-1R-positive tumor cells. In an *in vitro* kinase assay, PPP did not affect the INSR or compete with ATP [41]. Our findings are also supported by various reports that demonstrated dramatic increase in INSR and a decrease in IGF-1R during the transition from preadipocytes to adipocytes in the 3T3-L1 cell line [42-45].

IGF-1-dependent and -independent effects of IGFBPs on metabolism represent a rapidly growing field of research. IGFBP-1 was reported to inhibit IGF-1-stimulated glucose uptake but not insulin-stimulated glucose uptake in 3T3-L1 adipocytes [31]. IGFBP-3 can lead to insulin resistance in 3T3-L1 adipocytes as reported by Chan et al [46]. There is increasing evidence for the role of IGFBP-2 in regulating normal metabolism [47]. Low serum levels of IGFBP-2 are correlated with obesity [22], metabolic syndrome [18] and type 2 diabetes [48], whereas overexpression of IGFBP-2 protects against diabetes and obesity [22, 23]. Roles of IGFBP-2 on metabolism such as inhibition of adipogenesis and lipogenesis [49], enhancing insulin-stimulated glucose uptake in skeletal myotubes [50] and inhibition of preadipocyte differentiation *in vitro* [14] have been reported. However, the effects of IGFBP-2 on basal glucose uptake and the mechanisms underlying its IGF-1-independent role on glucose uptake are not well studied. Here, we reported significantly increased glucose uptake in 3T3-L1 adipocytes treated with 100 nM IGFBP-2. To our knowledge, this is the first report to show the stimulatory effects of IGFBP-2 on basal glucose uptake in adipocytes. In addition, our data showed non-significant effect for short and long-term treatment with IGFBP-2 on IGF-1 and IGF-1 LR3

stimulated glucose uptake. Adipocytes treated with IGFBP-2 for 24 h followed by 30 min stimulation with IGF-1 showed a trend increase in glucose uptake. Increased basal glucose uptake in control cells incubated with IGFBP-2 for 24 h may explain this increase. However, incubation with IGFBP-2 for 24 h exerted a significant additive effect on the insulin-stimulated glucose uptake which coincides with the study of Yau et al [50] who reported similar effect for IGFBP-2 in human skeletal muscle cells *in vitro*. It can be postulated that the additive increase in the acute insulin-induced stimulation of glucose uptake after long-term treatment with IGFBP-2 is due to the impact of IGFBP-2 on basal glucose uptake that is likely also mediated through different signaling pathways other than the PI3K/Akt pathway. Moreover, these findings provide a notion that IGFBP-2 binding to IGF-1 doesn't inhibit IGF-1 from exerting its biological role, at least on glucose uptake *in vitro.*

In addition to its ability to bind and modulate the activity of IGFs, IGFBP-2 can bind to proteoglycans [51] through two heparin-binding domains (HBDs) as well as to integrins through its integrin bonding motif, Gly-Arg-Asp (RGD) [51, 52]. This may explain, at least in part, the IGFR-independent IGFBP-2 activities [49].

Interestingly, neither S961 nor PPP blocked the stimulatory effect of IGFBP-2 on glucose uptake. Moreover, INSR knockdown even increased IGFBP-2 induced increase in glucose uptake. These findings indicate the involvement of other receptors or pathways in IGFBP-2 stimulated glucose uptake in 3T3-L1 adipocytes. This is concordant with the findings of Xi et al [53], who reported that IGFBP-2 stimulates AMPK *via* its own receptor.

Signaling *via* INSR and IGF-1R share many common signaling pathways at target cells. One of the common pathways in mediating glucose uptake and metabolism is the PI3K pathway [40, 54]. Insulin and IGF-1 are known to stimulate the activity of PI3K by triggering its phosphorylation at specific tyrosine residues by upstream components of the INSR and IGF-1R signaling pathways [55]. In the present study, the PI3K inhibitors, LY294002 and wortmannin reduced basal and, insulin, IGF-1 and even IGFBP-2 stimulated glucose uptake in adipocytes, pointing to the role of PI3K pathway in mediating the IGFBP-2 effect on glucose uptake. We, therefore, investigated the impact of short and

long-term treatment with IGFBP-2 on PI3K phosphorylation in 3T3-L1 adipocytes, using insulin and IGF-1 as controls. As expected, treatment of the adipocytes with either insulin or IGF-1 significantly increased PI3K phosphorylation. Similarly, IGFBP-2 induced marked increase in PI3K phosphorylation after both short and long-term treatment, confirming the involvement of PI3K activation in mediating IGFBP-2 effects.

