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Using real time RT-PCR analysis to determine gene expression patterns in RBL-2H3 cells in response to insulin, glucose and the anti-diabetic bis (maltolato)oxovanadium (IV)

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Abstract: *We used real time reverse transcription PCR (real time RT-PCR) to examine and compare relative expression levels of ten genes in rat basophilic leukemia (RBL-2H3) cells in response to treatment with either insulin, glucose, the anti-diabetic compound bis (maltolato) oxovanadium(IV) (BMOV) or a combination of these treatments. We found significant differences in the relative expression levels of seven key genes involved in insulin receptor and AMP-activated protein kinase (AMPK) signaling pathways that may prove important in the treatment of type 2 diabetes and metabolic syndrome. Six of these genes including InsR, GLUT4, Ampkβ1, Ampkβ2, Ampkγ1 and Ampkγ2 were significantly upregulated in response to treatment with insulin, glucose or the anti-diabetic compound BMOV. Interestingly, the Ampkα1 gene was significantly downregulated with treatment of glucose and even more so with overnight treatment of BMOV.*

Keywords: *Insulin receptor, AMP-activated protein kinase, BMOV, Diabetes, real time RT-PCR.*

Introduction:

Type 2 diabetes has also been referred to as non-insulin dependent diabetes mellitus (NIDDM) or adult onset diabetes mellitus. It is the most common form of diabetes; about 90-95% of people with diabetes have type 2. A major distinguishing characteristic of type 2 diabetes is a lack of sensitivity to insulin by the cells of the body, particularly fat and muscle cells (Kahn SE, 2003). The defective responsiveness to insulin may involve the insulin receptor in cell membranes. In addition to the genetic factors which are strongly associated with this

form of diabetes (Gerich J, 1998), there are other predisposing risk factors including central obesity (Scheen AJ, 2001), a previous history of gestational diabetes, physical inactivity, and certain ethnicities. The symptoms of type 2 diabetes develop gradually, and include fatigue, frequent urination, increased thirst and hunger, weight loss, blurred vision, and slow healing of wounds or sores.

Many researchers in the field of diabetes have tried to find orally active therapeutic compounds to use in place of insulin injections and other synthetic pharmaceuticals. Several metals, such as chromium (Wang et al. 2006, Chen et al. 2009), and vanadium (Thompson et al. 2009) have been found to produce insulin-like effects.

Metabolic diseases, like obesity and type 2 diabetes, are problems caused by a positive energy balance, and it was found that activators of an enzyme called AMP-activated protein kinase (AMPK) might be useful in treating such disorders (Winder and Hardie, 1999). It is well-understood now that pharmacological activation of AMPK improves blood glucose homeostasis, lipid profile, and blood pressure in insulin-resistant rodents (Viollet and Andrelli, 2011).

AMPK, a serine/threonine protein kinase, is an enzyme that monitors cellular energy status and responds to a variety of stresses causing ATP depletion. It is a heterotrimeric enzyme composed of a catalytic α subunit ($\alpha_1$and α_2) and regulatory β (β_1 and β_2) and γ subunits (γ_1, γ_2, and γ_3) (Stapleton et al. 1996, Woods et al. 1996). The genes encoding the three subunits of the kinase are found in essentially all eukaryotic genomes (Hardie, 2011). The catalytic subunits are encoded by two alternate genes in mammals (protein kinase, AMP activated, $\alpha_1$and α_2 catalytic subunits) (PRKAA1 and PRKAA2 in humans). The α_1 isoform is widely expressed, whereas α_2 is available more in tissues such as skeletal and cardiac muscle. The β_1 and β_2 subunits are encoded by two alternate genes in mammals (PRKAB1 and PRKAB2 in humans). Both are widely expressed, with β_1 expression is more obvious in some tissues, such as rodent liver (Thornton et al. 1998). The γ_1, γ_2, and γ_3 subunit isoforms are encoded by three alternate genes in humans (PRKAG1-3 in humans). The γ_1 isoform appears to be expressed ubiquitously, whereas γ_2, and γ_3 are most abundant in muscle.

When cells are exposed to metabolic stress because of glucose or oxygen deficiency, inhibition of ATP synthesis, or acceleration of

ATP consumption, AMPK will be activated by the increase in cellular ADP:ATP and/or AMP:ATP ratios, and by phosphorylation on threonine (Thr 172) of the catalytic subunit by upstream kinases. The finding that a serine-threonine protein kinase called LKB1 acts as an upstream kinase was important because LKB1 had previously been identified as the product of a tumor suppressor gene (Alessi et al. 2006), and AMPK was its first downstream target to be identified. On the contrary, the activity of calmodulin-dependent kinase kinases (CaMKK) on AMPK is very low in intact cells, it phosphorylates Thr 172 in response to treatments that increase intracellular Ca^{++} (Hawley et al. 2005).

Once activated, AMPK phosphorylates serine residues surrounded by a well-defined recognition motif in a variety of downstream targets (Gwinn et al. 2008). AMPK turns on catabolic pathways like activation of glucose transporter 4 (GLUT4)-mediated glucose uptake in muscles by phosphorylating TBC1D1, a Rab-GAP protein (one of the small GTPases) (Frøsig et al. 2010), activation of fatty acid uptake via translocation of the transporter CD36 to the plasma membrane (Habets et al, 2009), inhibition of liver glycogen synthesis by phosphorylation and inactivation of glycogen synthase-2 (Bultot et al, 2012), and inhibition of protein synthesis by directly phosphorylating Raptor, a subunit of the mammalian target of rapamycin (mTORC1) complex (Gwinn et al. 2008).

