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THE IMPACT OF ADENOSINE A2B RECEPTORS MODULATION ON PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA CO-ACTIVATOR 1-ALPHA AND TRANSCRIPTION FACTORS

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Keywords:

Adenosine A2B receptors, skeletal muscle, cAMP, NR4A and PGC-1α

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Abbreviations:
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-chlorophenzyl) iperazide-1-sulfonyl) phenyl]]-1-propyl xanthine; CGS 21680 : 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-52 –Nethyluron amide; DMSO: Dimethyl sulphoxide; P-value: Probability; NR4A; Nuclear Receptor Subfamily 4, Group A, PGC-1α; Peroxisome proliferator-activated receptor gamma (PPARgamma) coactivator 1alpha (PGC-1α)

ABSTRACT: Previous studies have demonstrated that the activation of adenosine A2B receptors increases adenosine 3'-cyclic monophosphate (cAMP) accumulation in rat skeletal muscle cells, which is probably an important yet unknown mechanism contributing to the regulation of skeletal muscle functions. via rat L6 skeletal muscle cells, it has been elucidated further potential molecular signaling responsible for adenosine A2B receptor modulations via quantitative real-time PCR assays (probe-based). The results of the present study has shown for the first time that NECA alters the expression of PGC-1α significantly in both one week and 19 hours starved skeletal muscle cells (*P<0.05* and *P<0.01*, respectively) (2.5 and -0.58 fold change compared to vehicle, respectively). Adenosine A2B receptors mediate NECA-modulated PGC-1α mRNA gene expression in skeletal muscle cells. To our knowledge, this is the first study demonstrating an induction of PGC-1α gene by CGS 21680 significantly (*P<0.001*) (around 1.8 fold change compared to vehicle). In the current study, NECA (10 μM) increases NR4A1 and NR4A3 mRNA gene expression significantly (*P<0.05*) (around 2.7 and 5.2 -fold change to vehicle, respectively), which are blocked by a selective adenosine A2B receptor antagonist, PSB 603. This current study identifies the adenosine A2B receptors as a significant regulator of PGC-1α, NR4A1 and NR4A3 mRNA gene expression in skeletal muscle, thereby pointing to its therapeutic potential. In summary, it has been observed the selective, potentially functional expression of adenosine A2 receptors in skeletal muscle cells. Whether adenosine A2 receptor mediated functional responses play a role in skeletal muscle pathophysiology is yet to be elucidated.

INTRODUCTION: Skeletal muscle has, due to its large mass, a central role to systemic energy homeostasis and whole body metabolism, being a major site for systemic glucose and lipid metabolism [1, 2]. Moreover, it is a major site involved in peripheral insulin resistance; it clears the majority (70-80%) of ingested glucose [3-5]. Indeed, skeletal muscle potentially plays a crucial role in metabolic diseases [6] as this tissue has an ability to oxidize glucose and fatty acid substrates and also has a high degree of metabolic activity [1].

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Moreover, skeletal muscle also potentially plays an active role in the inflammatory process by secreting cytokines [7]. There are many signals that can regulate the ordinary and extra-ordinary functions of skeletal muscles and one such signal is the activation of adenosine receptors by the endogenous adenosine.

Adenosine, as a local metabolic factor, is a ubiquitous homeostasis regulator. Adenosine is released from most tissues in the body and cells in culture. Extracellular adenosine may arise from intracellular adenosine or from breakdown of the adenine nucleotides, such as adenosine triphosphate (ATP), outside the cell [8]. Adenosine has been demonstrated to regulate many physiological functions of the cardiovascular system [9] and of most tissues, including skeletal muscle [10].

With regard to skeletal muscle, it has been proposed that adenosine regulates many important physiological actions, including the synergistic effect of contraction and insulin stimulated glucose uptake via adenosine receptors [10].

Adenosine acts on cell surface receptors that are coupled to intracellular signaling cascades. There are four subtypes of adenosine receptors termed adenosine A1 , A2A, A2B and A3 receptors based on pharmacology and the adenylate cyclase systems [11]. The adenosine A1 and A3 receptors inhibit the production cAMP through coupling to Gi. The adenosine A2A and A2B subtypes are coupled to Gs to stimulate adenylate cyclase. Furthermore, the adenosine A2B receptors, which has the lowest affinity (Ki > 1 uM) of all subtypes for native adenosine, is also coupled to Gq [12]. Adenosine receptors are expressed in various tissues and cell types, including skeletal muscle [13-15]. Moreover, adenosine A2B receptors are found to increase cAMP concentration in skeletal muscle myotubes [15, 16]. A key regulatory pathway in skeletal muscle that might be responsible for the modulation of skeletal muscle metabolism in response to adenosine is the adenosine A2B receptor signaling pathway [16].

One of the possible downstream signalling targets of cAMP in skeletal muscle cells is peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a tissue-specific and inducible transcriptional coactivator for several nuclear receptors [17, 18]. PGC-1α is a strong activator of mitochondrial biogenesis and oxidative metabolism [19]. Moreover, PGC-1α plays a key role in energy metabolism [20]. PGC-1α mRNA is predominantly expressed in heart, brown adipose tissue, kidney, skeletal muscle, and liver, and its expression is induced in response to stimuli known to regulate metabolic activity.

PGC-1α is one of the key regulatory factors in the active skeletal muscle [21]. Reduced muscle activity is associated with impaired PGC-1α expression [21]. PGC-1α has also been suggested to have additional roles in regulating inflammatory processes [21] and to be associated with myoblast differentiation [22]. It is highly likely that PGC-1α is involved in diseases such as diabetes and obesity. In particular, its regulatory function in lipid metabolism makes it a promising target for medical intervention in the treatment of Type 2 diabetes and obesity [23]. Another possible downstream signalling targets of cAMP in skeletal muscle cells is NR4A [24]. NR4A is a subfamily of the orphan nuclear receptor (NR) superfamily, which consists of three members, namely Nur77 (NR4A1), Nurr1 (NR4A2) and NOR1 (NR4A3) [25]. More recently, several studies have demonstrated that NR4A receptors as key transcriptional regulators are implicated in various biological processes, such as inflammation, lipid and glucose metabolism, insulin sensitivity, energy balance and cell proliferation and differentiation [26-31]. These studies have focused on NR4A mainly in the liver, adipose and skeletal muscle [32-35].

There is growing evidence to suggest that the modulation of PGC-1α and NR4A leads to an increase in the gene expression of intracellular downstream signalling pathways that, it has been suggested, participate in the regulation of inflammation, glucose and fatty acid metabolism and cell growth in skeletal muscle [24, 28, 36-38]. Cross-talk between adenosine A2B receptors and (PGC-1α and/or NR4A) signalling may potentially represent an important yet unknown mechanism contributing to the regulation of skeletal muscle functions. Thus further studies, including those on the L6 skeletal muscle cells as a cell model to understand the molecular signalling involved in the cross-talk, are highly significant.

The aim of this work was to elucidate the potential signalling underlying the adenosine A2B receptor modulation on PGC-1α and NR4A mRNA gene expression and the potential involvement of the cAMP pathway in adenosine A2B receptor modulation.