Given that IGFBP-2 activates PI3K, we tested its effect on the downstream signaling molecules Akt and AMPK, and GLUT-4 translocation. As a result of PI3K activation, insulin and IGF-1 stimulated the phosphorylation of Akt. These findings are in agreement with the studies of Karlsson et al [56] and Zhang et al [57]. Moreover, IGF-1 significantly increased AMPK phosphorylation. IGF-1 has been previously shown to stimulate the phosphorylation of AMPK at its alpha subunit [58]. On the other hand, insulin didn't affect the level of p-AMPK indicating that insulin mainly uses the PI3K pathway to exert its effects on glucose metabolism. Our findings are in agreement with Shen et al [59] who clearly showed that insulin doesn't stimulate AMPK. In the same context, pharmacological activation of AMPK increases glucose uptake in skeletal muscles of subjects with type 2 diabetes [60] by an insulin-independent mechanism [61].

Similarly, IGFBP-2 produced a significant increase in Akt phosphorylation which is attributed to its stimulatory effect on PI3K. Concordant data were reported by Yau et al [50] in human skeletal muscle cells. The surface proteoglycan receptor-type protein tyrosine phosphatase β (RPTPβ) has been identified as a functionally active cell surface receptor that links IGFBP-2 and the activation of Akt [62]. IGFBP-2 binds RPTPβ through its HBD, resulting in inhibition of RPTPβ phosphatase activity and subsequently phosphatase and tensin homolog (PTEN) suppression [62]. PTEN is known to prevent Akt activation by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3). The study of Shen et al [62] showed that IGFBP-2$^{-/-}$ mice had increased RPTPβ activity and impaired Akt activation, changes that were reversed by administration of IGFBP-2.

In addition, both short and long-term treatment of the adipocytes with IGFBP-2 induced significant increase in AMPK phosphorylation. IGFBP-2 and IGF-1 have been recently reported by Xi et al [53] to induce stimulatory effects on AMPK in osteoblasts. Our results were further confirmed through testing the effect of AMPK inhibitor, Compound C, on IGFBP-2 stimulated glucose uptake. Incubation of the adipocytes with Compound C significantly abolished IGFBP-2 induced glucose uptake. Taken together, AMPK activation plays potential role in mediating IGFBP-2 stimulated glucose uptake in 3T3-L1 adipocytes.

One of the major metabolic changes elicited by AMPK activation is the promotion of glucose uptake [63]. AMPK induces glucose uptake either acutely through GLUT-4 translocation or in the longer term *via* up-regulation of GLUT-4 expression [63]. Here, we show that treatment of the 3T3-L1 adipocytes with IGFBP-2 for 30 min stimulates GLUT-4 translocation to the plasma membrane. This effect is attributed to the ability of IGFBP-2 to activate AMPK. In addition, we show a significant increase in the phosphorylation of the Rab-GAP protein TBC1D1 by IGFBP-2. Therefore, the mechanism underlying the IGFBP-2 impact on GLUT-4 translocation and the subsequent promotion of glucose uptake involves the phosphorylation of TBC1D1 at least partly through AMPK-pathway activation. This effect is similar to the complementary regulation of TBC1D1 by insulin and AMPK activators [64, 65]. Increased TBC1D1 phosphorylation and GLUT-4 translocation by IGFBP-2 could also be directly mediated by Akt activation. In skeletal muscle of rodents, Akt phosphorylates TBC1D1 [66] which promote the hydrolysis of guanosine-5'-triphosphate on GLUT-4-containing vesicles [67].

The atypical protein kinase PKCζ/λ/GLUT-4 is another signaling pathway we thought to have a role in mediating the positive effect of IGFBP-2 on glucose uptake in adipocytes. In our study, insulin, IGF-1 and IGFBP-2 induced a significant increase in PKCζ/λ phosphorylation. Since, PKCζ/λ is dependent on PI3K activation [29, 68], it was expected to be activated in adipocytes treated with insulin, IGF-1 and IGFBP-2 because of their ability to activate PI3K. Following activation, PI3K signaling diverges into Akt-dependent and PKCζ/λ-mediated pathways [69]. PKCζ/λ is known to play little or no role in

mediating insulin effects on glucose uptake in 3T3-L1 adipocytes [70], which may explain the IGFBP-2 induced additive increase in the insulin-induced glucose uptake after long-term treatment in our present study. However, further studies using inhibitors or gene silencing are needed to explore the precise involvement of PKCζ/λ in mediating the IGFBP-2 induced increase in glucose uptake and GLUT-4 translocation. One of the limitations of our study was the IGFBP-2 concentrations used to elicit a significant impact on glucose uptake. IGFBP-2 increased glucose uptake at concentrations 7-10-fold higher than those described in humans. Therefore, further *in vivo* studies are needed to explore the precise impact of physiological concentrations of IGFBP-2 on glucose utilization in humans.

