

The Impact of Adenosine A2B Receptors Modulation on Nuclear Receptors (NR4A) Gene Expression

MANSOUR HADDAD*

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

*Corresponding author E-mail: Dr.man.haddad@gmail.com

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ABSTRACT

Adenosine is a ubiquitous signalling molecule, of which the majority of the effects are mediated by interaction with its four G-protein-coupled receptors (A1, A2A, A2B and A3) ¹. It has been proposed that adenosine modulates different functional activities in skeletal muscle cells. Previous studies have demonstrated that the activation of adenosine A2B receptors increases adenosine 3'-cyclic monophosphate (cAMP) accumulation in rat skeletal muscle cells ², which is probably an important yet unknown mechanism contributing to the regulation of skeletal muscle functions. Using rat L6 skeletal muscle cells, I elucidate further potential molecular signalling responsible for adenosine A2B receptor modulations. Skeletal muscle cells are commonly employed cells, and have been previously described as expressing adenosine receptors ³. Quantitative real-time PCR assays (probe-based) are used to evaluate the gene expression profiles of adenosine A2B receptor signalling. In this study, I show that adenosine A2B receptors are functionally expressed in skeletal muscle cells, as indicated by the fact that the activation of adenosine A2B receptors increases the NR4A mRNA gene expression level using qRT-PCR. Previous studies have shown that the nonselective adenosine A2B receptor agonist NECA, but not the adenosine A2A receptor selective agonist CGS 21680, induces cAMP accumulations ². The effect of NECA is blocked by the selective antagonist of adenosine A2B receptors, PSB 603 ². In the current study, NECA (10 μ M) increases NR4A1, NR4A2 and NR4A3 mRNA gene expression significantly (a 5.9-, 2.9- and 2.4-fold change to vehicle, respectively), which are blocked by a selective adenosine A2B receptor antagonist, PSB 603 (100 nM). This current study identifies the adenosine A2B receptors as a significant regulator of NR4A mRNA gene expression in skeletal muscle, thereby pointing to its therapeutic potential. In summary, I observe the selective, potentially functional expression of adenosine A2B receptors in skeletal muscle cells. Whether adenosine A2B receptor-mediated functional responses play a role in skeletal muscle pathophysiology is yet to be elucidated.

Key words: Adenosine A2B receptors, NECA, PSB603, CGS 21680, NR4A, skeletal muscle cells.

INTRODUCTION

Adenosine has been demonstrated to regulate many physiological functions of the cardiovascular system ⁴ and of most tissues, including skeletal muscle ⁵. 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 ⁵. The cellular activation of these adenosine receptors, in particular the activation of adenosine A2B receptors, in skeletal muscle is,

however, not known. Adenosine receptors belonging to the superfamily of G-protein coupled receptors have been classified into A1, A2A, A2B and A3 and are involved in the regulation of adenylate cyclase ¹. Adenosine A1 and A3 receptors inhibit cAMP production via Gi, whereas adenosine A2A and A2B receptors stimulate it via Gs ⁶. Adenosine receptors are expressed in various tissues and cell types, including skeletal muscle ³⁻⁷. Moreover, adenosine A2B receptors are found to increase cAMP concentration in skeletal muscle myotubes ^{2,8}.

One of the possible downstream signalling targets of cAMP in skeletal muscle cells is NR4A⁹. NR4A is a subfamily of the orphan nuclear receptor (NR) superfamily, which consists of three members, namely Nur77 (NR4A1), Nurr1 (NR4A2) and NOR1 (NR4A3)¹⁰. 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 differentiation¹¹⁻¹⁶. These studies have focused on NR4A mainly in the liver, adipose and skeletal muscle¹⁷⁻²⁰.

