IJPSR (2016), Vol. 7, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 June, 2016; received in revised form, 14 October, 2016; accepted, 19 October, 2016; published 01 December, 2016

THE IMPACT OF ADENOSINE A2B RECEPTORS ON GLYCOLYSIS AND INSULIN RESISTANCE IN SKELETAL MUSCLE

Mansour Haddad *

Department of Clinical Sciences, Faculty of Pharmacy, Philadelphia University, Amman, Jordan.

Keywords:

Adenosine A2B receptors, skeletal muscle, PDK4, HKII, PFK and metabolism

Correspondence to Author:
Mansour Haddad

Assistant Professor,
Department of Clinical Sciences,
Faculty of Pharmacy, Philadelphia University, Amman, Jordan.

E-mail: Dr.man.haddad@gmail.com

Abbreviations:
Interleukin-6: IL-6; Pyruvate dehydrogenase kinase 4: PDK4; Hexokinase II: HKII; Phosphofructokinase: PFK; cAMP: Cyclic-adenosine monophosphate; cDNA: Complementary DNA; mRNA: messenger ribonucleic acid; PCR: Polymerase chain reaction; NECA: 5'-N-ethylcarboxamido adenosine; PSB 603: 8-[4-[4-(4-chloro phenzyl) piperazide - 1 - sulfonyl) phenyl]]-1-propylxanthine; CGS21680: 2-(4-[2-carboxyethyl]-phenethylamino) adeno sine-52 -Nethyluronamide; DMSO: Di methyl sulphoxide; P-value: Probability

ABSTRACT: Adenosine has been proposed as a key factor regulating the metabolic balance between energy supply and demand in skeletal muscle. Adenosine A2B receptors are expressed across multiple tissues including skeletal muscle. Previously, it has been identified the robust (in vitro) induction of the nuclear receptors (NR4A) subgroup following adenosine A2B receptors stimulation in rat skeletal muscle cells. This may be concomitant with changes in the expression of genes involved in the regulation of nutrient metabolism. Considering that skeletal muscle is a primary tissue responsible for glucose disposal, glucose oxidation and insulin resistance, this study investigated a potential functional role for adenosine A2B receptors in rat L6 skeletal muscle myotubes in terms of glycolysis (HKII and PFK), and insulin resistance/glucose oxidation (PDK4) using a transcriptional approach (QRT-PCR (Probe-based)). For the first time in rat skeletal muscle cells, the results of the present study have shown that stable adenosine analogue NECA alters the expression of a number of genes (HKII, and PFK). Moreover, this is the first study demonstrating that selective adenosine A2B receptors antagonist/inverse agonist PSB 603 alters the expression of a number of genes (HKII, PFK, and PDK4). To our knowledge, this is the first study also demonstrating an alteration of PFK gene by selective adenosine A2A receptors agonist CGS 21680. Adenosine A2B receptors mediate both NECA (adenosine-5'N-ethylcarboxamide)-induced HKII, and NECA-inhibited PFK mRNA gene expression in skeletal muscle cells. These results indicate that adenosine A2B receptors play a critical role in the regulation of metabolic function in skeletal muscle. Collectively, these data reveal adenosine A2B receptors as a novel target to consider in our understanding of metabolic function and risk for the development of metabolic-based diseases.

INTRODUCTION: Adenosine is a key extracellular signalling molecule that is released from all tissues and cells including fat, pancreas, liver, and muscle, particularly under inflammation, intense exercise, stress, or during cell damage [1]. Adenosine plays an important regulatory role in several aspects of tissue function by activating four G-protein-coupled receptors, A1, A2A, A2B and A3 adenosine receptors [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(12). 4917-26

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (12). 4917-26

Accumulating evidence highlights a critical role for the adenosine system in the regulation of glucose homeostasis, insulin resistance/sensitivity, the pathophysiology of type 2 diabetes mellitus [3]. Moreover, serum adenosine deaminase level was also shown to be elevated in nonobese type II Diabetes Mellitus [4, 5]. Earlier studies have clearly shown that adenosine system signaling, in particular adenosine A2B receptors, increases inflammation, in particular interleukin-6, in skeletal muscle cells (data not published yet). However, its molecular signaling role in glucose metabolism in skeletal muscle is less clear.

Adenosine is a strong modulator of gene expression, which underpins some of its potential

expected effects in skeletal muscle in terms of glucose metabolism. A number of studies have suggested that activation of the adenosine A2B receptors may play an important role in glucose homeostasis. Activation of A2B receptors on rat skeletal muscle cells has been shown to also induce the release of the inflammatory cytokine interleukin (IL-6) (data not published yet). Scientists at Eisai have provided evidence that the A2B receptor antagonists and/or mixed adenosine A2B/A1 receptor antagonists may be useful in the treatment of diabetes. Eisai found in a separate study that inhibition of glucose production was best correlated with the adenosine A2B receptor affinity of the compounds used in a diabetes model [6, 7].

Therefore, the above evidence supports the notion that adenosine plays a role in diabetes, and that its effects may be, at least in part, mediated through the adenosine A2B receptors. Although the adenosine A2B receptor antagonists described in the literature are currently under investigation in order to fully define the role of adenosine A2B receptors in disease state, early in vitro and in vivo experiments suggest that adenosine A2B receptor antagonists may be beneficial for the role cancer [8]-A2B mediates angiogenesis [9], asthma-A2B mediates inflammatory cytokine release [10], diabetic retinopathy-A2B mediates angiogenesis [9], diabetes-A2B mediates gluconeogenesis [6, 7] and inflammation in skeletal muscle (data not published yet).

