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Adenosine A_1 receptors do not play a major role in the regulation of lipogenic gene expression in hepatocytes

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ABSTRACT

Activation of adenosine A_1 receptors was reported to promote fatty acid synthesis in AML-12 cells, by increasing the expression of SREBP-$_{1c}$ (sterol regulatory binding protein 1c) and FAS (fatty acid synthase). Since these findings have important therapeutic implications for the discovery of adenosine A_1 receptor agonists, further studies were undertaken to determine the expression and functional relevance of adenosine A_1 receptor in the liver. To that end, we used two classes of distinct adenosine A_1 receptor agonists: CPA (N^6-cyclopentyladenosine), a full agonist and GS-9667 (2-{6-[((1R,2R)-2-hydroxycyclopentyl)-amino]purin-9-yl}(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]-oxolane-3,4-diol), a partial agonist. Treatment of AML-12 cells, HepG2 cells and primary human hepatocytes with either CPA or GS-9667 did not increase the gene expression of SREBP-$_{1c}$ or FAS. Furthermore, in AML-12 and HepG2 cells, CPA did not antagonize forskolin-stimulated cAMP production, a characteristic of adenosine A_1 receptor activation, indicating that these cells lack adenosine A_1 receptor function. Consistent with this finding, adenosine A_1 receptor gene expression was found to be very low and adenosine A_1 receptor protein levels were hardly detectable by radioligand binding assays in hepatic cell lines such as AML-12 and HepG2 as well as in both mouse and human liver tissues. Finally, acute treatment with adenosine A_1 receptor agonist GS-9667 had no significant effect on gene expression of both SREBP-$_{1c}$ and FAS in livers of Sprague Dawley rats. Taken together, our data suggest that the expression of adenosine A_1 receptor is too low to play a major role in the regulation of lipogenic gene expression in hepatocytes.

1. Introduction

Adenosine is a nucleoside generated from tissues and cells including hepatocytes, adipocytes, and neural cells. It regulates a variety of physiological processes through interactions with cell-surface G protein-coupled adenosine receptors of which there are four known subtypes: A_1, A_{2A}, A_{2B}, and A_3. Adenosine A_1 receptors are highly expressed in brain, heart and adipose tissue (Morey et al., 1998; Reppert et al., 1991; Saggerson and Jamal, 1990; Weber et al., 1990; Zhang et al., 1997). Activation of adenosine A_1 receptor in adipose tissue is known to have anti-lipolytic effects resulting in reduction of free fatty acid levels in the circulation, which is suggested to have beneficial effects in the treatment of dyslipidemia and type II diabetes (Dhalla et al., 2009a; Dong et al., 2001; Schoelch et al., 2004).

Recently it was reported that activation of adenosine A_1 receptor mediates activation of transcription factor sterol regulatory element-binding protein 1c (SREBP-$_{1c}$), resulting in enhanced expression of lipogenic genes such as fatty acid synthase (FAS) and promoting fatty acid synthesis, leading to accumulation of intracellular lipids in AML-12 cells, a mouse hepatic cell line (Peng et al., 2009). The authors proposed that this function of adenosine A_1 receptors contributes to the ethanol-induced up-regulation of lipogenic gene expression and fatty liver in the *in vivo* mouse model. However, a large body of evidence accumulated to date has also demonstrated that chronic treatment of ethanol can increase hepatic lipogenesis both *in vitro* and *in vivo* through many other signaling pathways as well (Crabb and Liangpunsakul, 2006; Purohit et al., 2009; Shklyaev et al., 2003; Yamauchi et al., 2007; You and Crabb, 2004; Zhou et al., 2001). Therefore, it cannot be excluded that the increase of hepatic lipogenic gene expression in *in vivo* ethanol model is a result of activation of multiple signaling pathways in addition to the activation of adenosine receptors. Furthermore, several previous studies demonstrated that adenosine A_1 receptors are poorly expressed in liver (Dixon et al., 1996; Mahan et al., 1991; Reppert et al., 1991), suggesting only a minor role, if at all, in the regulation in hepatic lipogenic processes by adenosine A_1 receptors.