There is growing evidence to suggest that the activation of NR4A leads to an increase in the gene expression of intracellular downstream signalling pathways that, it has been suggested, participate in the regulation of glucose and fatty acid metabolism and cell growth in skeletal muscle^{9,13,21-23}. Cross-talk between adenosine A2B receptors and 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 NR4A mRNA gene expression and the potential involvement of the cAMP pathway in adenosine A2B receptor modulation.

Abbreviations

cAMP: Cyclic-adenosine monophosphate; cDNA: Complementary DNA; mRNA: messenger ribonucleic acid; PCR: Polymerase chain reaction; NECA: 5'-N-ethylcarboxamidoadenosine; PSB603: 8-[4-[4-(4-chlorophenyl) piperazine-1-sulfonyl] phenyl]-1-propylxanthine; CGS21680: 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-5' -N-ethyluronamide; DMSO: Dimethyl sulphoxide; P-value: Probability; NR4A: Nuclear Receptor Subfamily 4, Group A.

MATERIALS AND METHODS

NECA, forskolin, PSB603 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% CO₂. 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 cm² flasks were cultured for a further 14 days (to allow myotube formation), according to the protocol mentioned in²⁴ with slight modifications (Figure 2). 70%-90% confluent myotubes (approximately 2 weeks in culture) were serum-starved (incubated in Ham-F 10 medium alone) for 7 days. Then, cells (Figure 2) were treated for 1 hour with vehicle (0.1% DMSO), NECA 10 µM, PSB 603 100 nM, and 1µM Forskolin 100nM, 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) (2mL per flask).

RNA extraction and cDNA synthesis

Rat L6 skeletal muscle cells (in 25cm² 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 μ L 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 μ L (13 μ L Maxima Probe qPCR Master Mix 2X reagent, 1.5 μ L forward primer (10 μ M), 1.5 μ L reverse primer (10 mM), 2.5 μ L Probe (2 μ M), 5 μ L water, and 5 μ L cDNA). The qRT-PCR analysis was performed using a 7500 fast 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 synthesized 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%.

Data analysis

Data are expressed as means \pm 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 levels was set at $P < 0.05$.

Table 1: List of gene primer and probe sequences

Gene	Sequences (5' \rightarrow 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'- GACGCAACGCCAGAGAC -3' Reverse primer 5'- TAGAACTGCTGCACGTGCTCA -3'	92
TATA-BOX	Probe 5'- TCCCAAGCGTTTGTCTGCAGTCA -3' Forward Primer 5'- TTCGTGCCAGAAATGCTGAA-3' Reverse Primer 5'- GTTCGTGGCTCTTATTCTCATG -3'	73

RESULTS

NECA stimulates NR4A mRNA expression in skeletal muscle cells

To assess whether stimulation of adenosine A2 receptors could induce NR4A mRNA gene expression in rat L6 skeletal muscle cells, the effects of NECA were relatively quantified using a non-selective adenosine receptor agonist on NR4A mRNA gene expression by qRT-PCR (probe-based). Starved skeletal muscle cells were incubated with NECA (10 μ M) for one hour, and mRNA gene expression of NR4A was subsequently quantified.

Incubation of L6 skeletal muscle cells with 10 μ M of the non-selective adenosine analogue NECA increases ($p < 0.05$) mRNA gene expression of NR4A1, NR4A2, and NR4A3 (5.9-, 2.9-, and 2.5-fold change compared to vehicle, respectively) (Figure 3).

Adenosine A2B receptors mediates NECA-induced NR4A mRNA gene expression in skeletal muscle cells

To determine which subtype of adenosine A2 receptors mediate the increase NR4A mRNA gene expression level, the adenosine receptor