A key regulatory pathway in skeletal muscle that may be responsible for the modulation of muscle metabolism in response to adenosine is the adenosine A2B receptor signaling pathway [11, 12]. As adenosine A2B receptor activation accumulated cAMP level and increased NR4A expression in skeletal muscle cells [11-13], it is possible, therefore, that adenosine A2B receptors affect NR4A (subtype of nuclear receptors) through the cAMP pathway in skeletal muscle, and consequently, adenosine A2B receptors might modulate glucose metabolism in skeletal muscle tissue.

This is supported by the fact that these transcription factors (NR4A) regulate the gene expression of proteins responsible for glucose metabolism through modulating key metabolic genes in skeletal muscle (Chao et al., 2007; Kanzleiter et al., 2010; Lessard et al., 2009). Taken together, modulation of adenosine A2B receptor by ligands might affect glucose utilization in skeletal muscle. Moreover, previous study suggested that the adenosine A2B receptors contribute to inflammation by increasing IL-6 expression in skeletal muscle cells (data not published yet).

Thus, the implication of this is that adenosine A2B receptors might be recommended as a therapeutic target in diabetes. Taken the inflammatory role of adenosine A2B receptors in skeletal muscle (data not published yet), metabolic functions of skeletal muscle tissue in terms of glucose metabolism [14], and the association between inflammation and diabetes [15], it is crucial to examine the specific effects of adenosine A2B receptors modulation on expression of glucose metabolic genes (**Table 1**).

TABLE 1: GENETIC REGULATORS OF SKELETAL MUSCLE METABOLISM AND THE CONSEQUENCES OF T2DM ON THEIR EXPRESSION AND FUNCTIONS

Gene	Effect of obesity and T2DM on expression	Metabolic consequences of obesity and T2DM on changing gene expression	Main metabolic functions in skeletal muscle	References
PDK4	Increase	Increase fatty acid oxidation	Inhibits conversion of pyruvate to acetyl CoA leading to preferential fatty acid oxidation	16, 17
HKII	Decrease	Increase insulin resistance	Glycolysis	18, 19
PFK	Normal-(diabetes)	------	Glycolysis	20, 21

MATERIALS AND METHODS:

Materials: NECA, forskolin, PSB 603 and CGS21680 were obtained from Tocris Bioscience, UK; dimethyl sulphoxide reagent was sourced from Santa Cruz, USA; and Trizol and charcoal stripped serum were brought from Applied Biosystem, USA. Maxima Probe qPCR Master Mix (2X) and Thermo Scientific Revert Aid First Strand cDNA Synthesis were obtained from Thermo Scientific Company, USA.

RNeasy Mini Total RNA Purification kits and RNase-Free DNase Set were brought from Qiagen, Germany. Fetal bovine serum (FBS) was supplied by Capricorn Scientific, USA. Horse serum was from Sigma company, Germany. Ham-F 10 was sourced from PAA Company, USA. Dulbecco's modified essential medium (DMEM) was from Caisson, USA.

Cell Culture: Rat L6 skeletal muscle and myoblast cell line were originally obtained from the American Type Culture Collection (USA). Cells were maintained as an attached monolayer culture in DMEM with high glucose (4500 mg/L) and L-glutamate supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 µg/ml penicillin-streptomycin. Cells were incubated at 37 °C in a 90% humidified atmosphere of 5% CO_2. The cells were passaged upon reaching a state of approximately 60%-70% confluency, and the medium was changed three times per week (**Fig. 1**).

Confluent cells in 25 cm^2 flasks were cultured for a further 14 days (to allow myotube formation), according to the protocol mentioned in [22] with slight modifications (**Fig. 2**). 70%-90% confluent myotubes (approximately 2 weeks in culture) were serum-starved (incubated in Ham-F 10 medium alone) for 19 hours or 7 days. Then, cells (Figure 2) were treated for 1 hour with vehicle (0.1% DMSO), NECA 100nM and 10µM, PSB 603 100nM, 1 µM and 10 µM, Forskolin 100 nM, NECA and PSB 603 (cells were pre-treated with PSB 603 for 10 minutes prior to the addition of NECA). Following treatment, cells were washed with ice cold PBS, then lysed with TRIzol(Invitrogen product name) (2 mL per flask).

RNA extraction and cDNA synthesis: Rat L6 skeletal muscle cells (in 25cm^2 flasks) were scraped in 2 ml of ice cold TRIzol (Applied Biosystems, USA) and RNA was then isolated according to the manufacturer's instructions. Total RNA clean-up and on-column DNAse digestion was performed using RNeasy purification columns (Qiagen, Germany). RNA concentration and purity was determined using a spectrophotometer (JENWAY Genova Nano). For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis (Thermo Scientific, USA) in a total volume of 20 µL for 5 minutes at 25 °C, followed by 1 hour at 42 °C, and the reaction was terminated at 70 °C for 5 minutes.