MATERIALS AND METHODS:

Materials: NECA, forskolin, PSB 603 and CGS 21680 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 RevertAid 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 cell line 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**.

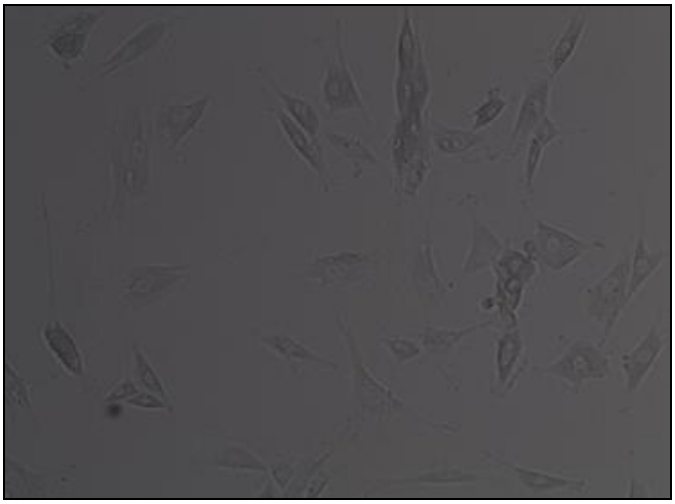
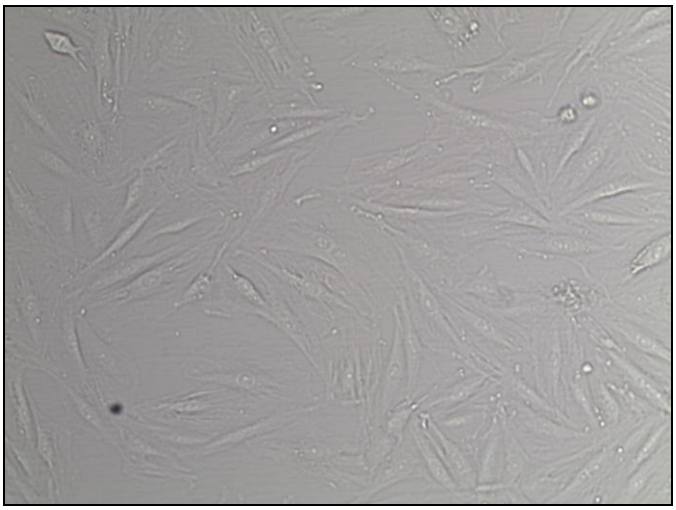


FIG. 1: REPRESENTATIVE MYOBLASTS DERIVED FROM PASSAGE NUMBER 7. Myoblasts taken after 1 day seeding into 25cm^2 (10X), scale bar=100 µm.

Confluent cells in 25 cm^2 flasks were cultured for a further 14 days (to allow myotube formation), according to the protocol mentioned in [5] 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 (**Fig. 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).



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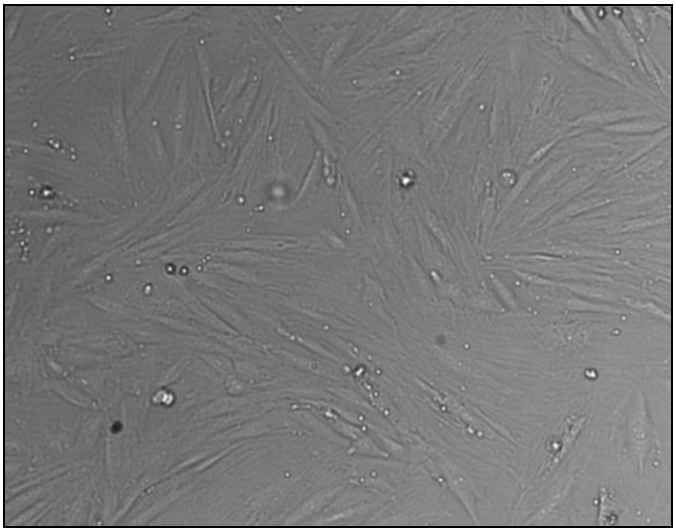

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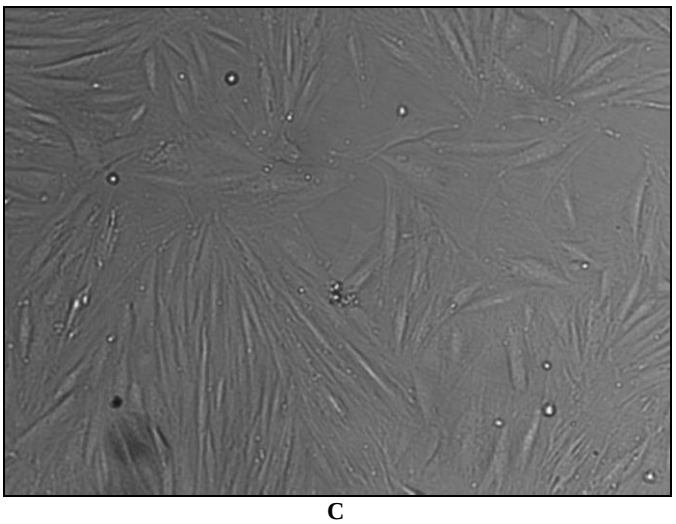

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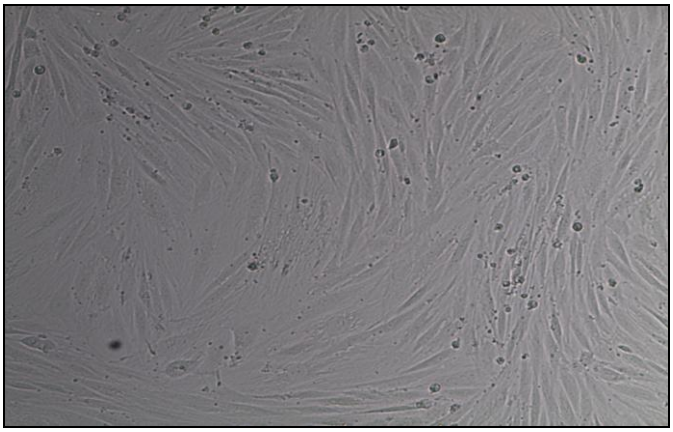

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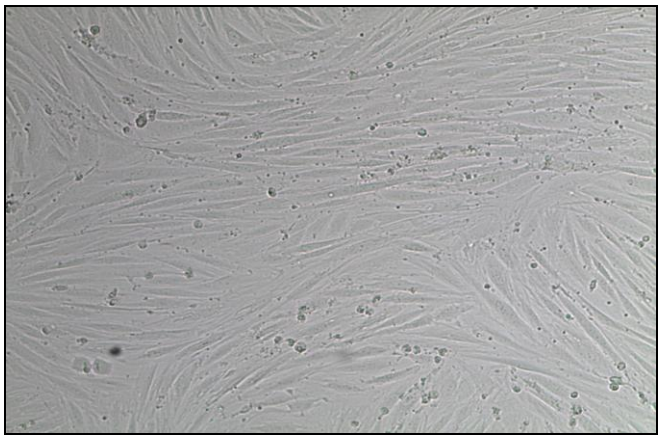

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FIG. 2: REPRESENTATIVE MYOBLASTS/MYOTUBES DERIVED FROM PASSAGE NUMBER 7. A) myoblasts taken at (Ham-F10, 10% FBS, 1% P/S) during 3-4 days of tissue culture (10X). B) myoblasts taken at (Ham-F10, 6% Hoarse serum, 1% P/S) during 4-5 days of tissue culture (10X). C) myoblasts taken at (Ham-F10, 2% Hoarse serum, 1% P/S) during 6-7 days of tissue culture (10X). D) myotubes taken at (Ham-F10, 2% charcoal serum, 1% P/S) during 11-12 days of tissue culture (10X). E) myotubes taken at (Ham-F10, 1% P/S) after 16 hours starvation (10X).