Vanadium-containing compounds are known to exert insulin enhancing effects (Willsky et al. 2011, Barrio et al. 2010). One coordination complex, bis(ethylmaltolato) oxovanadium(IV) (BEOV), and several salts have been used in clinical trials for the treatment of diabetes (Thompson et al. 2009). BEOV is closely related to bis(maltolato)oxovanadium(IV) (BMOV), which has been the subject of numerous studies (Vardatsikos et al. 2009). Vanadium coordination complexes such as BEOV and BMOV, along with vanadium-containing salts, improve both insulin sensitivity and glucose homeostasis in animal models of type I (Karmaker et al. 2007) and type II (Adachi et al. 2006) diabetes mellitus. The effects of various organic ligands in vanadium-containing compounds have been investigated indirectly in both animal and cellular model systems.

After delivery to a target cell, the insulin-enhancing effects of vanadium compounds are generally attributed to inhibition of protein tyrosine phosphatases with inhibition constants ranging from pM to

μM, including protein tyrosine phosphatase 1B (McLauchlan et al. 2010).

In this study we aimed to examine the expression levels of a number of key genes involved in IR and AMPK signaling pathways in response to treatments with insulin, glucose and/or BMOV. We report significant differences in relative expression levels compared to untreated RBL-2H3 cells in seven of the ten genes that were examined.

Materials and methods:

2.1 Cell culture and animal tissues

2H3 rat basophilic leukemia (RBL-2H3) were used because they already express insulin receptors on their surfaces and because we have previously reported that these cells positively respond to the effects of various vanadium containing compounds. RBL-2H3 cells were purchased from ATCC (Manassas, VA) and maintained in cell medium that contained Minimum Essential Medium (MEM) with Earle's Balanced Salts (Thermo Scientific, Logan, UT), 10% fetal bovine serum (FBS) (Gemini BioProducts, Woodland, CA), 200 mM L-glutamine, 10,000 U/mL penicillin G, 10 μg/mL streptomycin, and 25 μg/mL Fungizone (Gemini BioProducts). In some experiments, cells were incubated in supplemented MEM without FBS overnight to remove a source of insulin and other growth factors. Four biological replicates of RBL-2H3 cells were collected on separate days after appropriate treatment protocols and then kept frozen at -80°C. The number of cells collected per sample ranged from 4×10^6 to 32×10^6 cells. Rat liver and skeletal muscle tissues (Animal population health institute, CSU) were processed and RNA isolation was made.

2.2 Treatment of cells with insulin, glucose and/or BMOV

RBL-2H3 cells were treated in 60 cm^2 dishes (VWR International, Radnor, PA) at 37°C with media alone for one hour (untreated), or media containing 200 nM insulin for one hour, 10 μM vanadium (BMOV) for one hour, 10 μM BMOV overnight, 16 mM glucose for 48 hours or a combination of the above mentioned treatments. For protocols combining glucose and an additional treatment condition, cells were first treated with 16 mM glucose for 48 hours prior to insulin or BMOV treatment. Insulin from bovine pancreas was

purchased from Sigma-Aldrich (St. Louis, MO). BMOV was obtained from Dr. Debbie Crans research lab (Chemistry department, CSU**).**

2.3 Total RNA isolation

Total RNA was isolated using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions and followed by DNAse digestion with RNAse-free DNAse treatment (Qiagen) to remove any residual genomic DNA. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE) and samples were then stored at -80°C.

2.4 Reverse transcription of mRNAs

Messenger RNA was reverse transcribed into cDNA using the qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD) in a Veriti 96-well Thermo Cycler (Applied Biosystems). Briefly, 5 µl of RNA was combined with nuclease free water and qScript SuperMix. RNA was reverse transcribed by incubating the samples at 22°C for 5 minutes, 42 °C for 30 minutes, and 82°C for 5 minutes. cDNA was used immediately for real time PCR analysis.

2.5 Primer design and validation

Primers for ten rat genes, *Insr, Igf1r, GLUT4, Ampkα1, Ampkα2, Ampkβ1, Ampkβ2, Ampkγ1, Ampkγ2 and Ampkγ3,* were designed with the MGI (Mouse Genome Informatics) online database and NCBI BLAST. Oligonucleotide primers were purchased from (eurofins, Huntsville, AL). The primer sequences for the eleven genes examined are shown in Table (1) below. To ensure that only the target gene sequence was amplified, the primers were tested for efficiency by making a standard curve against pooled samples of rat liver and skeletal muscle tissues that express the genes of interest, therefore serving as a positive control. In addition PCR products were run on a 2% agarose gel and visualized using ethidium bromide. A Qiaquick Gel Extraction Kit was used to extract and purify DNA of 70 bp to 10 kb from a standard 2% agarose gel. This step was done to test for the accurate sequencing of the specified primers.

Table 1: Primer sequences for the examined genes

Gene symbol	Name, synonym	Genbank accession number	Primer sequence (Forward) (5'- 3')	Primer sequence (Reverse) (5'- 3')
18srRNA	18s ribosomal RNA	X56974	GAGGCCCTGTAATTGGAATGAG	GCAGCAACTTTAATATACGCTATTGG
Insr	Insulin receptor	NM_017071	TGTGGCAAGAGCCAAAGGAA	GCTCCAGGGCAAAATGCTTC
Igfr1	Insulin-like growth factor 1 receptor	NM_052807	TTCCCATGCCTTGGTCTCCT	AGTCCCACAGCTGCTGCAAG
GLUT4	Facilitated glucose transporter 4, Slc2a4	NM_012751	AGTCATCAACGCCCCACAGA	CGGAGAGAGCCCAAAGGGTA
Ampkα1	Protein kinase, amp-activated, alpha1catalytic subunit, Prkaa1	NM_019142	TGTGACAAGCACATTTTCCAA	CCGATCTCTGTGGAGTAGCAG
Ampkα2	Protein kinase, amp-activated, alpha2catalytic subunit, Prkaa2	NM_023991	CACAGGGACCTGAAGCCAGA	GGCGATCCACAGCTAAGTTCG
Ampkβ1	Protein kinase, amp-activated, beta1catalytic subunit, Prkab1	NM_031976	GGCCCAAGATCCTGATGGAC	GGGGGCTTTCTCATTCACCTC
Ampkβ2	Protein kinase, amp-activated, beta2catalytic subunit, Prkab2	NM_022627	GATGGGTTGCGCTAAGGA	CCCTCACCTCCTCCAAGTTAT
Ampkγ1	Protein kinase, amp-activated, gamma1catalytic	NM_013010	GGCAGCACGAACACCGTTA	GCTCCAAGCTGGTGGTATTTG