In summary, this study shows that IGFBP-2 stimulates glucose uptake in 3T3-L1 adipocytes and that synergistic activation of Akt and AMPK mediates the modulatory effect of IGFBP-2. The PI3K/PKCζ/λ/GLUT-4 signaling is here shown to mediate the IGFBP-2 induced increase in glucose uptake. Furthermore, we showed that IGFBP-2 induced glucose uptake is independent of its binding to IGF-1, INSR and IGF-1R. Our findings highly strength the potential and novel role for IGFBP-2 in glucose metabolism.

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Authors' contribution

AMA conceived the study and acquired funding for the experiment. BA and AMM performed experiments and analyzed the results. BA, AMM and AMA wrote the manuscript. AFHP, ALB and JS revised and commented on the manuscript. All authors read and approved the final manuscript.

Competing interests:
The authors have declared that no competing interests exist.

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Tables:

Table 1. Primers used for qRT-PCR.

Gene	Sequence (5'-3')
INSR	F: GTACTGGGAGAGGCAAGCAG
	R: ACTGGCCGAGTCGTCATACT
36B4	F: TCATCCAGCAGGTGTTTGACA
	R: GGCACCGAGGCAACAGTT

Table 2. List of antibodies used.

Antibody	Species	Supplier	Catalog number
Anti-Phospho-AMPKα (Thr172)	Rabbit	Cell Signaling Technology	2535
Anti-AMPKα	Rabbit	Cell Signaling Technology	2532
Anti-Phospho-Akt (Ser473)	Rabbit	Cell Signaling Technology	9271
Anti-Akt	Rabbit	Cell Signaling Technology	9272
Anti-Phospho-(Tyr) p85 PI3K	Rabbit	Cell Signaling Technology	3821
Anti-PI3K p85	Rabbit	Cell Signaling Technology	4292
Anti-Phospho-TBC1D1 (Ser237)	Rabbit	Millipore	07-2268
Anti-TBC1D1	Rabbit	Cell Signaling Technology	5929
Anti-Phospho-PKCζ/λ (Thr410/403)	Rabbit	Cell Signaling Technology	9378
Anti-PKCζ	Mouse	Santa Cruz Biotechnology	SC-17781
Anti-GLUT-4	Rabbit	Sigma	G4173
Anti-Na^+,K^+-ATPase	Rabbit	Cell Signaling Technology	3010
Anti-GAPDH	Rabbit	Cell Signaling Technology	2118
Goat anti-rabbit IgG HRP-linked	Goat	Cell Signaling Technology	4074
Horse anti-mouse IgG HRP-linked	Horse	Cell Signaling Technology	4076

Figure legends

Figure 1. Effects of IGFBP-2 on insulin and IGF-1 stimulated glucose uptake in 3T3-L1 adipocytes. (**A-D**) Dose dependent effects of insulin, IGF-1, IGF-1 LR3 and IGFBP-2 on glucose uptake in 3T3-L1 adipocytes. *P<0.05, **P<0.01 and ***P<0.001 versus control. (**E**&**F**) effect of IGFBP-2 (100 nM) (black bars) on basal and insulin (20 nM), IGF-1 (20 nM) and IGF-1 LR3 (20 nM)-induced glucose uptake (white bars) after short and long-term incubations. *P<0.05 and **P<0.01. Each experiment was performed with three technical replicates and total number of experiments was three. The glucose uptake values are percentage of the controls. The results are presented as mean ± SEM.

Figure 2. IGFBP-2 stimulates glucose uptake in insulin receptor and IGF-1 receptor-independent mechanism. (**A**) Effect of the insulin receptor (INSR) blocker S961 (black bars) on basal and insulin, IGF-1, IGF-1 LR3 and IGFBP-2 induced glucose uptake (white bars). (**B**) Effect of the IGF-1 receptor blocker PPP (black bars) on basal and insulin, IGF-1, IGF-1 LR3 and IGFBP-2 induced glucose uptake (white bars). Differentiated 3T3-L1 adipocytes were incubated with 100 nM S961 for 2 h or 60 nM PPP for 4 h before treatment with insulin, IGF-1, IGF-1 LR3 or IGFBP-2 for 30 min. (**C-D**) Relative mRNA expression, normalized to 36B4, and western blot analysis of INSR following siRNA transfection, respectively. (**E-F**) Effect of 30 min treatment with insulin, IGF-1 and IGFBP-2 on glucose uptake in control siRNA and INSR siRNA transfected 3T3-L1 adipocytes. Each experiment was performed with three technical replicates and total number of experiments was three. The glucose uptake values are percentage of the controls. The results are presented as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001.