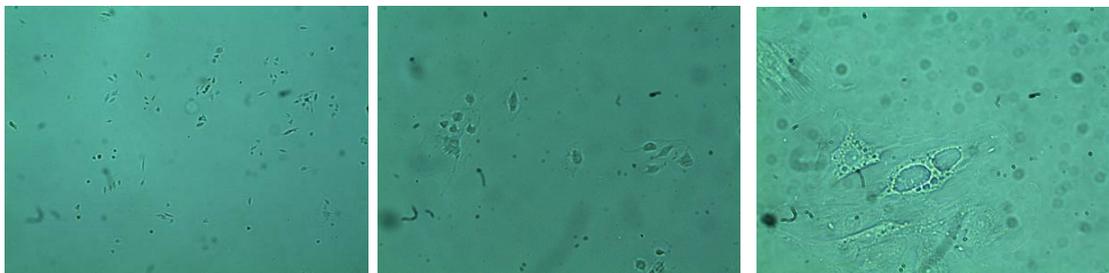
agonist CGS21680 (subtype A_{2A} selective) was used. The concentration applied for CGS 21680 could selectively activate the indicated subtype ($K_i = 27$ nM)²⁵.

Furthermore, since no selective agonist exists for adenosine A_{2B} receptors, the effect of a selective antagonist to the adenosine A_{2B} receptors (PSB 603) was investigated. PSB 603 exhibits a strong affinity to adenosine A_{2B} receptors and very weak affinity to three other adenosine receptors subtypes. Adenosine A_{2B} receptors display >17000-fold selectivity over other adenosine receptors (K_i values: 0.553, > 10000, > 10000, and > 10000 nM for A_{2B}, A₁, A_{2A}, and A₃ receptors, respectively)²⁶.

As shown in Figure 3, NECA (10 μ M) increases the mRNA gene expression level of

NR4A significantly ($P < 0.05$). In contrast, the adenosine A_{2A} receptor selective agonist CGS21680 (100 nM) does not cause a significant increase in the mRNA gene expression level of NR4A.

To investigate the effect of a selective adenosine A_{2B} receptor antagonist, PSB 603, rat L6 skeletal muscle cells were incubated with 10 μ M NECA for one hour in combination with PSB 603 (which was added to cells 10 minutes prior to adding NECA) in concentrations of 100 nM and 1 μ M, which blocks all NR4A mRNA gene expression of NR4A1, 2, and 3 significantly ($P < 0.05$) at a concentration equal to 100 nM (see Figure 3). It is interesting to note that PSB 603 at a concentration of 1 μ M does not block NECA-induced NR4A3 mRNA gene expression. However, it blocks the effect



**Fig. 1: Representative myoblast derived from L6 skeletal muscle (passage number 4).
A) Myoblast taken during the first three days of tissue culture (4x magnification),
B) Myoblast taken during the first three days of tissue culture (10x magnification),
and C) Myoblast taken during the first three days of tissue culture (40x magnification)**

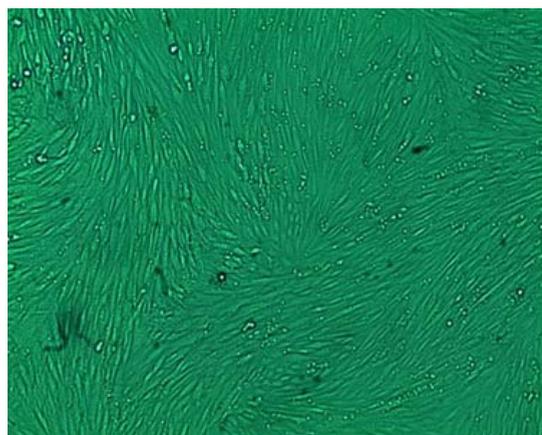


Fig. 2: Representative myotubes derived from passage number 5 (at 14 days of tissue culture)

of NECA on NR4A1 and NR4A2 mRNA gene expression at the same concentration.

Collectively, these results indicate that adenosine A_{2B} receptors are the functionally expressed receptors of adenosine A₂ receptors in skeletal muscle, whereas no functional expression of the adenosine A_{2A} receptors was detected using mRNA gene expression levels for NR4A as a functional readout.