	subunit, Prkag1			
Ampk γ2	**Protein kinase, amp-activated, gamma2catalytic subunit, Prkag2**	**NM_184051 XM_231276**	**AGCTCATCCAGGTTCTGCTTC**	**ATACTTACCCACAAAAGAATCCTCAAG**
Ampk γ3	**Protein kinase, amp-activated, gamma3catalytic subunit, Prkag3**	**NM_001106921 XM_001057174 XM_237293**	**GGAAGCAGAGCCACCAGGTT**	**CAGCCAAGGGAACAGCTTGA**

2.6 Real-time PCR quantification of mRNA

The relative expression level of ten genes involved in IR and AMPK signaling (*Insr, Igf1r, GLUT4, Ampkα1, Ampkα2, Ampkβ1, Ampkβ2, Ampkγ1, Ampkγ2* and *Ampkγ3*) and one gene used as an internal control (18s rRNA) was examined using real-time qPCR. The reason behind using18s rRNA as an internal control is that it shows less variance in expression across a variety of treatment conditions than β-actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH). Amplification efficiencies were determined using a 10 fold serial dilution series. Analysis was performed in 10 µl reactions containing SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.5 µM gene specific forward and reverse primer, and cDNA using the LightCycler 480 Real-Time PCR System (Roche). Eva green dye is a fluorescent nucleic acid dye that is not toxic, with safe handling and easy disposal. It is far brighter than SYBR green 1 for detecting amplification due to novel DNA binding mechanisms. It is stable during storage and under PCR conditions, in addition, electrophoretically separated PCR products can be visualized directly via a UV box without the need for another gel stain. To ensure highly specific amplification, Eva green is used because it has the ability to bind and detect any dsDNA generated during amplification. The reaction conditions were as follows: 94°C for 5 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 15 seconds, and then 72°C for 3 minutes. A melt curve analysis was performed to confirm amplification of single PCR products.

2.7 Analysis

The relative expression level of transcripts was determined using the comparative Ct method previously described by Schmittgen and Livak by calculating the geometric mean of 18s rRNA expression values, using this as a normalization factor. Statistical differences were assessed at $p < 0.05$ using a Student's t-test. The level of each mRNA expression was calculated by the formula: $2^{(-\Delta Ct)}$ where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{18s}$. Fold change between treated and untreated samples was calculated as $2^{-\Delta\Delta Ct} = [(Ct_{\text{gene of interest}} - Ct_{18s})\text{ treated} - (Ct_{\text{gene of interest}} - Ct_{18s})\text{ untreated}]$ as previously described (Schmittgen and Livak, 2008).

Results:

1- Gene expression of *Insr* in response to treatments with insulin, glucose and/or BMOV:

We are interested in studying the effects of insulin, glucose and/or BMOV on the expression of *Insr* gene. Because of that, 2H3 cells were exposed to 200 nM insulin for one hour, 10 μM vanadium (BMOV) for one hour, 10 μM BMOV overnight, 16 mM glucose for 48 hours or a combination of the above mentioned treatments. We found that insulin was the only treatment that has a profound effect on the expression of *Insr gene*, as illustrated in Figure (1), and Table (2) below.