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 real-time PCR system (Applied Biosystems, USA). Gene expression was determined relative to reference 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%.

TABLE 1: LIST OF GENE PRIMER AND PROBE SEQUENCES

Gene	Sequences (5' →3')	Amplicon size (bp)
NR4A1	Probe 5'- CTTTATCCTCCGCCTGGCCTACCGA - 3' Forward primer 5' - TGTTGCTAGAGTCCGCCTTTC -3' Reverse primer 5' - CAGGCCTGAGCAGAAGATGAG -3'	95
NR4A2	Probe 5'- TACGCTTAGCATACAGGTCCAACCCAGTG -3' Forward Primer 5'- CCAAAGCCGATCAGGACCT -3' Reverse primer 5'- GACCACCCCATTGCAAAAGAT -3'	116
NR4A3	Probe: 5'- ACTGTCCCACCGACCAGGCCACT -3' Forward Primer: 5'- GACGCAACGCCCAGAGAC -3' Reverse primer 5'- TAGAACTGCTGCACGTGCTCA -3'	92
PGC-1α	Probe 5'-TGGAACTCTCTGGAACTGCAGGCCTAACT-3' Forward-Primer 5'-TTCCCCATTTGAGAACAAGACTATT-3' Reverse-Primer 5'-GTTATCTTGGTTGGCTTTATGAGGA-3'	100
TATA-BOX	Probe 5'- TCCCAAGCGGTTTGCTGCAGTCA -3' Forward Primer 5'- TTCGTGCCAGAAATGCTGAA-3' Reverse Primer 5'- GTTCGTGGCTCTCTTATTCTCATG -3'	73

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 AND DISCUSSIONS:

Effects of NECA, CGS 21680, and PSB 603 on PGC-1α mRNA gene expression in skeletal muscle: Incubation of one week starved L6 skeletal muscle cells with 10 μM of the non-selective adenosine analogue NECA increases mRNA gene expression of PGC-1α significantly (P<0.05) (around 2.5 fold change compared to vehicle) (**Fig. 3**). This effect of NECA is significantly blocked by PSB 603 at a concentration equal to 100 nM and 1 μM (**Fig. 3**). However, incubation the same cells with the adenosine A2A receptor selective agonist CGS 21680 (100 nM) or the selective adenosine A2B receptor antagonist/ inverse agonist, PSB 603 (100 nM or 1 μM) for one hour does not cause a significant change in the mRNA gene expression level of PGC-1α (**Fig. 3**). Indeed, adenosine A2B receptors mediates NECA-induced the mRNA gene expression of PGC-1α in these skeletal muscle cells.

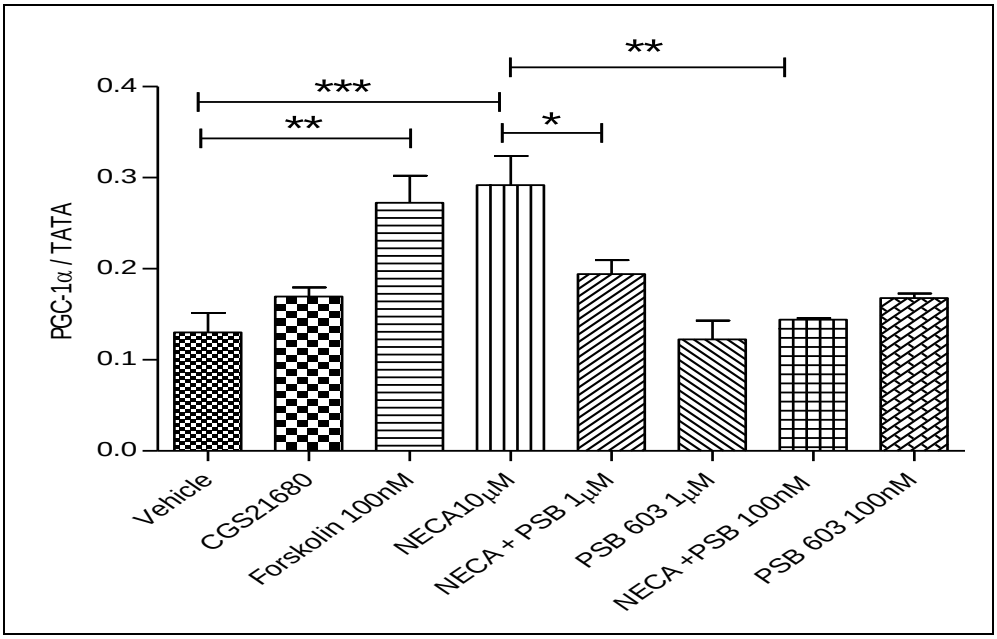


FIG. 3: EFFECTS OF NECA, CGS 21680 AND PSB 603 ON THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARGAMMA) COACTIVATOR 1ALPHA (PGC-1α) gENE EXPRESSION IN RAT L6 SKELETAL MUSCLE MYOTUBES USING CHARCOAL SERUM. Rat L6 skeletal muscle myotubes were stimulated for the indicated time from (1 hours (starvation 7 days)) and PGC-1α mRNA levels, relative to TATA-Box, was measured by quantitative real time PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (100 nM and 10 μM), PSB 603 (100nM, 1μM and 10 μM), CGS 21680 (100 nM) and forskolin (100 nM). Stimulation was performed up to 1 hour. 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.

In contrast to one week starved skeletal muscle cells, incubation the 19 hours starved rat L6 skeletal muscle cells with 10 μM NECA (but not 100 nM NECA) for one hour significantly down-regulates the mRNA expression level of PGC-1α (P<0.01) (around -0.58 fold change compared to vehicle), this effect of NECA is blocked by 10 μM PSB 603 significantly (P<0.001) (**Fig. 4-A**). This strongly indicates that this effect of NECA is adenosine A2B receptor dependent. Indeed, adenosine A2B receptors mediates NECA-inhibited the mRNA gene expression of PGC-1α in these skeletal muscle cells.

Incubation of 19 hours starved L6 skeletal muscle cells for 3 hours and 24 hours with 100 nM and 10 μM of the non-selective adenosine analogue NECA does not change mRNA gene expression of PGC-1α (**Fig. 4-B** and **4-C**).

Incubation of 19 hours starved L6 skeletal muscle cells for one, three or 24 hours with 10 μM of the selective adenosine antagonist/inverse agonist PSB 603 does not change mRNA gene expression of PGC-1α (**Fig. 4**).