The adenosine A_{2B} receptors inverse agonists, PSB 603 (at 100 nM and 1 μ M), mediates a significant decrease in baseline NR4A1 mRNA gene expression levels in skeletal muscle cells ($P < 0.05$; -2.3-fold change compared to vehicle). The inverse agonists, PSB-603, have no effect on

baseline NR4A2 and NR4A3 mRNA gene expression at either of the concentrations assessed (100 nM and 1 μ M) (Figure 3).

activation of adenosine A2B receptors in skeletal muscle by NECA increases the mRNA gene expression of NR4A (see above section).

Role of cAMP pathway in the expression of NR4A

In previous studies, activation of adenosine A2B receptors in skeletal muscle by NECA increased cAMP accumulation ^{2 8}, and

Experiments were conducted to investigate if the adenylyl cyclase pathway is involved in the activation of NR4A transcription profile in skeletal muscle cells. For this purpose,

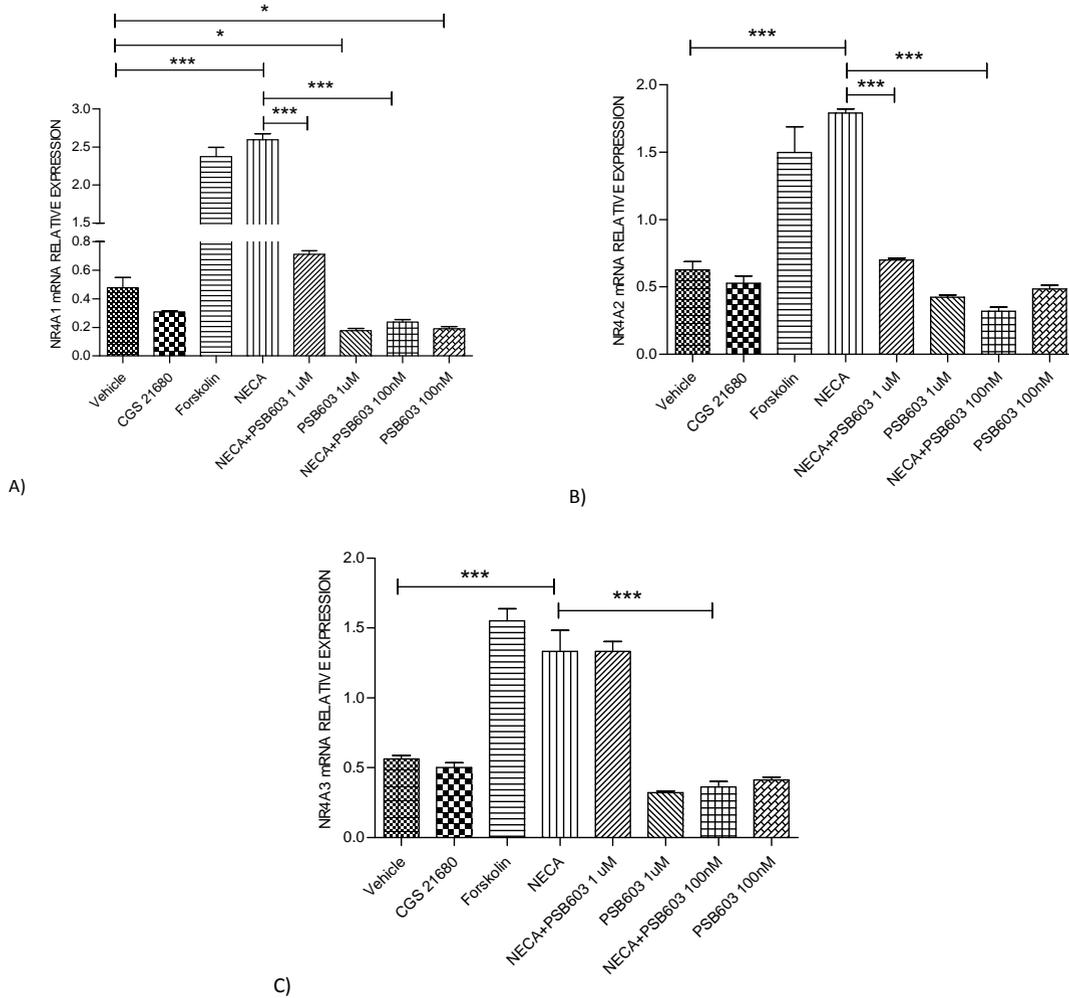


Fig. 3: Effects of NECA, CGS21680, and PSB 603 on NR4A1, NR4A2, and NR4A3 mRNA gene expression in rat L6 skeletal muscle myotubes using charcoal serum. Rat L6 skeletal muscle myotubes (70-90% confluent) were serum starved for 7 days and then stimulated for 1 hour. NR4A1 mRNA levels were measured relative to TATA-Box using real-time quantitative PCR (qRT-PCR); Stimulation was performed with vehicle (0.1% DMSO), NECA (10 μ M), PSB 603 (100nM and 1 μ M), CGS21680 (100 nM), and Forskolin (100 nM). A) mRNA relative expression of NR4A1, B) mRNA relative expression of NR4A2, and C) mRNA relative expression of NR4A3. Data were represented as means \pm 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

the adenylyl cyclase activator Forskolin was used in the present study to understand the potential role of the cAMP pathway in NECA-induced NR4A mRNA gene expression in skeletal muscle.

Forskolin (100 nM) increases the expression of NR4A significantly (an average 3.8-fold change compared to vehicle) (Figure 1), a result similar to that of NECA. These findings support the idea that the cAMP pathway plays an important role in NECA-induced NR4A mRNA gene expression.

DISCUSSION

The novel findings of this study are that the adenosine analogue NECA increases the mRNA gene expression of NR4A1, NR4A2 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 nuclear receptors (NR4A) in skeletal muscle, and it represents a novel signalling mechanism for the role of adenosine A2B receptors in skeletal muscle cells.