Taqman quantitative real-time PCR: The relative standard curve method based on Taqman quantitative real-time PCR (qRT-PCR) was used to quantify gene expression. Samples were prepared in a total reaction volume of 25 µL (13 µL Maxima Probe qPCR Master Mix 2X reagent, 1.5 µL forward primer (10 µM), 1.5 µL reverse primer (10 mM), 2.5 µL Probe (2 µM), 5 µL water, and 5 µLcDNA). The qRT-PCR analysis was performed using a 7500 real-time PCR system (Applied Biosystems, USA). Gene expression was determined relative to referenece gene, TATA. Primers and probes for all genes (**Table 1**) were designed using Primer Express software (Applied Biosystems, USA) and synthesised by Integrated DNA Technologies, Inc., USA. The standard curve method was used, with a slope between -3.2 and -3.6 and R^2 values of more than 99%, indicating that amplification efficiency was nearly 100%.

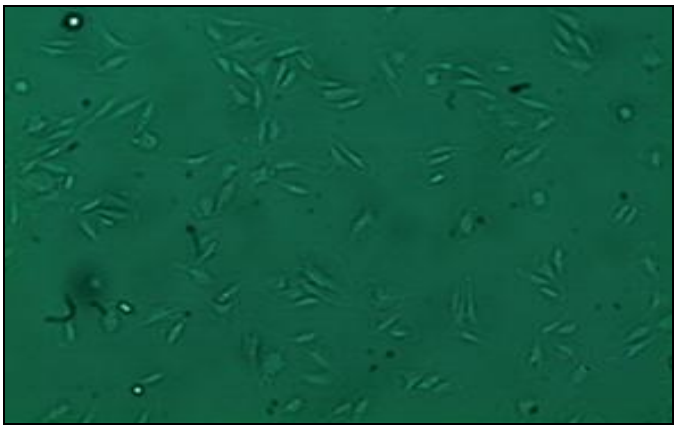

FIG. 1: REPRESENTATIVE MYOBLASTS DERIVED FROM PASSAGE NUMBER 7. MYOBLASTS TAKEN AFTER 1 DAY SEEDING INTO 25 CM^2 (10X).

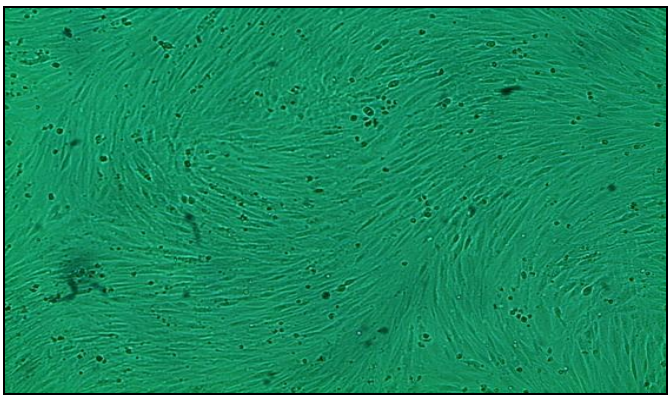

FIG. 2: REPRESENTATIVE MYOTUBES DERIVED FROM PASSAGE NUMBER 7 (AT 14 DAYS OF TISSUE CULTURE).

TABLE 1: LIST OF GENE PRIMER AND PROBE SEQUENCES

Gene	Sequences (5' →3')
PDK4	Probe 5'- CGTCGCCAGAATTAAAGCTCACACAAGTC - 3'
	Forward primer 5' - AGCAGTAGTCGAAGATGCCTTTG -3'
	Reverse primer 5' - ATGTGGTGAAGGTGTGAAGGAA -3'
HKII	Probe 5'-AGTTCCTGTCTCAGATAGAGAGCGACTGCCT-3'
	Forward primer 5' - GCATCTCAGAGCGCCTCAAG -3'
	Reverse primer 5' - GATGGCACGAACCTGTAGCA -3'
PFK	Probe 5'-CTGCCCTGCACCGCATTGTAGAGATC-3'
	Forward-Primer-5'- TGGCACTGATATGACCATTGGT-3'
	Reverse-Primer-5'- TGAGCGGTGGTGGTGATG-3'
TATA-BOX	Probe 5'- TCCCAAGCGGTTTGCTGCAGTCA -3'
	Forward Primer 5'- TTCGTGCCAGAAATGCTGAA-3'
	Reverse Primer 5'- GTTCGTGGCTCTCTTATTCTCATG -3'

Data analysis: Data are expressed as means ± standard error of mean (SEM) of triplicate or quadruplicate wells generated from at least three independent experimental group. All mRNA data were analysed using one-way ANOVA with a Tukey test. Analysis was performed using GraphPad Prism, version 5.03 (GraphPad Software Inc). The level of statistical significance was set at $p<0.05$.

RESULTS

NECA does not alter PDK4, stimulates HKII, and inhibits PFK mRNA gene expression in skeletal muscle cells: To assess whether stimulation of adenosine A2 receptors could modulate PDK4, HKII, and PFK mRNA gene expression in rat L6 skeletal muscle cells, the effects of NECA were relatively quantified using a non-selective adenosine receptor agonist on above genes mRNA expression by qRT-PCR (probe-based). 19 hours or one week starved skeletal muscle cells were incubated with NECA (100 nM and/or 10 μM) for one, three and/or 24 hours, and mRNA gene expression of PDK4, HKII, and PFK were subsequently quantified.