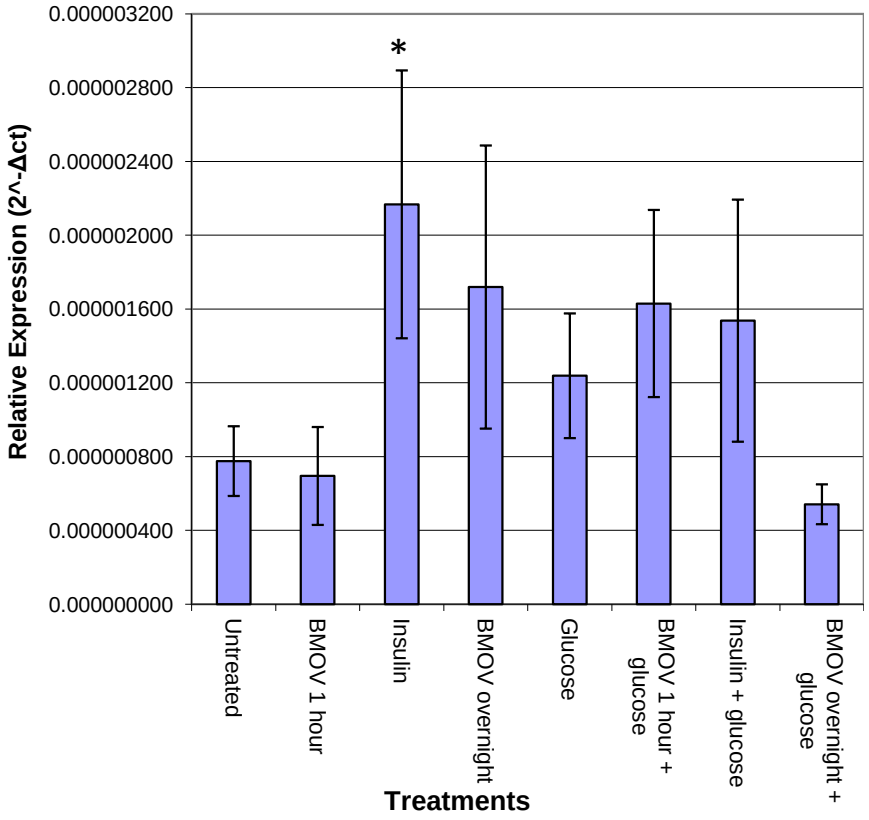

Figure 1: Relative expression of Insulin receptor in response to insulin, glucose, and/or BMOV

Table 2: Significant differently expressed genes compared to untreated 2H3-RBL cells from real time RT-PCR experiments

Gene	Treatment(s)	Fold increase	Fold decrease	P-value
Insr	**Insulin**	**2.39**		**0.05**
GLUT4	**BMOV 1hour +glucose** **BMOVovernight+glucose**	**3.17** **2.94**		**0.03** **0.015**
AMPKα1	**Glucose** **BMOVovernight**		**5.88** **3.79**	**0.01** **0.04**

AMPKβ1	**Insulin** **Glucose** **Insulin+glucose** **BMOVovernight** **BMOV1hour+glucose** **BMOVovernight+glucose**	**2.82** **1.60** **2.07** **1.91** **1.90** **1.45**		**0.008** **0.01** **0.01** **0.01** **0.02** **0.02**
AMPKβ2	**Insulin** **Glucose** **BMOVovernight** **BMOVovernight+glucose**	**4.01** **1.79** **2.32** **1.48**		**0.001** **0.007** **0.0005** **0.003**
AMPKγ1	**Insulin** **Glucose** **Insulin+glucose** **BMOVovernight** **BMOV1hour+glucose** **BMOVovernight+glucose**	**2.79** **1.41** **1.64** **1.66** **1.58** **1.27**		**0.002** **0.02** **0.02** **0.01** **0.05** **0.02**
AMPKγ2	**Insulin** **Glucose** **Insulin+glucose** **BMOVovernight** **BMOV1hour+glucose**	**3.08** **1.72** **3.18** **1.91** **1.89**		**0.01** **0.01** **0.004** **0.04** **0.03**

2- Gene expression of GLUT4:

Our findings indicated that the greatest effect on GLUT4 gene expression was observed when 2H3 cells were exposed to glucose and BMOV regardless on the time of exposure, as implicated in Figure (2) and Table (2).

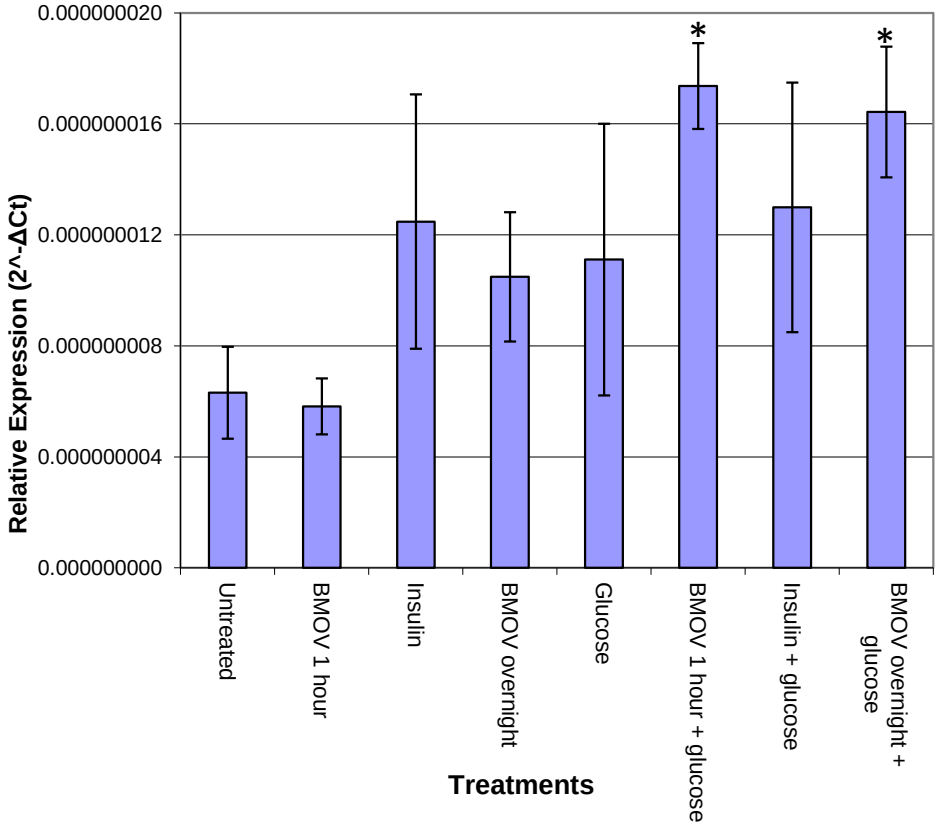

Figure 2: Relative expression of GLUT4 in RBL-2H3 cells in response to various treatments compa**red to untreated cells**

3- Gene expression of AMPK subunits:

To explore mRNA expression of AMPK catalytic and regulatory subunit isoforms, the relative expression of each subunit isoform was determined by qRT-PCR. No expression of α2 and γ3 mRNAs was detected in RBL- 2H3 cells. The main expressed subunit isoforms are

α1, β1, β2, γ1, and γ2. Figure (3) and Table (2) showed that the relative expression of α1 isoform was decreased by 5.88 and 3.79 fold when the cells were exposed to glucose and BMOV overnight, respectively. Insulin, glucose, and/or BMOV have great positive effect on the expression of AMPK β1 as indicated in Figure (4). The relative expression of AMPK β2 was increased in response to insulin, glucose, BMOV overnight alone or in association with glucose as shown in Figure (5) and Table (2). The effect of the various treatments on the relative expression of AMPK γ1 subunit was indicated in Figure (6), with the fold increase illustrated in Table (2). The effects on AMPK γ2 was shown in both Figure (7) and Table (2).