Our results show the following. (I) The nonselective adenosine receptor agonist NECA increases the expression of NR4A, 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 receptors²⁵. This was the rationale for determining the effect of this agonist at a concentration of 100 nM. (II) The effects of NECA on NR4A expression are blocked by a selective antagonist of the adenosine A2B receptor subtype, PSB 603. Collectively, these findings provide strong evidence for the role of the adenosine A2B receptors in up-regulating the expression of NR4A caused by NECA. (III) PSB 603 down-regulates NR4A1 mRNA gene expression.

Our contention that the activatory effects of NECA are mediated via adenosine A2B receptors is supported by the approved subclassification of adenosine A2A and A2B receptors. Indeed, numerous studies have shown that the relative potencies of CGS21680 and NECA can be used as

a reference to differentiate A2A from adenosine A2B receptors.

In the present study, the potential role of the cAMP pathway is indicated in the NECA-induced mRNA gene expression of NR4A in skeletal muscle. Several findings have supported the role of the cAMP pathway. (I) In previous studies, NECA increases cAMP accumulation in skeletal muscle cells^{2,8} and also, in the present study, NECA increases the mRNA gene expressions of NR4A, 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 accumulation² and in the present studies forskolin increases the mRNA gene expressions of NR4A.

Both adenosine A2 receptors are coupled to stimulatory Gs proteins, which leads to the production of cAMP²⁷. However, in the previous study, the selective adenosine A2A receptors agonist CGS21680 fails to stimulate cAMP production in skeletal muscle cells² and, in the present study, CGS21680 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 study². As cAMP in turn has downstream signalling on NR4A in skeletal muscle⁹, and adenosine A2B receptors are positively coupled with adenylyl cyclase and their activation results in a significant increase in cAMP levels in a previous study², then the activatory effects of NECA on NR4A mRNA gene expression in skeletal muscle cells are most likely mediated largely via the second messenger cAMP. Moreover, in the present study, compared with CGS21680, NECA is effective in terms of mimicking the activatory effects of forskolin for NR4A mRNA gene expression, which further substantiates our conclusion that the activatory effects of NECA are mediated via adenosine A2B receptors.