Incubation of one week starved L6 skeletal muscle cells with 10 μM of the non-selective adenosine analogue NECA does not change mRNA gene expression of PDK4 and PFK mRNA gene expression, and indicates that there is an upward trend in mRNA gene expression of HKII statistically insignificant (around 1.2-fold change compared to vehicle) (**Fig. 3**). Incubation of 19 hours starved L6 skeletal muscle cells for one hour with 100 nM and 10 μM of the non-selective adenosine analogue NECA (100 nM and 10 μM) activates mRNA gene expression of HKII significantly ($P<0.001$ and $P<0.05$, respectively) (around 1.1 and 1.25 fold change, respectively), and does not change mRNA gene expression of PDK4, and PFK (**Fig. 3**). Incubation of 19 hours starved L6 skeletal muscle cells for 3 hours with either 100 nM or 10 μM of the non-selective adenosine analogue NECA does not change mRNA gene expression of PDK4, HKII, and PFK (**Fig. 3**).

Incubation of 19 hours starved L6 skeletal muscle cells for 24 hours with either 100 nM or 10 μM of the non-selective adenosine analogue NECA does not change mRNA gene expression of PDK4. Moreover, incubation the 19 hours starved cells for 24 hours with NECA 100nM (but not 10 μM) down-regulates the mRNA expression of PFK significantly ($P<0.01$) (around -0.67 fold change compared to vehicle). In addition, incubation of 19 hours starved L6 skeletal muscle cells for 24 hours with 10 μM (but not 100 nM) of the non-selective adenosine analogue NECA increases mRNA gene expression of HKII significantly ($P<0.01$) (around 1.5 fold change compared to vehicle) (**Fig. 3).**

PSB 603 modulates PFK, and inhibits PDK4 and HKII: Interestingly, incubation of one week starved L6 skeletal muscle cells with PSB 603 (100 nM and 1 μM) inhibits PDK4 mRNA gene expression significantly ($P<0.01$) (around -0.9 fold change compared to vehicle), and with PSB 603 (1 μM) inhibits HKII mRNA gene expression in that skeletal muscle cells significantly ($P<0.01$) (around -0.59 fold change compared to vehicle). In contrast, incubation of one week starved L6 skeletal muscle cells with PSB 603 (100 nM and 1 μM) significantly increases mRNA gene expression of PFK ($P<0.001$ and $P<0.01$, respectively) (around

1.4 and 1.6 fold change compared to vehicle, respectively) (**Fig. 4**).

Incubation of 19 hours starved L6 skeletal muscle cells for one hour with 10 μM of the selective adenosine antagonist/inverse agonist PSB 603 mediates a significant increase in baseline of HKII mRNA gene expression levels in skeletal muscle cells ($P<0.05$) (around 1.3 fold change compared to vehicle) at one hour incubation time (**Fig. 4**). However, that does not change mRNA gene expression of PDK4, and PFK (**Fig. 4**).

Incubation of 19 hours starved L6 skeletal muscle cells for 3 hours with 10 μM of the selective adenosine antagonist PSB 603 does not change mRNA gene expression of PDK4, HKII, and PFK (**Fig. 4**).

Incubation of 19 hours starved L6 skeletal muscle cells for 24 hours with 10 μM of PSB 603 mediates mediates a significant decrease in baseline of PFK mRNA gene expression levels ($P<0.01$) (around -0.66 fold change compared to vehicle). However, that does not change mRNA gene expression of PDK4, and HKII in same skeletal muscle cells at 24 hours incubation time (**Fig. 4**).

Adenosine A2B receptors mediate both NECA-induced HKII, and NECA-inhibited PFK mRNA gene expression in skeletal muscle cells: To determine which subtype of adenosine A2 receptors mediate the modulation of HKII, and PFK mRNA gene expression level, the adenosine receptor agonist CGS21680 (subtype A2A selective) was used. The concentration applied for CGS 21680 could selectively activate the indicated subtype (Ki = 27 nM) [23].

Incubation the one week starved skeletal muscle cells with the adenosine A2A receptor selective agonist CGS21680 (100 nM) for one hour does not cause a significant change in the mRNA gene expression level of PDK4, HKII, and PFK in rat L6 skeletal muscle cells (**Fig. 3**).

Incubation the 19 hours starved skeletal muscle cells with CGS21680 (100 nM) for one hour does not cause a significant change in the mRNA gene expression level of PDK4, HKII, and PFK in rat L6 skeletal muscle cells (**Fig. 4).**

Incubation the 19 hours starved skeletal muscle cells with CGS21680 (100 nM) for 3 hours does not cause a significant change in the mRNA gene expression level of PDK4, HKII, and PFK in rat L6 skeletal muscle cells (**Fig. 4**).

Incubation the 19 hours starved skeletal muscle cells with CGS21680 (100 nM) for 24 hours does not cause a significant change in the mRNA gene expression level of PDK4, and HKII in rat L6 skeletal muscle cells. However, CGS21680 (100 nM) increases the mRNA gene expression level of PFK significantly ($P<0.001$) (around 1.3 fold change compared to vehicle) at the same incubation time (**Fig. 4**).

To investigate whether the effect of NECA is mediated by adenosine A2B receptors, a selective adenosine A2B receptor antagonist/inverse agonist, PSB 603 was incubated in skeletal muscle cells before 10 minutes of addition the NECA.