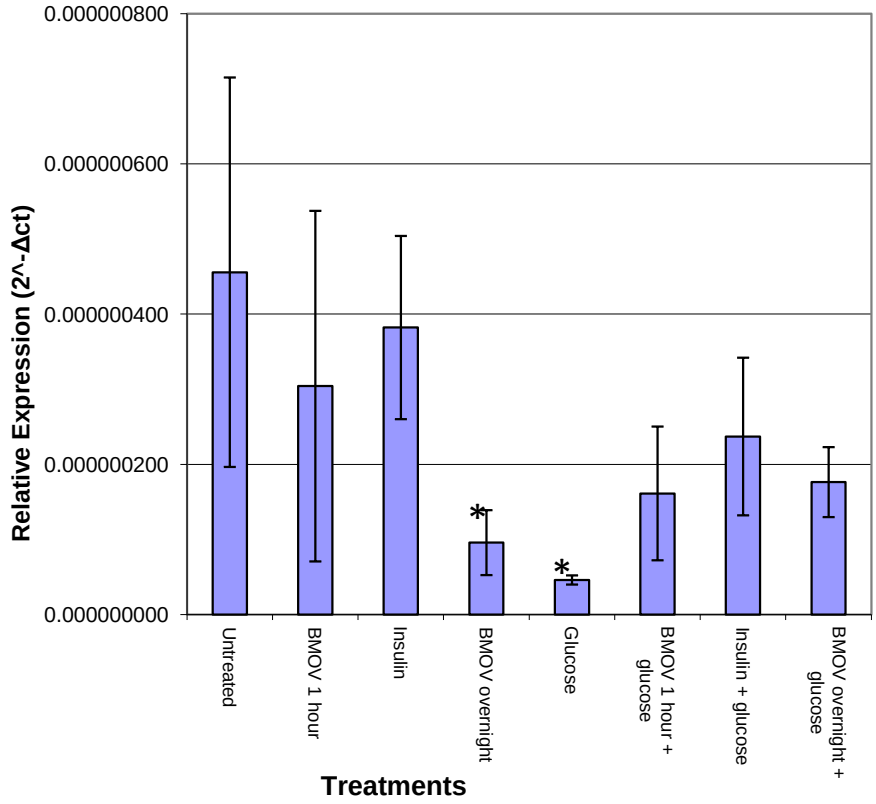

Figure 3: Effects of different treatments on the relative expression of AMPKα1

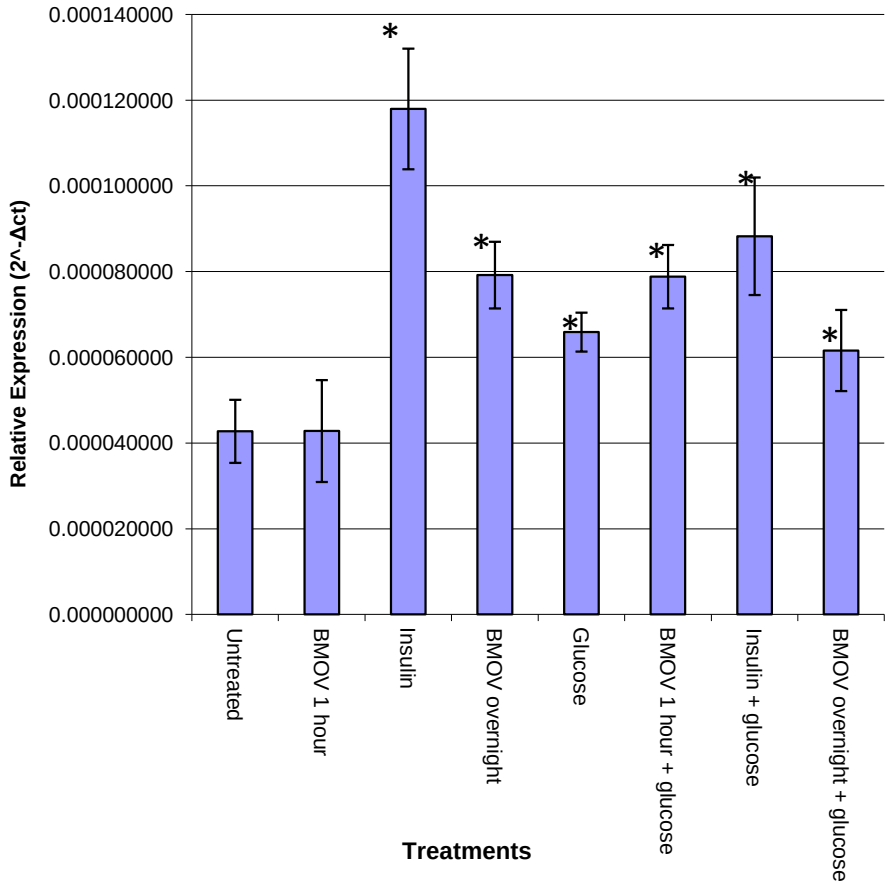

Figure 4: Relative expression of AMPKβ1 in response to different treatments

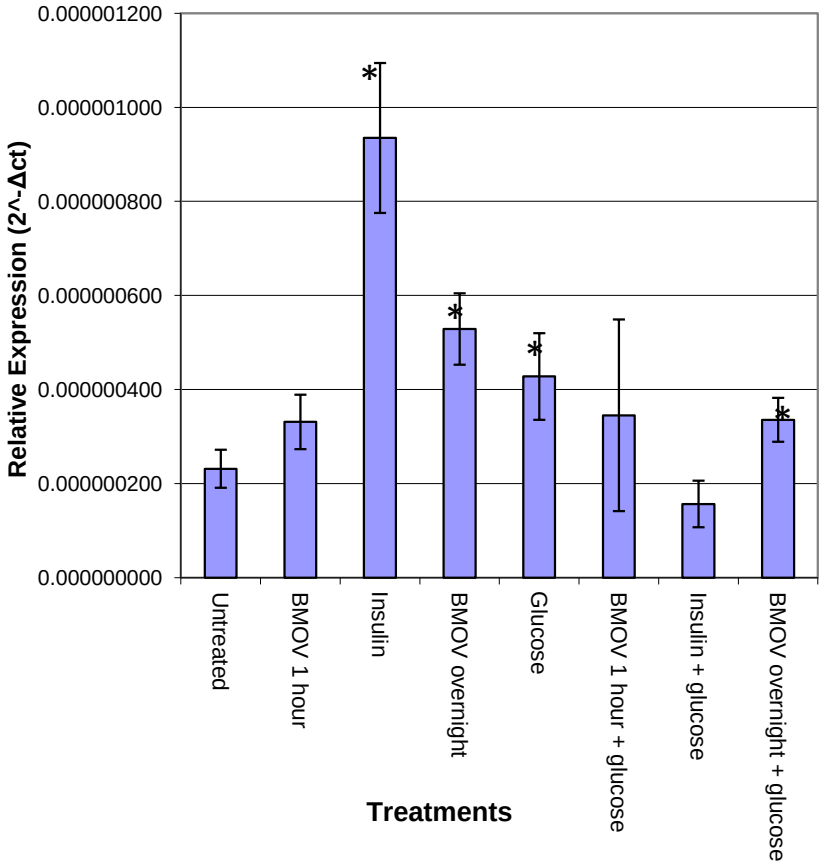

Figure 5: Response of AMPKβ2 expression to different treatments

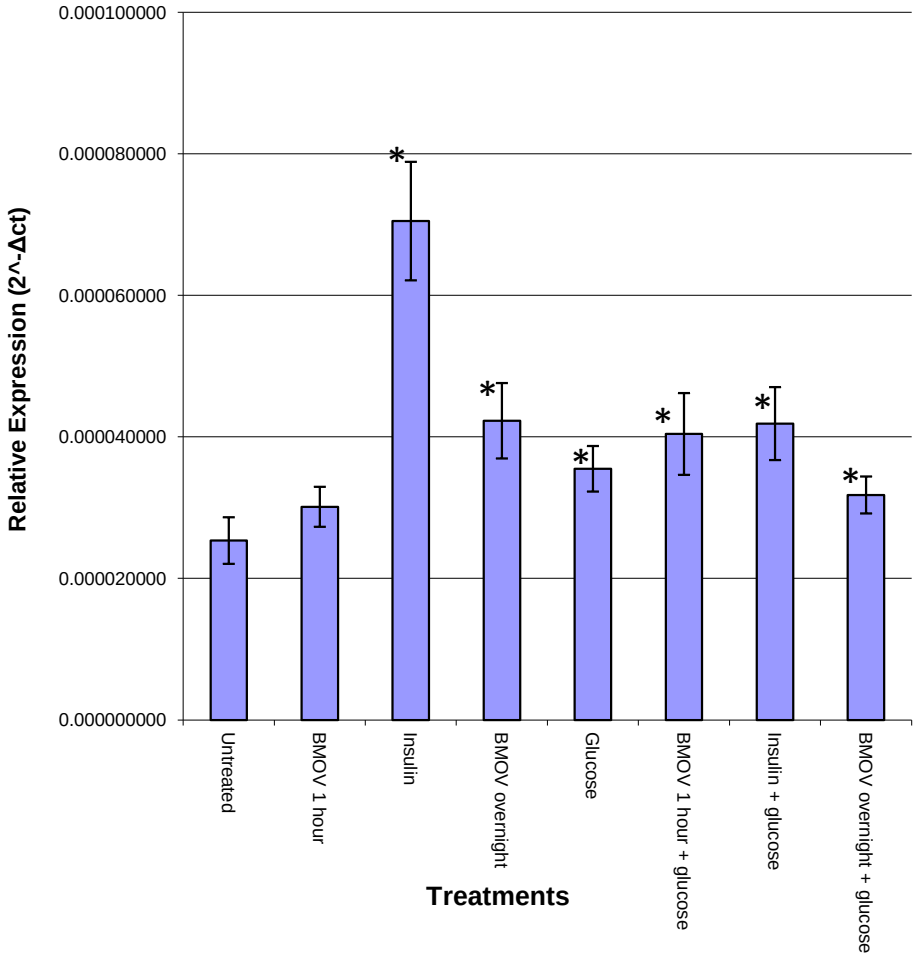

Figure 6: Relative expression of AMPKγ1 in RBL-2H3 cells

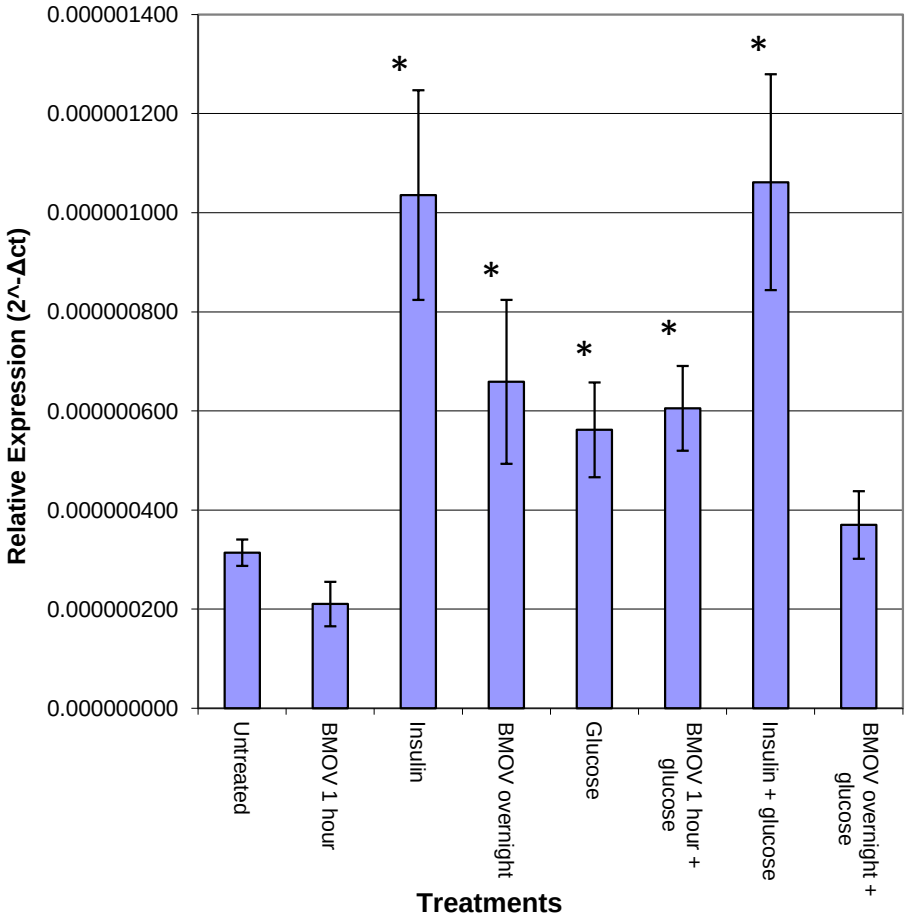

Figure 7: Relative expression of AMPKγ2 in RBL-2H3 cells. *: clinically significant

Discussion:

Traditional management of the symptoms of type 1 and advanced type 2 diabetes is by insulin injections. Many problems involving physical and mental pain, and some side effects can be caused by insulin injections. Many researches are carried out in order to find orally active therapeutic compounds that could replace insulin injections in the near future.

The insulin receptor (IR) is a member of the large family of receptor tyrosine kinases and has a covalent dimeric α2β2 organization. Activation of IR stimulates signal transduction pathways that lead to multiple biological processes such as glucose and lipid uptake and metabolism, gene expression, protein synthesis and cell growth, multiplication and survival. Previously, our data suggested the involvement of specialized, nanoscale membrane microdomains in insulin-mediated insulin receptor (IR) signaling and suggested that the spatial association of key signaling