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^{2,8}. In agreement with the cAMP assay in the previous skeletal muscle studies^{2,8}, 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 cells²⁸. In several different tissues, activation of NR4A has been found to be associated with an increased gene expression of several metabolic genes²⁹ and specifically in skeletal muscle; NR4A activation has been shown to be involved in muscle development and growth, fatty acid oxidation and glucose metabolism^{23,30}. 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.

Adenosine receptors, in particular adenosine A2B receptors, have been previously demonstrated to be regulators of various cellular responses involved in glucose homeostasis and obesity³¹⁻³². 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^{11,33}, it is possible that adenosine A2B receptor modulation plays a potential therapeutic role in metabolic diseases such as diabetes.

Recently, I have demonstrated that NR4A3 mRNA gene expression to NECA could not be antagonized by the adenosine A2B receptor selective antagonist PSB 603 (1 μ M). In contrast, NR4A3 mRNA gene expression to NECA is fully sensitive to antagonism by PSB 603 (100 nM). Indeed, the concentration ratio between the NECA (10 μ M) and PSB 603 (1 μ M) is an important issue to consider in this case. These data suggest that PSB 603 may have an allosteric mechanism of action or the concentration of PSB 603 may give a response as a ligand through other receptors. Interestingly, NECA evokes a significant elevation of NR4A1 mRNA gene expression, an effect blocked by PSB 603. Furthermore, the inhibitory effect of PSB 603 on basal levels of NR4A mRNA gene expression could be interpreted to mean that adenosine A2B receptors in this tissue exhibit constitutive activity. PSB 603 has been reported to be an inverse agonist in 22Rv1 prostate cancer cells expressing constitutively activated human adenosine A2B receptors in which the suppression of cAMP production in these cells is reversed by PSB 603³⁴. However, there is nothing in

the literature that has reported data about constitutively activated rat adenosine A2B receptors.

A2B adenosine receptor activation is found to increase NR4A expression in skeletal muscle in this study; it is possible, therefore, that A2B receptors might modulate fat and glucose metabolism in skeletal muscle tissue. This is supported by the fact that I) NR4A has been shown to be reduced in the skeletal muscle of diabetic animals¹¹, II) NR4A is associated with genes related to glucose and fatty acid utilization through up-regulating the mRNA expression of PDK4, FOXO1, PGC-1 α and lipin-1 α ²¹, III) NR4A null mice after high-fat feeding compared with wild-type animals have been shown to exhibit decreased mRNA expression of GLUT4 and PDK4 and Lipin 1 α and impaired insulin receptor substrate 1 (IRS-1) phosphorylation and insulin resistance in skeletal muscle, and slower blood glucose clearance, increased body weight and decreased energy usage³³, IV) in C2C12 cells, C2C12 siRNA-NR4A cells have been shown to decrease the mRNA expression of fatty acid translocase (CD36/fat), uncoupling protein-3 (UCP3) and GLUT4 compared to wild-type native C2C12 cells³⁵ and V) in C2C12 cells transfected with adenovirus-mediated NR4A expression, non-insulin glucose uptake have been shown to increase significantly compared to in normal C2C12 cells³⁰. Taken together, the modulation of the A2B adenosine receptor by ligands might affect glucose and fatty acid utilization in skeletal muscle. Therefore, the implication of this is that it is recommended that A2B adenosine receptor agonists/antagonists be investigated as a therapeutic option in diabetes or obesity.

CONCLUSIONS

These findings demonstrate that NECA increases the mRNA expression of NR4A mostly via the Gs-adenosine A2B receptor signalling pathway. Moreover, PSB 603 down-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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