Figure 3. IGFBP-2 stimulates glucose uptake in a PI3K-dependent manner. (**A**) Insulin, IGF-1 and IGFBP-2 increase the phosphorylation of PI3K. 3T3-L1 cells were cultured and differentiated in 24-well plates for glucose uptake assay and in 6-well plates for western blotting analysis. The results are presented as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 versus control. (**B & C**) The PI3K inhibitors, LY294002 and wortmannin (black bars), significantly reduce basal as well as insulin, IGF-1

and IGFBP-2-induced glucose uptake (white bars). Differentiated 3T3-L1 adipocytes were incubated with 100 µM LY294002 for 1 h or 200 nM wortmannin for 30 min before treatment with insulin, IGF-1 or IGFBP-2 for 30 min. The results are presented as mean ± SEM. ***P<0.001. Each experiment was performed with three technical replicates and total number of experiments was three.

Figure 4. IGFBP-2 stimulates Akt and AMPK activation and thus increased GLUT-4 translocation. (**A**) Insulin, IGF-1 and IGFBP-2 significantly increase Akt Ser473 phosphorylation, (**B**) IGF-1 and IGFBP-2 but not insulin increase AMPK Thr172 phosphorylation. *P<0.05, **P<0.01 and ***P<0.001 versus control. (**C**) The AMPK inhibitor, Compound C, abolishes IGFBP-2-induced glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated with 200 µM Compound C for 20 min before treatment with IGFBP-2 for 30 min. *P<0.05 and ***P<0.001. Insulin, IGF-1 and IGFBP-2 significantly increase TBC1D1 Ser237 phosphorylation (**D**) and GLUT-4 translocation (**E**). *P<0.05, **P<0.01 and ***P<0.001 versus control. Each experiment was performed with three technical replicates and total number of experiments was three. The glucose uptake values are percentage of the controls. The results are presented as mean ± SEM.

Figure 5. IGFBP-2 stimulates PKCζ/λ Thr410/403 phosphorylation in 3T3-L1 adipocytes. Insulin, IGF-1 and IGFBP-2 increase the phosphorylation of PKCζ/λ. Each experiment was performed with three technical replicates and total number of experiments was three. The results are presented as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 versus control.