Incubation the 19 hours starved skeletal muscle cells with CGS 21680 (100 nM) for one hour or three hours does not cause a significant change in the mRNA gene expression level of PGC-1α. However, incubation the 19 hours starved skeletal muscle cells with CGS 21680 (100 nM) for 24 hours increases the mRNA gene expression level of PGC-1α significantly (P<0.001) (around 1.8 fold change compared to vehicle) (**Fig.4**). Incubation the 19 hours starved muscle cells with 100 nM or 10 μM NECA for 3 hours or 24 hours does not significantly change mRNA expression level of PGC-1α. However, incubation the same cells for the same time with NECA 100 nM or 10 μM after 10 minutes of addition PSB 603 10 μM modulates mRNA expression level significantly compared to vehicle. Interestingly, pre-treatment the same cells with 10 μM PSB 603 for 10 minutes prior the addition of 100 nM or 10 μM NECA for 3 hours significantly down-regulates (P<0.05, P<0.01, respectively) and for 24 hours up-regulates (P<0.01 and P<0.001, respectively) the mRNA expression of PGC-1α. Moreover, there is a significant difference in the mRNA gene expression level of PGC-1α between incubation that cells with PSB 603 (10 μM) alone compared with NECA (100 nM (but not 10 μM)) and PSB 603 (10 μM) (**Fig.4**).

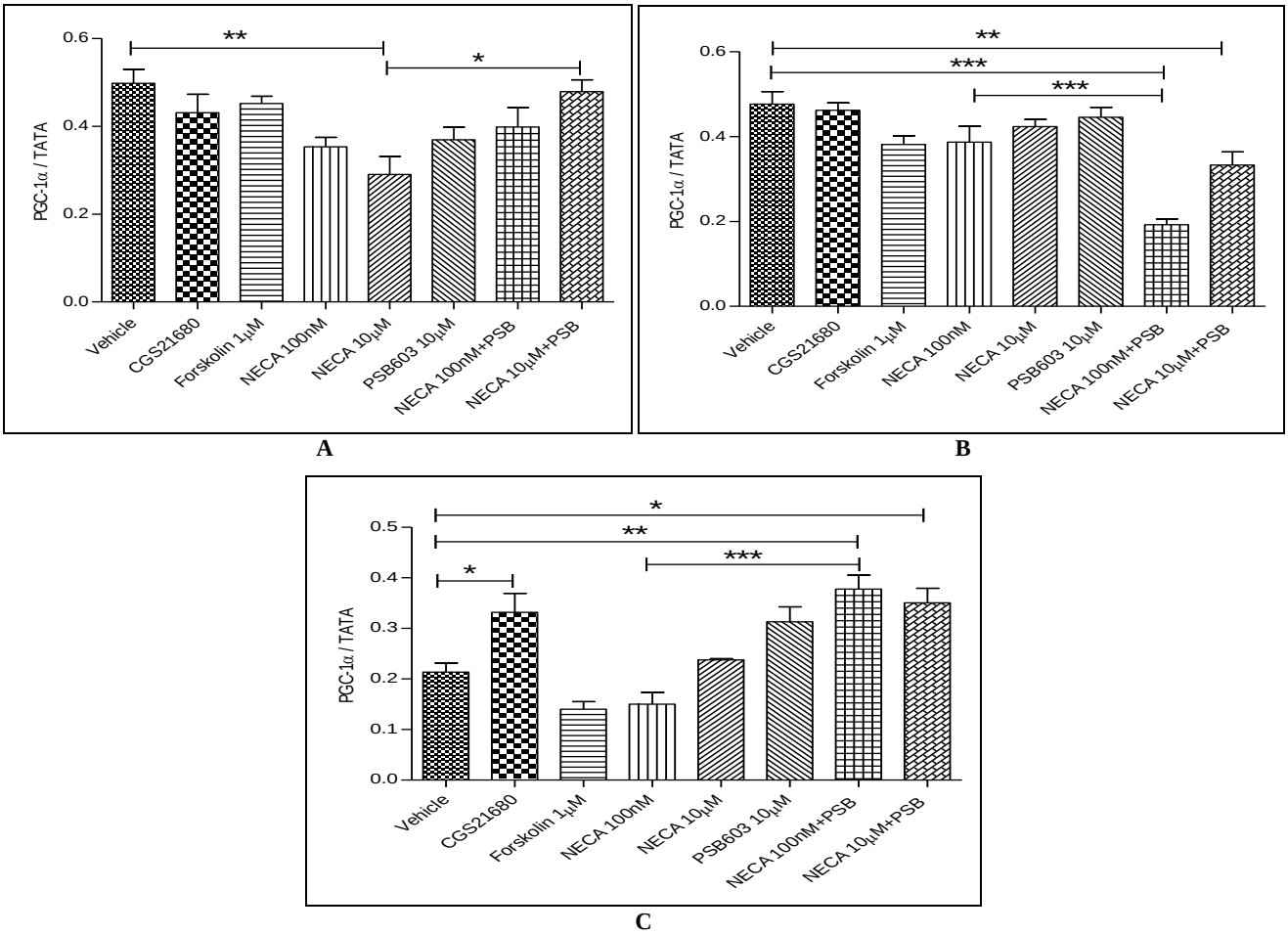


FIG. 4: EFFECTS OF NECA, CGS 21680 AND PSB 603 ON PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARGAMMA) COACTIVATOR 1ALPHA (PGC-1α) GENE EXPRESSION IN RAT L6 SKELETAL MUSCLE MYOTUBES USING CHARCOAL SERUM. Rat L6 skeletal muscle myotubes were stimulated for the indicated time from (1 hours (starvation 19 hours)) to 3 hours (starvation 19 hours) and PGC-1α mRNA levels, relative to TATA-Box, was measured by quantitative real time PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (100 nM and 10 μM), PSB 603 (100nM, 1μM and 10 μM), CGS 21680 (100 nM) and forskolin (1 μM). A) Stimulation was performed up to 1 hour. B) Stimulation was performed for up to 3 hours. C) Stimulation was performed 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.

Effects of NECA, CGS 21680, and PSB 603 on NR4R1 mRNA gene expression: It has been previously shown that adenosine A2B receptors mediates NECA-induced NR4A1 in one week starved skeletal muscle cells [39]. However, the one week starved skeletal muscle cells is not physiological relevant. Therefore, NR4A1 mRNA expression was investigated using 19 hours starved skeletal muscle cells (**Fig. 5-I**).

In the current study, treatment the 19 hours starved cells with CGS 21680 (100 nM) for 1 hour does not affect the NR4A1 gene expression. However, that cells treated with NECA (100 nM and 10 μM) for 1 hour show a significant increase in mRNA expression (NR4A1/TATA) ratio compared to vehicle ($P<0.01$ and $P<0.05$, respectively) (around 1.78 and 2.7 fold change compared to vehicle, respectively). Pre-treatment of the myotubes with 10 μM PSB 603 for 10 minutes prior the addition of 10 μM NECA inhibited this effect significantly (**Fig. 5-I**). In other words, the influence of NECA on NR4A1 is adenosine A2B receptor dependent. Indeed, adenosine A2B receptors mediates NECA-induced NR4A1 mRNA expression in rat L6 skeletal muscle cells.