GS-9667 (previously CVT-3619) is a selective and partial adenosine A_1 receptor agonist (A_1 agonist), which activates adenosine A_1 receptors with partial efficacy relative to the full agonist CPA (Fatholahi et al., 2006). GS-9667 is currently under investigation as an anti-lipolytic agent for the potential treatment of dyslipidemia, insulin resistance and diabetes (Dhalla et al., 2007, 2009b). The therapeutic potential of GS-9667 and other A_1 agonists would be greatly reduced if activation of adenosine A_1 receptors would induce hepatic

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lipogenesis as described by Peng et al. (2009). Therefore, the goal of this study was to determine whether partial A_1 agonist GS-9667 affects hepatic lipogenic gene expression *in vitro* and *in vivo*. We also examined adenosine A_1 receptor expression in hepatocytes and liver tissues. Our results provide evidence to conclude that A_1 agonists do not contribute notably to the regulation of hepatic lipogenic gene expression.

2. Materials and methods

2.1. Materials

DPCPX (1,3-dipropyl-8-cyclopentylxanthine), R-(−)-PIA [(−)-N^6-(2-phenylisopropyl)-adenosine], CPA and fatty-acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]-DPCPX (specific activity: 120 Ci/mmol) was purchased from Perkin Elmer (Boston, MA). G-418 (Geneticin) was purchased from Invitrogen (Carlsbad, CA). cAMP assay kit was purchased from DiscoveRx (Fremont, CA). TO901317 was purchased from Cayman Chemical (Ann Arbor, MI). GS-9667 was synthesized at Gilead Sciences, Inc. All other reagents were obtained from Sigma-Aldrich or VWR (Brisbane, CA). Mice (7–10 weeks old) were purchased from Jackson Laboratory. Human liver samples were from 35 to 50 year old patients without metabolic diseases and obtained from the National Disease Research Interchange (NDRI). We acknowledge use of human samples procured by the NDRI approved protocol # 704541 under review board with support from NIH grant 5 U42 RR006042. We confirm that consent for research donation was obtained from each donor before proceeding.

2.2. Cell culture

AML-12, HepG2, Huh7, Hep3B and CHO (Chinese hamster ovary) cells were obtained from The American Type Culture Collection (ATCC, Manassas, VA). AML-12 cells (passage 2–8 times) were maintained in DMEM-Ham's F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU penicillin, 50 μg/ml streptomycin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone. Three human hepatic cell lines, HepG2 (passage 4–10 times), Huh7 and Hep3B cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU penicillin, 50 μg streptomycin. CHO cells stably expressing the human adenosine A_1 receptor (referred to as CHO-A_1) were maintained in F12K medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU penicillin, 50 μg/ml streptomycin and 0.5 mg/ml G-418 (Yang et al., 2007). All cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator and split according to the provider's recommendation. Primary human hepatocytes from 52 to 58 year old Caucasian donors were provided by Invitrogen. All donors were non-smokers and did not drink alcohol regularly. Fresh suspension of human hepatocytes were seeded on collagen I-coated 6-well plates at 0.3×10^6 density in William E medium supplemented with a thawing/plating cocktail (5% FBS, 1 μM Dexamethasone, 1% penicillin/streptomycin, 4 μg/ml human recombinant insulin, 2 mM GlutaMAX™, and 15 mM HEPES) (Invitrogen) and incubated for 3 days at 37°C in a humidified 5% CO_2/95% air incubator before the experiments.