proteins such as IRS-1 within these microdomains provides an efficient mechanism for promoting IR signal transduction (Winter et al. 2012). Insulin reduce insulin receptor lateral diffusion and result in increased association of IR with specialized, detergent-resistant membrane structures (Winter et al. 2012). In this study, we were the first to show that IR mRNA expression in RBL- 2H3 cells was greatly affected by 200 nM insulin, in which insulin produced an increase in the expression by 2.39 fold compared to untreated cells. Several lines of evidence suggest that insulin induce IR activation by stimulating the autophosphorylation of these receptors. (Ropelle et al. 2009) showed that intracerebroventricular insulin induced increase in IR phosphorylation in the hypothalamus from both control and diet-induced obese (DIO) rats. In addition, (Jensen et al. 2007) indicated that insulin was able to tyrosine-phosphorylate the insulin receptor when L6 cells expressing human insulin receptor were incubated with increasing amounts of insulin for various times. To analyze gene expression profiles following IR activation by insulin using microarray technology, L6-hIR rat myoblasts were stimulated with 100 nM insulin for 3 hours. The researchers found that almost half of the genes differentially regulated by insulin were involved in cell proliferation and growth (Jensen et al. 2008). Insulin either selectively regulated the expression of these genes or was a more potent regulator. They also found that half of the differentially regulated genes interact with the genes involved with the MAPK (mitogen-activated protein kinase) pathway.