The effect of NECA in one week starved L6 skeletal muscle cells for one hour on mRNA expression of HKII is significantly blocked by PSB 603 at a concentration equal to 100 nM and 1 μM (**Fig. 3**). Interestingly, incubation of one week starved L6 skeletal muscle cells for one hour with NECA (10 μM) after 10 minutes of addition the 100 nM PSB 603 block the effect of PSB 603-induced the mRNA expression of PFK significantly ($P < 0.001$) (**Fig. 3**).

Regarding PDK4; interestingly, pre-treatment the 19 hours starved cells with 10 μM PSB 603 for 10 minutes prior the addition of 100 nM (but not 10 μM) NECA for 3 hours significantly up-regulates ($P<0.05$) mRNA expression of PDK4 while pre-treatment the same cells with 10 μM PSB 603 for 10 minutes prior the addition of 100 nM or 10 μM NECA for 24 hours significantly down-regulates ($P<0.001$ and $P<0.05$, respectively) mRNA expression of PDK4. Moreover, there is a significant difference in the mRNA gene expression level of PDK4 between incubation that cells for 3 hours with NECA (100 nM (but not 10 μM)) and PSB 603 (10 μM) compared with PSB 603 (10 μM) alone.

In addition, there is a significant difference in the mRNA gene expression level of PDK4 between incubation that cells with NECA (10 μM (but not 100 nM)) and PSB 603 (10 μM) compared with PSB 603 (10 μM) alone.

Role of cAMP pathway in the expression of PDK4, HKII, and PFK mRNA gene expression: In previous studies, activation of adenosine A2B receptors in skeletal muscle by NECA increased cAMP accumulation [11, 13] and increased the mRNA gene expression of NR4A [12]. In this current study, activation of adenosine A2B receptors in skeletal muscle by NECA does not change PDK4, stimulates HKII, and inhibits PFK mRNA gene expression in skeletal muscle cells.

Experiments were conducted to investigate if the adenylyl cyclase pathway is involved in the activation of those genes transcription profile in skeletal muscle cells. For this purpose, the adenylyl cyclase activator Forskolin was used in the present study to understand the potential role of the cAMP pathway in NECA-induces HKII mRNA gene expression, and NECA-inhibits PFK mRNA gene expression in skeletal muscle cells.

Incubation of one week starved cells with forskolin (100 nM) increases the expression of HKII significantly ($P<0.01$) (**Fig. 3**), a result similar to that of NECA. These findings support the idea that the cAMP pathway plays an important role in NECA-induces HKII mRNA gene expression. In contrast, forskolin (100 nM) does not cause any change in the expression of PDK4, and PFK.

It is interesting to note that treatment the 19 hours starved cells with forskolin (1 μM) for one hour down-regulates the mRNA expression of PDK4. In line with NECA effect on the mRNA expression of HKII, incubation of the 19 hours with forskolin (1 μM) for one, 3 and 24 hours up-regulates mRNA expression of HKII significantly (around 12.8, 2.5 and 1.4 fold change compared to vehicle, respectively). It is noteworthy that the fold change is the highest when the duration of incubation time is one hour. Indeed, there is gradual decrease in mRNA expression of HKII while increasing the incubating time of forskolin. Interesting, treatment the 19 hours skeletal muscle cells with forskolin (1 μM) for 1, 3 and 24 hours did not alter the mRNA expression of PFK.