Treatment the 19 hours starved cells with NECA (100 nM and 10 μM) for 24 hours increases mRNA expression of NR4A1 compared to vehicle significantly ($P<0.05$ and $P<0.01$, respectively) (1.55 and 1.4 fold change compared to vehicle, respectively). This effect of NECA 100 nM (but not 10 μM) was blocked by PSB 603 (10 μM). Interestingly, the treatment of skeletal muscle cells with 10 μM PSB 603 for 1 hour and 24 hours (but, not 3 hours) significantly ($P<0.05$) increases the mRNA expression (NR4A1/TATA) ratio compared to vehicle (around 2.5 and 1.5 fold change compared to vehicle, respectively) (**Fig. 5-I**).

Effects of NECA, CGS 21680, and PSB 603 on NR4R2 mRNA gene expression: It has been previously shown that adenosine A2B receptors mediates NECA-induced NR4A2 in one week starved skeletal muscle cells [39]. However, the one week starved skeletal muscle cells is not physiological relevant. Therefore, NR4A2 mRNA expression was investigated using 19 hours starved skeletal muscle cells (**Fig. 5-II**).

Treatment the 19 hours starved skeletal muscle cells with either CGS 21680 (100 nM) or NECA (10 μM) does not increase the mRNA expression of NR4A2 compared to vehicle (**Fig. 6**). There is a significant difference in mRNA expression of NR4A2 between treatment the cells for one hour with NECA (10 μM) alone and treatment that cells with PSB 603 10 minutes before addition of NECA. Moreover, there is a significant difference in mRNA expression of NR4A2 between treatment the cells for three hours with NECA (100 nM) alone and treatment that cells with PSB 603 10 minutes before addition of NECA (**Fig. 5-II**).

Effects of NECA, CGS 21680, and PSB 603 on NR4R3 mRNA gene expression: It has been previously shown that adenosine A2B receptors mediates NECA-induced NR4A3 in one week starved skeletal muscle cells [39]. However, the one week starved skeletal muscle cells is not physiological relevant. Therefore, NR4A3 mRNA expression was investigated using 19 hours starved skeletal muscle cells (**Fig. 5-III**).

Treatment the myotubes with NECA (100 nM and 10 μM) for 1 hour significantly up-regulates NR4A3 gene expression ($P<0.05$) (around 2.8 and 5.2 fold change compared to vehicle, respectively). Pretreatment of the myotubes with 10 μM PSB 603 for 10 minutes prior the addition of 10 μM NECA inhibits this effect significantly. Adenosine A2B receptors mediates NECA-induced NR4A3 mRNA expression in rat L6 skeletal muscle cells. Moreover, treatment the cells with NECA (10 μM) for 3 hours significantly up-regulates NR4A3 gene expression ($P<0.001$) (around 1.4 fold change compared to vehicle). However, this response was not inhibited by PSB 603 (10 μM) (**Fig. 5-III**).

Treatment the cells with NECA (100 nM) for 24 hours significantly up-regulates NR4A3 gene expression ($P<0.05$) (1.5 fold change compared to vehicle). However, this response was not inhibited by PSB 603 (10 μM). Treatment the cells with either NECA (100 nM) or PSB 603 (10 μM) for 24 hours does not increase mRNA expression of NR4A3. However, treatment the cells with PSB 603 10 minutes before the addition of NECA (10 μM) up-regulate the mRNA expression of NR4A3 **(Fig. 5-III).**

It is worth note that the effect of PSB 603 (1 hour (but not for 3 hours and 24 hours)) activates the mRNA expression of NR4A3 significantly (P<0.05) (around 7.7 fold change compared to vehicle).

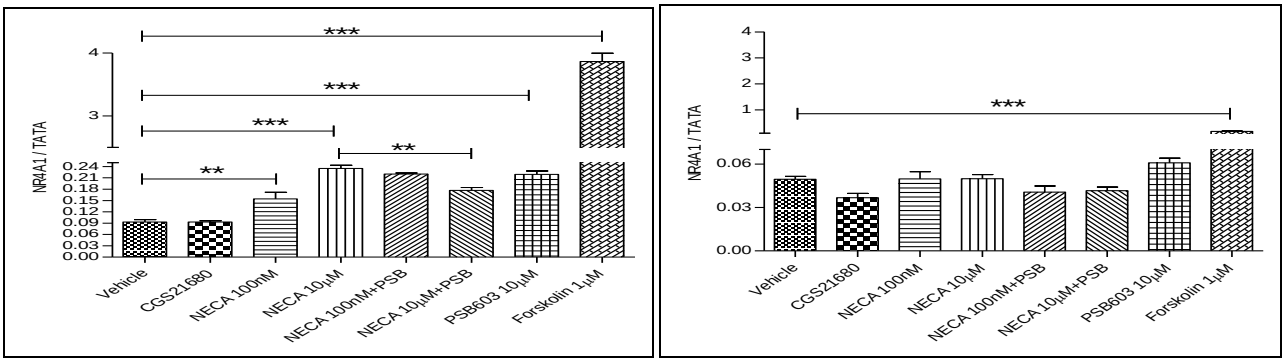
Role of cAMP pathway in the expression of PGC-1α, NR4A1 and NR4A3 mRNA gene expression: In previous studies, activation of adenosine A2B receptors in skeletal muscle by NECA increased cAMP accumulation [15, 16]. In this current study, activation of adenosine A2B receptors in skeletal muscle by NECA modulates the mRNA expression of PGC-1α and increases the mRNA gene expression of NR4A1 and NR4A3. 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-modulates PGC-1α, NECA-induced NR4A1 and NR4A3 mRNA gene expression in skeletal muscle cells.

Incubation of one week starved cells with forskolin (100 nM) increases the expression of PGC-1α 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 PGC-1α mRNA gene expression. Interesting, treatment the 19 hours skeletal muscle cells with forskolin (1 μM) for 1, 3 and 24 hours did not change the mRNA expression of PGC-1α. Indeed, it is worth to note that treatment the 19 hours starved cells with forskolin (1 μM) for 1 hour and 3 hours (but, not for 24 hours) increased mRNA expression of NR4A1 significantly (P<0.05) (**Fig. 4**).

There is gradual decrease on the effect of forskolin on NR4A1 mRNA expression for 1, 3 and 24 hours (P<0.05, P<0.05 and P<0.001, respectively) (around 44.8, 3.6 and 1.1 fold change compared to vehicle, respectively) (**Fig. 5-I**).

Interesting, treatment the cells with forskolin for 24 hours down-regulates the mRNA expression of NR4A2 significantly. On the other hand, treatment that cells with forskolin for one hour and 3 hours up-regulate the mRNA expression of NR4A2 significantly (**Fig. 5-II**).