Figure 1

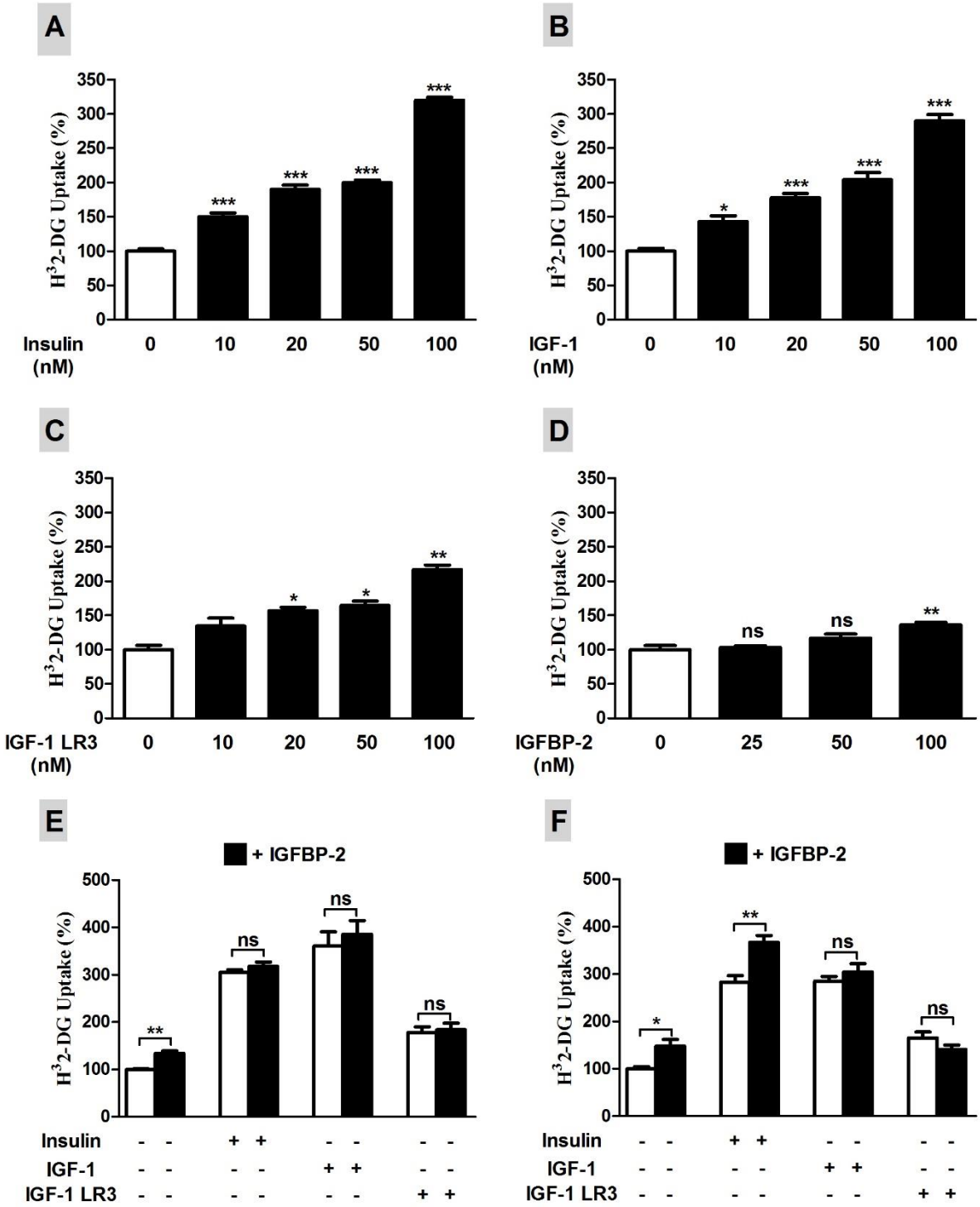

A
B
C
D
E
F
$H^3$2-DG Uptake (%)
Insulin (nM)
IGF-1 (nM)
IGF-1 LR3 (nM)
IGFBP-2 (nM)
+ IGFBP-2
ns
Insulin
IGF-1
IGF-1 LR3