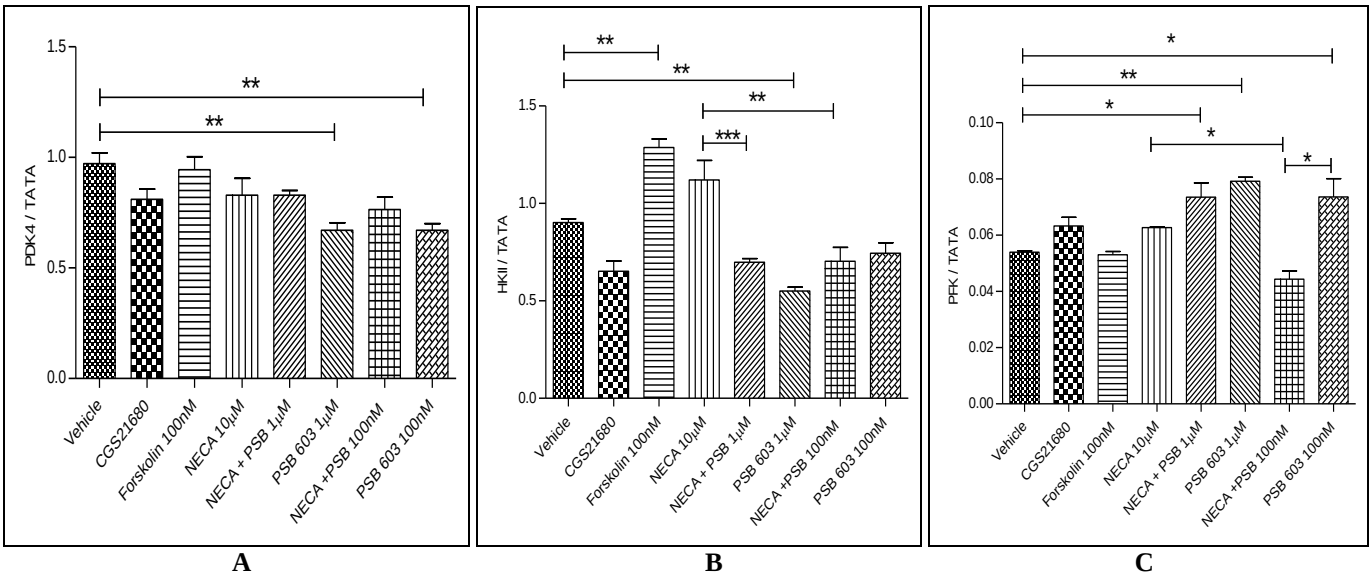

FIG. 3: EFFECTS OF NECA, CGS21680, AND PSB 603 ON PYRUVATE DEHYDROGENASE KINASE 4 (PDK4), HEXOKINASE II (HKII), AND PHOSPHOFRUCTOKINASE (PFK). Rat L6 skeletal muscle myotubes (70-90% confluent) were serum starved for 7 days and then stimulated for 1 hour. mRNA levels of those genes were measured relative to TATA-Box using real-time quantitative PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (10 μM), PSB 603 (100nM and 1 μM), CGS21680 (100 nM), and Forskolin (100 nM). mRNA relative expression of PDK4 (Figure 3-A), HKII (Figure 3-B), and PFK (Figure 3-C). Data were represented as means ± standard error of mean (SEM) of at least three independent experimental groups. * denotes $p<0.05$, ** denotes $P<0.01$ and *** denotes $p<0.001$. Data were analysed using a one-way ANOVA test followed by a Tukey test.