Treatment the 19 hours starved cells with forskolin (1 μM) for 1, 3 and 24 hours up-regulate mRNA expression of NR4A3 significantly (P<0.05) (around 53.6, 22.9 and 1.59 fold change compared to vehicle, respectively) (**Fig. 5-III).**



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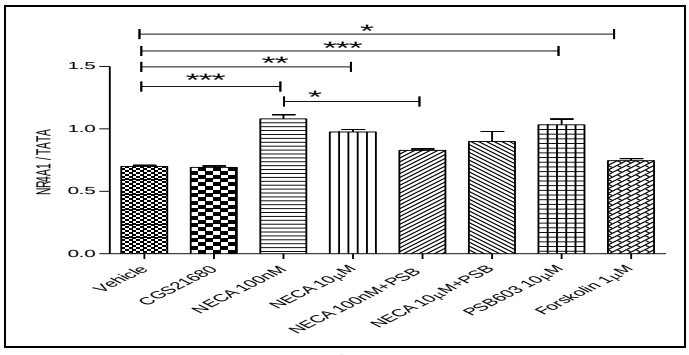

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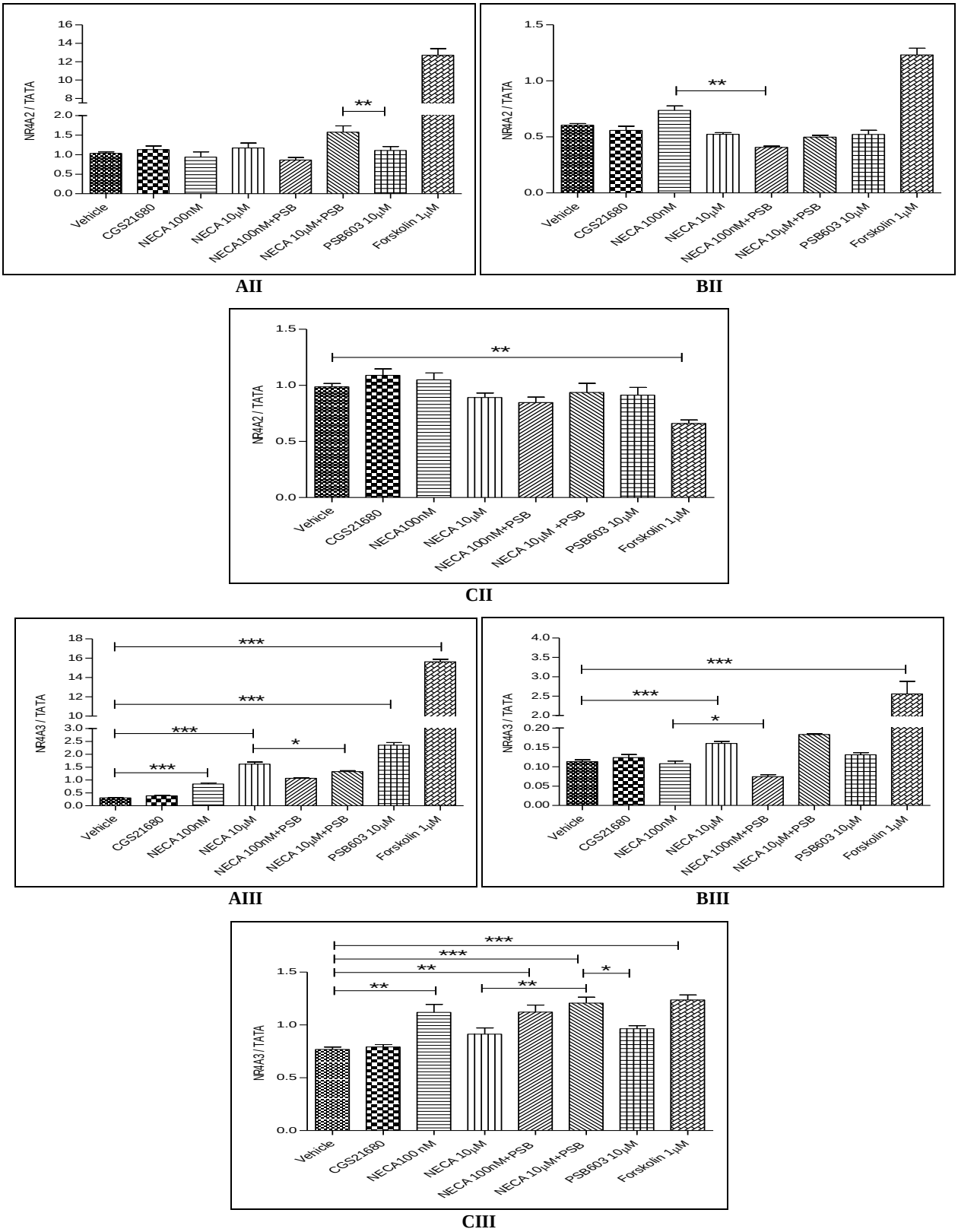

FIG. 5: EFFECTS OF NECA, CGS 21680 AND PSB 603 ON NUCLEAR RECEPTOR SUBFAMILY 4, GROUP A, MEMBER 1, 2 AND 2 (NR4A1, NR4A2 AND NR4A3) GENE EXPRESSION IN RAT L6 SKELETAL MUSCLE MYOTUBES USING CHARCOAL SERUM. Rat L6 skeletal muscle myotubes were stimulated for the indicated time for 1 hour, 3 hours and 24 hours and NR4A1 mRNA levels, relative to TATA-Box, was measured by quantitative real time PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (100 nM and 10 µM), PSB 603 (10 µM), CGS 21680 (100 nM) and forskolin (1 µM). 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.

The novel findings of this study are that the adenosine analogue NECA for one hour modulates the mRNA gene expression of PGC-1α in skeletal muscle cells and that such an effect by NECA is mediated by the adenosine A2B receptor subtype. Moreover, CGS 21680 for 24 hours increases mRNA gene expression of PGC-1α in the 19 hours starved skeletal muscle cells, and that such an effect by CGS 21680 are potentially mediated by the adenosine A2 receptor subtype. Furthermore in this study, the adenosine analogue NECA increases mRNA gene expression of NR4A1 and NR4A3 in skeletal muscle cells, and that such an effect by NECA is mediated by the adenosine A2B receptor subtype. To our knowledge, this is the first study on the effect of NECA and its adenosine A2B receptor subtype on PGC-1α and nuclear receptors (NR4A1 and NR4A3) in skeletal muscle, and it represents a novel signalling mechanism for the role of at least adenosine A2B receptors in skeletal muscle cells.