Figure 2

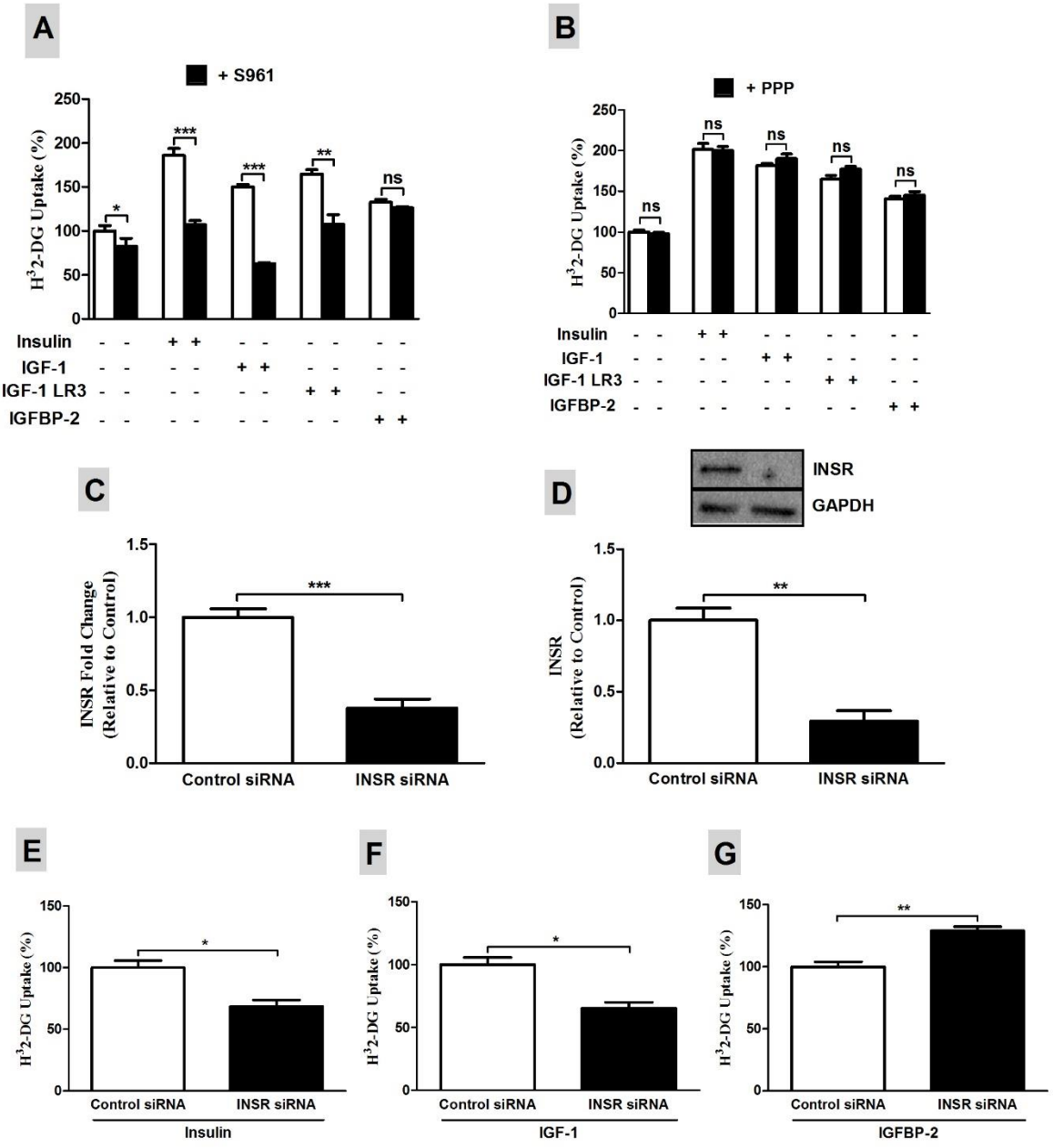

A
+ S961
H32-DG Uptake (%)
Insulin
IGF-1
IGF-1 LR3
IGFBP-2
B
+ PPP
H32-DG Uptake (%)
Insulin
IGF-1
IGF-1 LR3
IGFBP-2
C
INSR Fold Change (Relative to Control)
Control siRNA
INSR siRNA
D
INSR
GAPDH
INSR (Relative to Control)
Control siRNA
INSR siRNA
E
H32-DG Uptake (%)
Control siRNA
INSR siRNA
Insulin
F
H32-DG Uptake (%)
Control siRNA
INSR siRNA
IGF-1
G
H32-DG Uptake (%)
Control siRNA
INSR siRNA
IGFBP-2