The facilitated glucose transporters (GLUTs) are located on the surface of all cells, these transporters are designated GLUT1 to GLUT12, based on the order in which they were identified. GLUT4 catalyzes the rate-limiting step for glucose uptake and metabolism in skeletal muscles and fat. Because of the fact that the mechanism for

the insulin-enhancing effects of BMOV was not understood in details, it was postulated that vanadium containing compounds might improve glucose uptake by an increase in the intrinsic activity or enhanced targeting of GLUT4 to the cell surface. We were the first to indicate that GLUT4 mRNA expression was positively increased when RBL-2H3 cells were exposed to 10 µM BMOV for 1 hour or overnight. Other groups of researchers showed that in isolated rat adipocytes (Dubyak et al. 1980) and in cultured L-6 myotubes (Berger et al. 1994), sodium orthovanadate (NaOV) can stimulate GLUT-4 translocation to the cell surface. Vanadium exposure of STZ diabetic rats can restore the expression and/or cell surface translocation of GLUT-4 in skeletal muscle (Strout et al. 1990) and heart (Li SH and McNeill JH, 2001). Also, treatment of a nutritionally induced, insulin-resistant model (*p.obesus*) with vanadyl sulfate (VS) restored membrane-associated as well as total protein and mRNA content of GLUT-4 in the gastrocnemius muscle (Shafir et al. 2001).