These current results show the followings: (I) The nonselective adenosine receptor agonist NECA (but not CGS 21680) for one hour in one week starved skeletal muscle cells increases the expression of PGC-1α, whereas NECA (but not CGS 21680) for one hour in 19 hours starved skeletal muscle cells decreases the expression of PGC-1α. (II) Selective agonists of adenosine A2A receptors CGS 21680 for 24 hours in 19 hours starved skeletal muscle cells (but not for one hour or three hours) increase the expression of PGC-1α. (III) The effects of NECA for one hour in both 19 hours and one week starved skeletal muscle cells on PGC-1α expression are blocked by a selective antagonist of the adenosine A2B receptor subtype, PSB 603. However, adenosine A2A receptors cannot be ruled out since CGS 21680 for 24 hours in 19 hours starved skeletal muscle cells increases the expression of PGC-1α, whereas NECA for same incubation time has no effect. (IV) The nonselective adenosine receptor agonist NECA increases the expression of NR4A3, whereas selective agonists of adenosine A2A receptors, CGS 21680, have no effect. This agonist is very potent and, at this concentration (100 nM), it fully activates its cognate receptors without significant activation of the adenosine A2B receptors [40]. This was the rationale for determining the effect of this agonist at a concentration of 100 nM. (V) The effects of NECA on NR4A1 and NR4A3 expression are blocked by a selective antagonist of the adenosine A2B receptor subtype, PSB 603. (V) PSB 603 for one hour up-regulates NR4A1 and NR4A3 mRNA gene expression. Collectively, these findings provide strong evidence for the role of at least the adenosine A2 receptors in modulating the expression of PGC-1α caused by NECA and/or CGS 21680 and for the role of the adenosine A2B receptors in up-regulating the expression of NR4A1 and NR4A3 caused by NECA.

Our contention that the activatory effects of NECA are mediated via adenosine A2B receptors is supported by the approved sub-classification of adenosine A2A and A2B receptors. Indeed, numerous studies have shown that the relative potencies of CGS 21680 and NECA can be used as a reference to differentiate A2A from adenosine A2B receptors. 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 physiological functions. 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.

In this current study; in the short-term (1 hour), NECA significantly modulates the expression of PGC-1α; there was a decrease in mRNA levels of PGC-1α in 19 hours starved cells, while there was an increase in mRNA levels of PGC-1α in one week starved cells. The reason behind the opposite effect of NECA is ascribed to the level of adenosine released from the cells (presumably activated the A2B receptors) or the starvation that might modulate the expression of regulatory genes, although further studies are required to test this issue. In chronic-term (24 hours), CGS 21680 (but not 100 nM NECA) increase mRNA expression of

PGC-1α. At this point, it cannot be ruled out the functional presence of adenosine A2A receptor in skeletal muscle cells. Interestingly, the effect of CGS 21680 is not reproduced by the effect of NECA, the implication of that is this effect might be modulated not only by adenosine A2 receptors, but also adenosine A1 or A3 receptors, although further investigations are required to test this issue.

Utilizing gene expression technology, it has been identified a number of genes potentially involved in lipid and glucose homeostasis, oxidative and energy metabolism, proliferation, differentiation and inflammation that respond to adenosine A2B receptors signaling. Accordingly, these studies support a role for the cross-talk between NR4A and adenosine A2B receptors signaling and another cross-talk between PGC-1α and adenosine A2 receptors signalling in skeletal muscle. This current investigation might potentially associate impaired adenosine A2 receptor response with modulated expression of genes that are critical regulators of glucose and lipid metabolism, proliferation, differentiation and inflammation in skeletal muscle. Consequently, it is possible that this association lead to whole-body metabolism and/or inflammation.

Even though cAMP elevation, PGC-1α and NR4A mRNA expression was increased in skeletal muscle in response to NECA in adenosine A2B receptors dependent manner (not adenosine A2A receptors). Surprising, selective adenosine A2A receptors CGS 21680 activate the mRNA expression of PGC-1α.Taken togather, it is highly possible that at least both adenosine A2A receptors and A2B receptors are involved also in regulation of metabolic genes (glucose and fatty acid metabolism (Randle cycle)) related to PGC-1α and NR4A.

It is well known that the NECA effect in **Fig. 3** is made on skeletal muscle cells that are starved for one week, and this is not physiologically relevant. However, the idea behind investigating such a condition is that starvation might change the rate of RNA synthesis for PGC-1α or the expression level of adenosine receptors [41, 42]. Moreover, it should be highlighted that the 3 hours and 24 hours NECA stimulations did not work, but the 1 hour did on 19 hour-starved cells (**Fig. 4).** The reason behind that the concentrations of NECA do not explain the disparate effects of various conditions, duration of exposure may be a pivotal factor. The demonstration of adenosine A2B receptors expression level/density in skeletal muscle cells might fulfill a necessary condition for the specific action of an adenosine A2B receptors activator to alter expression of adenosine A2B receptor–responsive genes.

In the present study, the potential role of the cAMP pathway is indicated in the NECA-induced mRNA gene expression of PGC-1α, NR4A1 and NR4A3 in skeletal muscle (**Fig. 3** and **5**). Several findings have supported the role of the cAMP pathway. (I) In previous studies, NECA increases cAMP accumulation in skeletal muscle cells [15, 16] and also, in the present study, NCEA increases the mRNA gene expressions of PGC-1α, NR4A1 and NR4A3, whereas the selective antagonists to adenosine A2B receptors PSB 603 block such effects by NECA. (II) In previous studies, the adenylyl cyclase activator forskolin increases cAMP accumulation [16] and in the present studies forskolin increases the mRNA gene expressions of NR4A. These data suggest that adenosine A2B receptors mediate PGC-1α, NR4A1 and NR4A3 mRNA gene expression potentially through a cAMP-dependent mechanism in rat skeletal muscle cells, although my future studies are recommended to validate and investigate the exact signaling mechanism.

Both adenosine A2 receptors are coupled to stimulatory Gs proteins, which leads to the production of cAMP [43]. In the previous study, the selective adenosine A2A receptors agonist CGS 21680 fails to stimulate cAMP production in skeletal muscle cells [16] and, in the present study, CGS 21680 fails to stimulate NR4A mRNA gene expression. On the other hand, the stimulation of skeletal muscle cells with NECA has been shown to elevate cAMP levels in a previous study [16]. As cAMP in turn has downstream signalling on PGC-1α and NR4A in skeletal muscle [24, 44], and adenosine A2B receptors are positively coupled with adenylyl cyclase and their activation results in a significant increase in cAMP levels in a previous study [16], then the activatory effects of NECA on PGC-1α, NR4A1 and NR4A3 mRNA gene expression in skeletal muscle cells are most likely

mediated largely via the second messenger cAMP. Moreover, in the present study, compared with CGS 21680, NECA is effective in terms of mimicking the activatory effects of forskolin for PGC-1α, NR4A1 and NR4A3 mRNA gene expression, which further substantiates our conclusion that the activatory effects of NECA are mediated via adenosine A2B receptors. On the other hand, using 19 hours starved cells, NECA for one hours inhibits PGC-1α and CGS 21680 for 24 hours activates the PGC-1α mRNA expression. However, forskolin does not alter the PGC-1α. Therefore, it is highly possible that adenosine A2B receptors and A2 receptors couples Gq-protein, although further studies are recommended.

Previous pharmacological experiments on cultured muscle cells have shown that the activation of the adenosine A2B receptor, but not the adenosine A1 and A2A receptors, modulates intracellular cAMP levels [15, 16]. In agreement with the cAMP assay in the previous skeletal muscle studies [15, 16], activation of the adenosine A2B receptor induces a strong induction of the NR4A in rat L6 skeletal muscle cells. This observation is in agreement with studies on smooth muscle cells [45]. In several different tissues, activation of NR4A has been found to be associated with an increased gene expression of several metabolic genes [46] and specifically in skeletal muscle; NR4A activation has been shown to be involved in muscle development and growth, fatty acid oxidation and glucose metabolism [38, 47]. It is thus likely that NECA has at least the above-mentioned effects in skeletal muscle and that these effects are possibly mediated via activation of adenosine A2B receptors.