2.3. Membrane preparation

Tissues were collected and snap frozen immediately at −80 °C until use. Tissues were minced several times in a 10 mM HEPES, 1 mM EDTA, pH 7.4 buffer containing protease inhibitor cocktail, and homogenized for 1 min using an Ultra-Turrax IKA T18 (IKA Works, Inc., Wilmington, NC). Membrane containing supernatants were filtered through 4 layers of gauze and collected in tubes on ice. Cultured cells in 150×25 mm dishes were washed once with PBS and then detached by scraping into HEPES-EDTA buffer and then homogenized for 1 min. The membranes of tissues or cells were isolated by centrifugation at 40,000×*g* for 30 min at 4°C. Membrane pellets were resuspended in 10 mM HEPES, 1 mM EDTA, pH 7.4 buffer with protease inhibitor cocktail and incubated at 37 °C with 2 U/ml adenosine deaminase (Roche Molecular Biochemicals, Nutley, NJ) for 30 min to eliminate endogenous adenosine. Membranes were further washed by centrifugation at 40,000×*g* for 30 min at 4°C. The crude membrane preparations were aliquoted and stored at −80°C. The protein concentration was measured by BioRad *DC* protein assay with BSA as standard.

2.4. Radioligand binding assay

Adenosine A_1 receptor saturation binding assays were carried out using membranes (at 80 μg/sample) and 8 different concentrations of [^{3}H]-DPCPX incubated at 21°C for 90 min in 150 μl of 10 mM HEPES, 1 mM EDTA buffer (pH 7.4) containing 1 U/ml adenosine deaminase. Non-specific binding was determined in the presence of 30 μM R-(−)-PIA or 1 μM DPCPX. The membrane bound and free radioligand were separated by filtration using a 96-well cell harvester. Radioactivity was counted at an efficiency of ~40% using the TopCount Instrument (Perkin Elmer, Boston, MA).

2.5. Measurement of cAMP production

AML-12, HepG2, and CHO-A_1 cells were seeded in 75 mm^2 flasks and grown 36–72 h to reach ~80% confluence. Cells were then washed once with PBS and detached with PBS containing 2 mM EDTA. Cells were pelleted at a speed of 250×*g* and resuspended in PBS. Adenosine deaminase (1 U/ml) was added to eliminate adenosine. Cells were loaded into 96-well plates (~6500 cells/well) and incubated with different concentrations of CPA for 2 min, and then 3 or 10 μM forskolin and 20 μM rolipram were added for 30 min at 37°C. The cAMP production was measured by using a DiscoveRx kit according to the manufacturer's instructions.

2.6. Quantitative real-time RT-PCR (qPCR)

AML-12 cells were seeded in 6-well plates and incubated in culture medium for 24 h. Compounds were added to AML-12 cells in DMEM-F12 medium with 2% FBS and 0.5% BSA and incubated for 24 h. HepG2 cells were seeded in 6-well plates and incubated in culture medium for 1 day. Compounds were added to HepG2 cells in DMEM with 0.5% BSA and incubated for 24 h. Fresh human hepatocytes were seeded on collagen I coated 6-well plates in William E medium supplemented with thawing/plating cocktail. Three days later, human hepatocytes were serum-starved with William E medium for 5 h, then treated with agents as indicated in William E medium for 24 h. Total RNA was extracted using a RNA isolation kit (Stratagene, La Jolla, CA). cDNA was then synthesized using an iScript Reverse Transcription kit (BioRad, Hercules, CA). Primers used for qPCR are shown in Table 1. qPCR was performed using SYBR Green PCR reagents (Applied Biosystems, Foster City, CA) on Stratagene Mx3000P (Agilent, Santa Clara, CA). Relative mRNA levels were calculated by the delta Ct values (threshold cycle time) and reported relative to the levels of β-actin or ribosomal protein S9.

2.7. In vivo experiments

Male Sprague Dawley rats 330–420 g from Charles River Laboratories Inc. (Wilmington, MA) were housed two per cage and maintained on a normal light/dark cycle. Prior to dosing, animals were fasted overnight. Vehicle or GS-9667 was administered subcutaneously (10 mg/kg, in 20% polyethyleneglycol) with 3 doses at 0, 2, and 4 h time points, respectively. Four animals per group were sacrificed at 6, 9 and 12 h after the first dosing under isofluorane anesthesia. Livers were excised and flash frozen in liquid N_2 for gene expression studies. All experimental procedures were performed under an approved