Figure 3

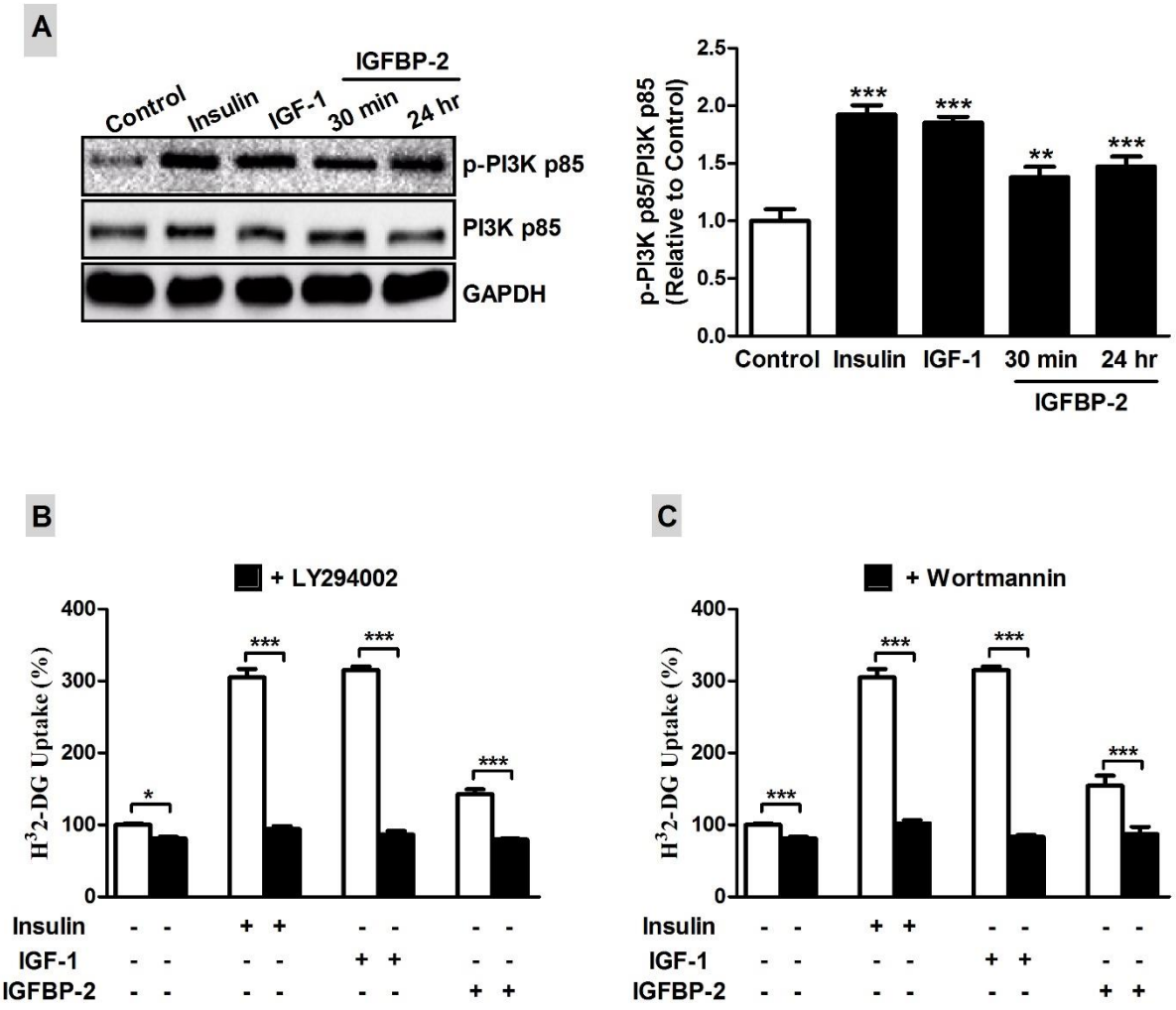

A
IGFBP-2
Control
Insulin
IGF-1
30 min
24 hr
p-PI3K p85
PI3K p85
GAPDH
p-PI3K p85/PI3K p85 (Relative to Control)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
**
***
Control
Insulin
IGF-1
30 min
24 hr
IGFBP-2
B
+ LY294002
H32-DG Uptake (%)
400
300
200
100
0
*
***
***
***
Insulin - - + + - - - -
IGF-1 - - - - + + - -
IGFBP-2 - - - - - - + +
C
+ Wortmannin
H32-DG Uptake (%)
400
300
200
100
0
***
***
***
***
Insulin - - + + - - - -
IGF-1 - - - - + + - -
IGFBP-2 - - - - - - + +

Figure 4

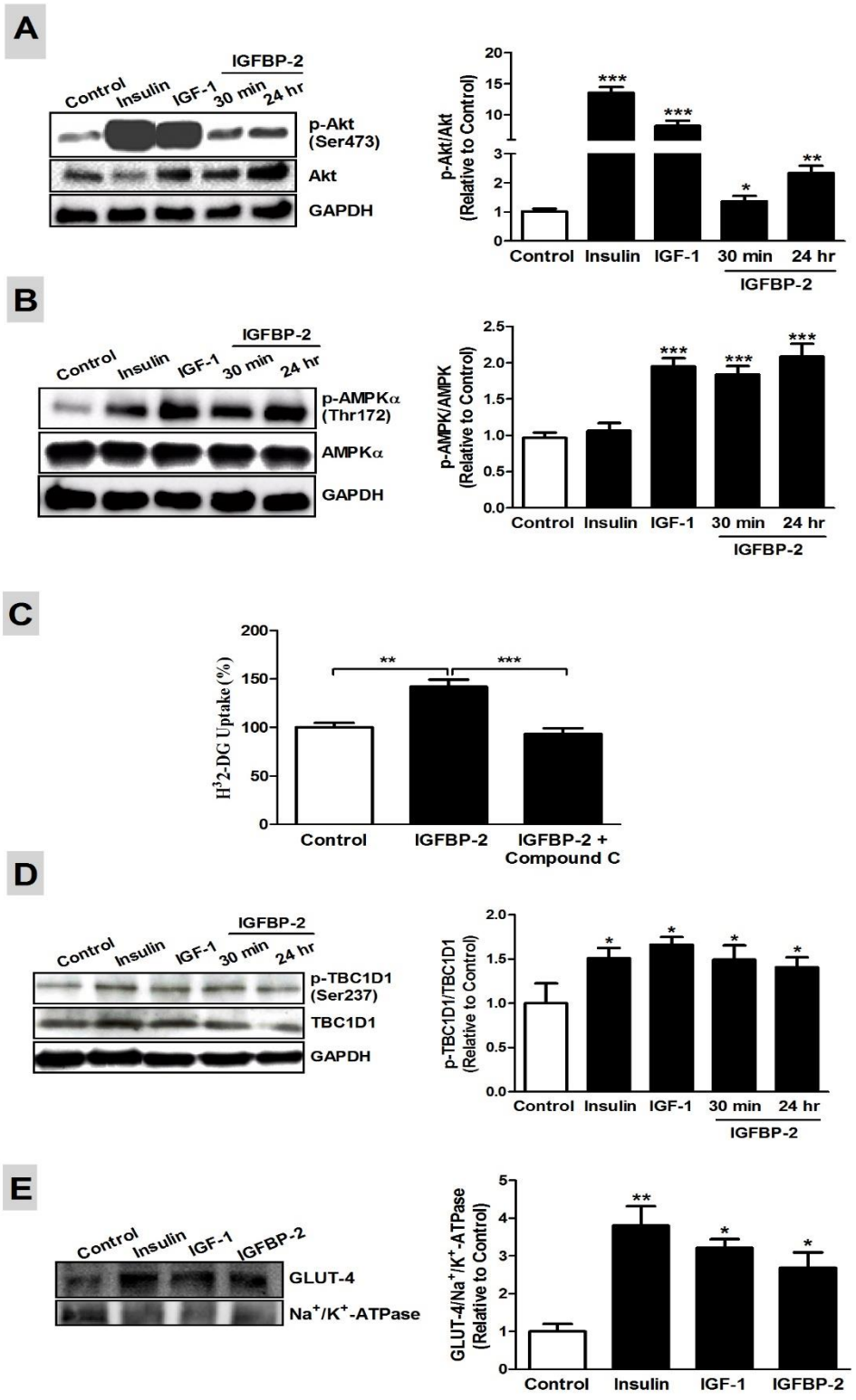
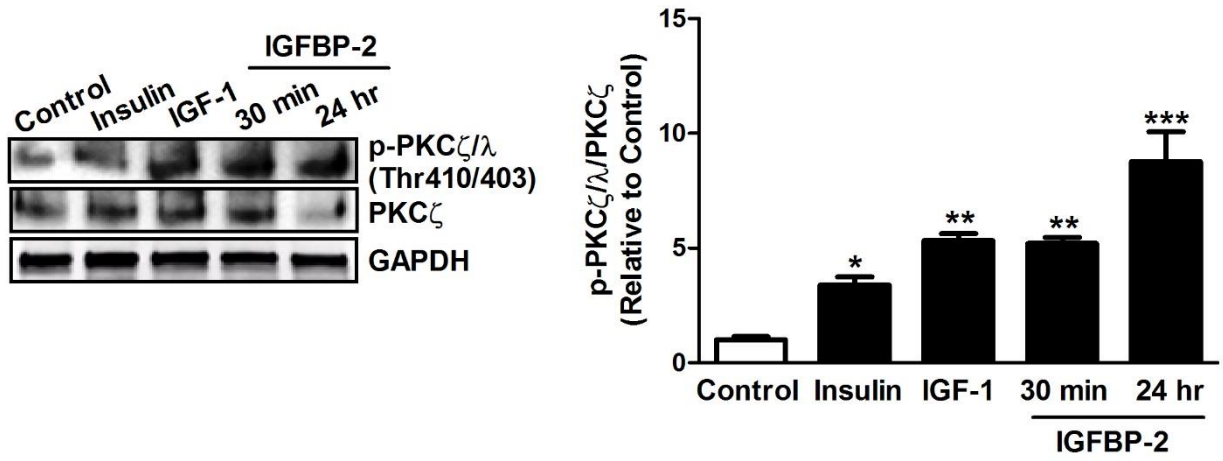