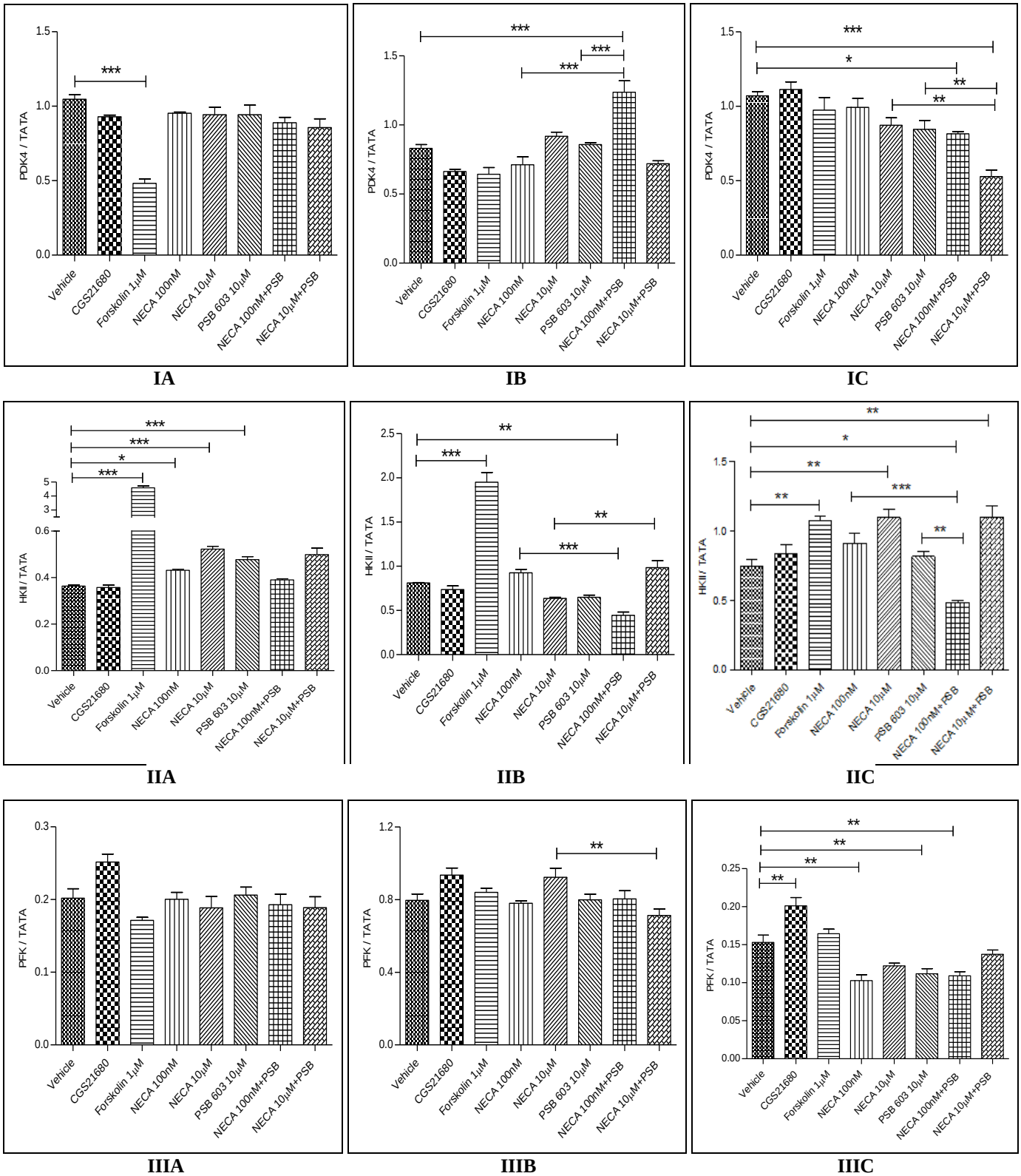

FIG. 4: EFFECTS OF NECA, CGS21680 AND PSB603 ON PYRUVATE DEHYDROGENASE KINASE 4 (PDK4), HEXOKINASE II (HKII), AND PHOSPHOFRUCTOKINASE (PFK) IN RAT L6 SKELETAL MUSCLE MYOTUBES USING CHARCOAL SERUM. Rat L6 skeletal muscle myotubes were stimulated for the indicated time from (1 hours (starvation 19 hours)) to 24 hours (starvation 19 hours) and mRNA levels of those genes, relative to TATA-Box, were measured by quantitative real time PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (100 nM and 10 µM), PSB603 (100nM, 1uM and 10 µM), CGS21680 (100 nM) and forskolin (100 nM). A) Stimulation was performed up to 1 hour. B) Stimulation was performed for up to 3 hours. C) Stimulation was performed for up to 24 hours. Data were represented as means ± standard error of mean (SEM) of three independent experimental group. (n=3; * denotes p<0.05, ** denotes P<0.01 and *** denotes p<0.001). Data were analyzed using one way ANOVA test followed by Tukey test.

DISCUSSION: In the present study, stable adenosine analogue modulates metabolic gene expression levels in cultures of rat skeletal muscle over a period of one-several hours. This effect is apparently mediated by at least A2B receptor subtypes and involves the activation of genes encoding proteins well known for their role in the regulation of glycolysis, glucose oxidation, insulin resistance and metabolism. The present pharmacological study is based on the use of a selective adenosine A2A receptor agonist, CGS 21680, and selective antagonist of adenosine A2B receptors, PSB 603. Hence, the lack of effect of CGS 21680 provides compelling evidence for the involvement of adenosine A2B receptors in the action of NECA. The results of the present study have shown for the first time that stable adenosine analogue alters the expression of genes involved in glycolysis (HKII and PFK). Moreover, this is the first study demonstrating that selective adenosine A2B receptor antagonist/inverse agonist, PSB 603 alters the expression of genes involved in glycolysis (HKII and PFK), and insulin resistance/glucose oxidation (PDK4). To our knowledge, this is the first study demonstrating an alteration of PFK gene by selective adenosine A2A receptors agonist, CGS 21680.

When this present study is considered in parallel with the role of adenosine A2B receptors in mediating inflammation in skeletal muscle (data not published yet), the current observations regarding adenosine A2B receptors signaling implies another important functional role for adenosine system, in particular adenosine A2B receptors, in mediating skeletal muscle metabolism. Indeed, this present study has demonstrated that adenosine system modulation, in particular adenosine A2B receptors, regulates the mRNA expression of genes that modulates skeletal muscle glycolysis, insulin resistance/sensitivity, and glucose oxidation.

Generally, the difference in the effect of NECA or PSB 603 between the one week starved cells and 19 hours starved cells is due to the duration of starvation that may play a role in gene expression modulation, including those metabolic genes [24]. Moreover, it is possible that the density of adenosine A2B and A2A receptor or extracellular adenosine accumulation differ between one week and 19 hours starved cells.

PDK4 inactivates the pyruvate dehydrogenase complex which is considered a crucial step for the inhibition of the whole oxidation of glucose [25]. To our knowledge, this is the first study demonstrating an inhibition of PDK4 gene by selective adenosine A2B receptors antagonist/inverse agonist, PSB 603 (100 nM and 1 uM). Assuming the modulation of PDK4 mRNA expression leads to modulation in PDK4 activity, these data in the present study are consistent with a suggested hypothesis that adenosine A2B receptors activation induces insulin resistance [26] which may be mediated in part directly by an increase of PDK4 activity and/or indirectly by cytokine production (data not published yet). Indeed, this hypothesis with the present study is generally in line with the previous study which suggested that adenosine analogue signaling through adenosine A2B receptors may also contribute to insulin resistance by induction the mRNA expression of IL-6 (data not published yet), and with another previous hyperinsulinemic euglycemic study which demonstrated that adenosine A2B receptors blocker signaling enhanced insulin sensitivity and glucose metabolism in skeletal muscle in KKAY mice [26].

Thus, inhibition of PDK4 is thought to be a primary means by which glucose oxidation is activated in the skeletal muscle cells exposed to PSB 603, presumably as part of an integrative response to consume glucose. Indeed, this data in the current study agrees with the effect of insulin on PDK4 mRNA expression in skeletal muscle [27]. In this regard, insulin's effect to suppress PDK4 expression was impaired in insulin-resistant states [28, 29]. Moreover, increased PDK4 expression (and decreased glucose oxidation) in muscle has been observed in insulin resistant states, such as high-fat–fed [28] or streptozotocin-induced diabetic [30] animals. Taken together, as skeletal muscle consumes 70% of whole body glucose , PSB 603 down regulate the mRNA expression of PDK4 (and presumably increase glucose oxidation), it is possible therefore that PSB 603 as selective adenosine A2B antagonist/inverse agonist is considered as a promising therapeutic agent for alleviating the level of glucose in whole body.