AMP-activated protein kinase (AMPK) is a stress-responsive enzyme involved in cellular response to an abnormal energy status. Given that AMPK is an important target for drugs used for treating type 2 diabetes, it is not surprising that many pharmaceutical companies have developed several pharmacologically active compounds for this purpose. Examples include metformin (El-Mir et al. 2000), thiazolidinediones which induces the release of adiponectin that activates AMPK and stimulate fat oxidation in liver and muscle (Yamauchi et al. 2002), A-769662 which is a thienopyridone compound developed by Abbott laboratories (Cool et al. 2006), salicylate (Hawley et al. 2012) and Phenobarbital (Hawley et al. 2010).

All of these examples has led us to the speculation that BMOV could exert its insulin-mimicking effects through regulation of AMPK, a step that may lead to a decrease in both glucose and triglyceride levels in plasma. In an attempt to test this hypothesis, RBL-2H3 cells were treated with various compounds including 200 nM insulin, 16 mM glucose and /or 10 µM BMOV for different lengths of time. We found that the level of AMPK α1 subunit expression was decreased by 5.88 fold when the cells were treated with 16 mM glucose for 48 hours. In regard to glucose treatment, (Salt et al. 1998) had previously shown that glucose regulated AMPK in HIT cells, in which, there was a clear

rise in the AMP/ATP ratio when medium glucose was decreased from 1 to 0.1 mM. The researchers proposed that incubation of HIT cells in low concentrations of glucose, which are not saturating for the glucose transporter, results in a reduction of glucose metabolism. Since these cells do not store enough quantities of glycogen, this leads to a decrease in ATP and a consequent increase in the ADP/ATP ratio. This causes phosphorylation of AMPK by AMPKK.

No expression of α2 mRNA was detected in 2H3 cells. This is in agreement to what was observed earlier in which α2 subunit is absent in cells of the blood and endothelial cell lineages (Hardie et al. 2012).

Vanadium salts have been seriously considered as a possible treatment for diabetes. Since inorganic vanadium compounds are poorly absorbed from the gastrointestinal tract and show multiple signs of toxicity, many attempts have been made to elevate the insulino-mimetic or insulin-enhancing activity of vanadium without increasing its toxicity. Several organically-chelated vanadium compounds such as bis (maltolato)oxovanadium (BMOV) have been synthesized. BMOV accumulates primarily in bone, liver, and kidney tissue (144). The more beneficial pharmacological effect of BMOV compared to that of inorganic vanadium has been attributed to increased absorption from the gastrointestinal tract due to its low molecular weight, acceptable lipophilic/hydrophilic balance, and the neutral charge of the vanadyl complex (Cohen et al. 1995). The major effects of BMOV on blood glucose and lipid levels were well established. It was administered to STZ-diabetic rats in the drinking water at a maximal concentration of 0.75 mg/ml for 6 months, this administration normalized plasma glucose levels in 8 out of 12 animals and restored elevated plasma lipid parameters and heart function in all diabetic treated rats (Yuen et al. 1993). In order to examine the effectiveness of organic vanadium in type II diabetes, BMOV has been used in *fa/fa* (fatty) Zucker rats. BMOV at a maximal concentration of 0.5 mg/ml for 14 weeks of treatment reduced plasma insulin levels from 180 to normal (50 μU/ml) by week 4. At the lower concentration, both plasma glucose and triglyceride levels were reduced in response to BMOV. An oral glucose tolerance test showed an improved glucose tolerance in fatty treated animals regardless of the concentration of BMOV (Wang et al. 2001).

Our study provides the first direct evidence that BMOV regulates AMPK. We found that BMOV could have positive or negative impact

on the different subunit isoforms of AMPK. The relative expression of α1 subunit was down regulated in the presence of BMOV, whereas, β1, β2, γ1, and γ2 expressions were increased when RBL-2H3 cells were incubated with BMOV either for 1 hour or 24 hours. BMOV produced its effect on AMPK subunits alone or in association with glucose. The dual effects of BMOV on AMPK subunits expression in RBL-2H3 cells was not unusual. It was previously shown that α1, β1, and γ1 subunits of AMPK began to increase with hypoxia at 1 hour, their expression at 6 hours reaching a level approximately 2 to 4 fold higher than that at 0 hour. No induction of α2, β2, γ2, and γ3 were observed under hypoxic conditions (Fukuyama et al. 2007).

In conclusion, based on the fact that AMPK has known effects on carbohydrate and lipid metabolism, it is not surprising that AMPK system is considered to be a major tool in the development and/or treatment of obesity, diabetes, and the metabolic syndrome. The different agents that do activate AMPK when applied to intact cells show that development of new activators is not impossible. Many of the existing agents activate AMPK by inhibiting ATP production, either by inhibiting oxidative phosphorylation or glycolysis, that leads to an increase in ADP:ATP and AMP:ATP ratios. In this work, we were the first group to explore new regulators of AMPK, that are insulin and BMOV. These two compounds can activate some subunit isoforms of AMPK. One explanation to this observation is that both compounds can decrease blood glucose level which will cause a depletion in the cellular level of ATP and an increase in AMP. An increase in AMP/ATP ratio is one of the causative agents of AMPK activation. Anyway, several researches with different methods and techniques are needed to prove the validity of such hypothesis.

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