A
IGFBP-2
Control
Insulin
IGF-1
30 min
24 hr
p-Akt (Ser473)
Akt
GAPDH
p-Akt/Akt (Relative to Control)
Control
Insulin
IGF-1
30 min
24 hr
IGFBP-2
B
p-AMPKα (Thr172)
AMPKα
GAPDH
p-AMPK/AMPK (Relative to Control)
C
H3-2-DG Uptake (%)
Control
IGFBP-2
IGFBP-2 + Compound C
D
p-TBC1D1 (Ser237)
TBC1D1
GAPDH
p-TBC1D1/TBC1D1 (Relative to Control)
E
GLUT-4
Na+/K+-ATPase
GLUT-4/Na+/K+-ATPase (Relative to Control)

Figure 5


IGFBP-2
Control
Insulin
IGF-1
30 min
24 hr
p-PKCζ/λ
(Thr410/403)
PKCζ
GAPDH
p-PKCζ/λ/PKCζ
(Relative to Control)
15
10
5
0
*
**
**
***
Control
Insulin
IGF-1
30 min
24 hr
IGFBP-2

Curriculum Vitae

Complete list of publications:

1**.** Biruhalem **Assefa,** Ayman M. Mahmoud, Andreas F. H. Pfeiffer, Andreas L. Birkenfeld, Joachim Spranger, and Ayman M. Arafat, “Insulin-Like Growth Factor (IGF) Binding Protein-2, Independently of IGF-1, Induces GLUT-4 Translocation and Glucose Uptake in 3T3-L1 Adipocytes,” Oxidative Medicine and Cellular Longevity, vol. 2017, Article ID 3035184, 13 pages, 2017. doi:10.1155/2017/3035184

2. Sarem Z, Bumke-Vogt C, Mahmoud AM, **Assefa B**, Weickert MO, Adamidou A, Bähr V, Frystyk J, Möhlig M, Spranger J, Lieske S, Birkenfeld AL, Pfeiffer AFH, Arafat AM . Glucagon Decreases IGF-1 Bioactivity in Humans, Independently of Insulin, by Modulating Its Binding Proteins. *J Clin Endocrinol Metab. 2017 Sep 1;102(9):3480-3490.* doi: 10.1210/jc.2017-00558.

3**. Assefa B**, Mahmoud A, Pfeiffer AFH, Birkenfeld AL, Spranger J, and Arafat AM. Differential effects of insulin like growth factor-1 binding proteins 1, 2 and 3 on glucose uptake in myotubes and 3T3-L1 adipocytes. *Manuscript in preparation*

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