Glycolysis is the main metabolic pathway that consumes glucose, where hexokinase and phosphofructokinase are rate-limiting enzymes of this process, functioning as indicators of this pathway flux [31]. In skeletal muscle cells, HKII mRNA expression was selectively increased by insulin, and this was associated with an increased rate of glucose utilization [32]. In the current study, during the one week starved skeletal muscle exposed to PSB 603 (1 hour), there is a down-regulation in mRNA expression of HKII. However, at the same time, there is up-regulation in mRNA expression of PFK.

In the current study, it seems that adenosine A2B receptors mediate acutely both NECA-induced HKII and NECA-inhibited PFK. The implication of this finding is that adenosine A2B receptors might be involved in an early molecular manifestation of a protective mechanism against hyperglycemia by a possible increase of intramuscular glycolysis, although further activity studies are required to test hypothesis. Moreover, during 19 hours starved cells exposed to 10 μM PSB 603, there is up-regulation (1 hour) and no change (24 hours) in mRNA expression of HKII. On the contrary, there is no change (1 hour) and down-regulation (24 hours) in mRNA expression of PFK. Indeed, there is some evidence in this present study that PSB 603 works in opposite coordinated scenario in regulation the mRNA expression of HKII and PFK, although further studies are required to certainly examine this hypothesis.

However, surprisingly in the present study, adenosine A2A receptors activation with the A2A selective agonist, CGS 21680 (24 hours) in L6 skeletal muscle myotubes results in significantly increased mRNA abundance of PFK. This effect of CGS 21680 is not reproduced by that of NECA (100 nM or 10 uM) at the same incubation time, suggesting that the effect of NECA is mediated by adenosine A2A and either A1 and/or A3 receptors. It is obvious that adenosine system, in particular at least adenosine A2 receptors, modulates the mRNA expression of key glycolytic enzymes (HKII and PFK). It is worth to note that that forskolin increase mRNA expression of HKII in similar to that of NECA. However, forskolin does not increase mRNA expression of PFK in similar to that of CGS 21680. This might support an evidence to Gs coupled for adenosine A2B receptors. However, Gq coupled (but not Gs coupled) for adenosine A2A receptors might be involved, although further studies are required to test this issue. At this point, we cannot rule out the functional presence of adenosine A2A receptor in skeletal muscle cells.

In general, the observation that adenosine A2B receptor blocker/inverse agonist down-regulated the expression of PDK4 and HKII and modulated the expression of PFK suggest that the capacity of skeletal muscle to modulate glycolysis and insulin resistance would also be regulated by adenosine system, in particular adenosine A2 receptors. Experiments primarily carried out in the present study suggest that transcriptional regulation of a number of key genes involved in glucose metabolism is responsible for the metabolic adaptation in response to adenosine receptor ligands in skeletal muscle. It is found in the current study that the mere addition of NECA and/or PSB 603 to the culture medium of rat skeletal muscle cells leads to a selective and coordinate induction of the expression of proteins involved in glycolysis, glucose oxidation, thereby showing that adenosine A2 receptors ligands themselves are able to modulate metabolic gene expression in skeletal muscle.

In summary, this analysis suggested that adenosine A2B receptors-mediated modulation of gene expression involved in glucose metabolism, is concomitant with the induction of the NR4A subgroup in skeletal muscle tissue. The non-selective adenosine receptors agonist, NECA also induced the expression of the NR4A family in skeletal muscle in adenosine A2B receptor dependent manner [12]. In the current study, it has been observed that the adenosine A2B receptor is accompanied by the expression of critical genes that regulated key aspects of glucose homeostasis in skeletal muscle.

CONCLUSIONS: Adenosine A2B receptors agonists/antagonists eventually possess multiple beneficial actions including boosting skeletal muscle glucose metabolism possibly through modulating mRNA expression of metabolic genes. Therefore, further studies are invited to evaluate the clinical potential of adenosine A2B receptors agonists/antagonists in both animals and humans.

It has been shown here that NECA treatment has profound effects on metabolic skeletal muscle cell gene expression, with a rapid response of key genes involved in nutrient oxidation and in other metabolic signaling pathways. This current study has identified that adenosine A2B expression is an important regulator of multiple intracellular processes that may influence metabolic function in rat skeletal muscle. Future experiments are critical to more clearly delineate the mechanisms by which adenosine A2B influences the intracellular functions of skeletal muscle. These findings significantly extend our understanding of adenosine A2B receptors physiology by considering mechanistic aspects outside of its classically known inflammatory role in skeletal muscle tissue. Future studies will be necessary to examine the therapeutic potential of manipulating adenosine A2B receptors expression and/or function in skeletal muscle as a means to prevent the development of metabolic diseases such as type II diabetes mellitus.

ACKNOWLEDGEMENT: I would like to thank Abdul Hameed Shoman Foundation for supporting scientific research in Jordan and for their kind generous financial support of this project. Indeed, this project was supported by grants from mainly Abdul Hameed Shoman Foundation (Grant number 12/2015) and Philadelphia University. Without this support, I could not perform this work.

DECLERATION OF INTEREST: The author has declared no conflict of interest.

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How to cite this article:
Haddad M: The impact of adenosine a2b receptors on glycolysis and insulin resistance in skeletal muscle. Int J Pharm Sci Res 2016; 7(12): 4917-26.doi: 10.13040/IJPSR.0975-8232.7(12). 4917-26.