The activatory effect of PSB 603 on basal levels of NR4A1 and NR4A3 mRNA gene expression could be interpreted to mean that the concentration used for PSB 603 is possibly able to activate other targets or it is possible that PSB 603 might act as an allosteric modulator for adenosine A2B receptors. It is interesting to note that PSB 603 might act as an allosteric modulator for adenosine A2B receptors in case of treatment the 19 hours starved skeletal muscle cells with PSB 603 and NECA (10 µM but not 100 nM) (cells were pre-treated with PSB 603 for 10 minutes prior to the addition of NECA) for 24 hours (but not 1 or 3 hours). In this situation, it seems that cAMP is not involved in the (NECA 10 µM and PSB 603)-induces NR4A3 expression as forskolin does not induce that. However, whether or not Gq-signalling or other downstream targets is involved in the regulation of (NECA for one hour and CGS 21680 for 24 hours)-induces IL-6 expression in 19 hours starved skeletal muscle cells needs to be investigated further. For example in the literature, the adenosine A2B receptors driven production of these pro-inflammatory molecules has been attributed to both Gs and Gq pathways [48, 49].

The current data in this study cannot rule out the potential role of adenosine A2B receptors in regulation of mRNA expression of NR4A2 in skeletal muscle cells. A2B adenosine receptor activation is found to increase NR4A1 and NR4A3 expression in skeletal muscle in this study; it is possible, therefore, that A2B receptors might at least modulate fat and glucose metabolism in skeletal muscle tissue. This is supported by several studies that indicated the potential role of NR4A in insulin resistance, glucose clearance and fatty acid metabolism in normal and diabetic models [26, 36, 46, 47, 50]. Taken these studies into consideration, the modulation of the A2B adenosine receptor by ligands might affect glucose and fatty acid utilization in skeletal muscle. Adenosine receptors, in particular adenosine A2B receptors, have been previously demonstrated to be regulators of various cellular responses involved in glucose homeostasis and obesity [51, 52] and inflammation (data not published yet).

As adenosine A2B receptors increase the mRNA gene expression of NR4A in skeletal muscle cells in this study, and NR4A, in particular NR4A1, modulates inflammation, insulin action and glucose transport [26, 50], it is possible that adenosine A2B receptor modulation plays a potential therapeutic role in metabolic diseases such as diabetes. Therefore, the implication of this is that it is recommended that A2B adenosine receptor agonists/antagonists be investigated as a therapeutic option in inflammatory disorders, diabetes or obesity.

Analysis of skeletal muscle PGC-1α reveals dysregulation of PGC-1α expression in skeletal muscle cells in response to modulation of at least

A2 receptors. And, this modulation of PGC-1α in skeletal muscle might be linked to the changes of inflammatory and metabolic genes. Of further interest in this study, it is found that CGS 21680 activates PGC-1α. Therefore, PGC-1α might be modulated by a bidirectional regulation link between adenosine A2A and A2B receptors signaling. Taken these data implicate a central role of adenosine receptor, in particular adenosine A2 receptors, in regulating the PGC-1α in skeletal muscle. This study further supports a strong association between the signaling of adenosine A2 receptors and the metabolic effect in skeletal muscle. Furthermore, it is shown in the current study that NECA induces PGC-1α. This is in agreement with previous results obtained in skeletal muscle in which Metformin, Pioglitazone and Clenbuterol induced PGC-1α [44, 53, 54]

As an important controller of energy homeostasis [55], PGC-1α expression in the in the current study is highly regulated at the transcriptional level in skeletal muscle in response to adenosine stable analogue. These novel findings are indicative of the fact that small changes in the signaling pathway important for energy and oxidative metabolism can have potent consequences overtime, and suggest that modest effects on PGC-1α activity can be crucial for human health. Understanding the signals that enable to modify PGC-1α may lead to ways to enhance PGC-1α and intervene in such diseases.

PGC-1α is a powerful regulator of energy metabolism in key tissues including skeletal muscle [55]. In skeletal muscle, PGC-1α has been suppressed the inflammatory cytokines such as IL-6 [56] suggesting interlink between inflammation and metabolism. Growing evidence supports the importance of inflammation in the metabolic disease [57]. In this study, adenosine A2 receptors activation modulates PGC-1α. Therefore, it is possible that adenosine A2 receptors modulate a link between inflammation and metabolism. This is supported by the previous study which indicates the adenosine A2B mediates NECA-induce IL-6 (data not shown).

When this present study is considered in parallel with the role of adenosine A2B receptors in mediating inflammation in skeletal muscle (data not shown), 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 might modulate skeletal muscle fatty acid transport and oxidation, glycolysis, insulin resistance/sensitivity, energy and oxidative metabolism. The differential regulation of transcription factors (NR4A1 and NR4A3) and co-activators (PGC-1α) involved in glucose and fat utilization in skeletal muscle during adenosine exposure may represent an important component of the molecular basis of the development of insulin resistance in metabolic conditions and diseases characterized by impaired lipid and glucose metabolism.

PGC-1α is a strong activator of mitochondrial biogenesis and oxidative metabolism [58, 59]. PGC-1α is a prominent regulator of myocellular energy metabolism orchestrating gene transcription programming in response to numerous environmental stimuli [60]. Moreover, it is widely acknowledged that mitochondrial metabolism (primary metabolic target of PGC-1α) disturbances are widely acknowledged contributors to type 2 diabetes development [61]. PGC-1α plays important roles in many physiological processes in skeletal muscle [62]. Moreover, the mRNA levels of PGC-1α were found be decreased in skeletal muscle from type 2 diabetics [63]. In the current study, the changes in PGC-1α levels might serve as a causal factor or as a mediator of the corresponding pathophysiologic processes. Therefore, modulation of adenosine A2 receptors might be a potential target for medical intervention.

CONCLUSIONS: In conclusion, these findings demonstrate that NECA-modulated the mRNA expression of PGC-1α through at least adenosine A2B receptors although there is some evidence for the role of adenosine A2A receptors in regulation of skeletal muscle response.

Therapeutic modulation of PGC-1α has a huge clinical potential for skeletal muscle disorders, and its related metabolic diseases. These findings in the current study strongly provide a selective targeting

of PGC-1α by modulation of adenosine A2 receptors. These findings also demonstrate that NECA increases the mRNA expression of NR4A mostly via the Gs-adenosine A2B receptor signalling pathway. Moreover, PSB 603 up-regulates the mRNA expression of NR4A1. The adenosine A2B receptors are functionally expressed in the skeletal muscle and signals through the cAMP-NR4A pathway. No evidence for a role of the adenosine A2A receptors in regulation of the skeletal muscle response is found. Thus, adenosine A2B receptor agonists/ antagonists/inverse agonists may be a potential agent for functional activities in skeletal muscle cells. Indeed, the adenosine A2B receptors are potentially of considerable interest as a new drug target for the treatment of metabolic and inflammatory diseases.

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