ALTERATION OF CYP3A1 mRNA LEVEL IN PRIMARY RAT HEPATOCYTES IN RESPONSE TO AMPK ACTIVATOR AICAR

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ABSTRACT

Background and Objective
AMP-activated protein kinase (AMPK) functions as a sensor of the intracellular energy status that can be stimulated by a synthetic activator, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), which is used to replicate the effect of physical exercise in hepatocyte embryoid bodies. This study investigated the effect of AICAR on the CYP3A1 mRNA expression in primary hepatocyte embryoid bodies derived from a rat liver.

Material and Methods
The primary hepatocytes were isolated from a male Sprague Dawley (SD) rat (215 g) and subjected to the following treatments: control without AICAR (CTL, n=3), 1 μM AICAR (n=3), 10 μM AICAR (n=3), and 100 μM AICAR (n=3). RNA was isolated and used as the template for synthesizing cDNA by reverse transcriptase to perform quantitative PCR (qPCR). The independent samples t-test was conducted to examine differences between groups. Statistical significance was set at \( p < 0.05 \).

Results
The qPCR analysis demonstrated that CYP3A1 mRNA expression in primary hepatocyte embryoid bodies significantly increased in the presence of 10 μM (\( t = 1.730, \ p < 0.05 \)) and 100 μM AICAR (\( t = 3.207, \ p < 0.05 \)) as compared to that in the control group hepatocytes. However, the observed increase of CYP3A1 mRNA in hepatocyte embryoid bodies was not statistically significant in the presence of 1 μM AICAR as the lowest test concentration.
Conclusion

In this study, we demonstrated that AICAR, an AMPK activator, can increase the expression of CYP3A1 mRNA in primary hepatocytes. Future studies should assess the effect of AICAR treatment on CYP3A4 in human hepatocytes.

Key words: 5-aminoimidazole–4–carboxamide–1–beta–D–ribofuranoside, AMP-activated protein kinase, CYP3A1

AMP-activated protein kinase (AMPK) contributes to maintaining the homeostasis of the body under various conditions including exercise and is activated by a stress-induced signal to preserve cellular homeostasis against changes in the environment outside of the body. Various studies have reported that endurance exercise activates AMPK. The synthetic compound 5-aminoimidazole–4–carboxamide–1–beta–D–ribofuranoside (AICAR) also causes the activation of AMPK, which suppresses the mechanistic target of rapamycin (mTOR), a muscle growth regulator.

Cytochromes P450 (CYP450s) are found in most organisms. The CYP450s are expressed in various body organs including the heart and other internal organs, and their highest expression levels are found in the liver. Certain types of CYP450s are found in subcellular compartments such as mitochondria and Golgi apparatus. Many human CYP450s metabolize endogenous substrates, control the biosynthesis of steroids and eicosanoids, and regulate vitamin D decomposition. Some CYP450s regulate the oxidation of xenobiotics such as drugs or environmental pollutants. They need NADPH-P450 reductase or interact with cytochrome b5 and electron donors to metabolize endogenous substrates and xenobiotics.

CYP450s can be induced or suppressed by certain dietary components or drugs, alcohol, and xenobiotics. Modified CYP450s expression affects the biotransformation rate of their substrates. In humans, CYP3A4 is expressed in the prostate, breast, large intestine, colon, and small intestine, but most of CYP3A4 is expressed in the liver. The human CYP3A4 is crucial for removing chemicals, and the rat CYP3A1 functions as its analog. CYP3A4 represents 30% of the total CYP450 protein content. Grapefruit, a tropical fruit, suppresses CYP3A4 expression, affecting the metabolism of 60–80% of drugs during clinical trials. Thus, drugs that are metabolized by CYP3A4 can induce side effects to the kidneys, liver, or other organs if grapefruits or grapefruit juice is consumed during treatment. Moreover, physical characteristics of aging such as decreased blood flow in the kidneys and liver as well as a change in liver mass can affect the pharmacokinetics of drugs, which increases drug-related risks in the elderly and, specifically, in older patients with vascular diseases that are at a high risk of severe symptoms. For instance, aging can reduce the clearance of antipyrine by increasing its half-life in the plasma. However, regular physical behaviours can reduce aging-related drug toxicity by effectively minimizing the risk of age-related illness and improving drug clearance.

The expression of CYP450s is affected by endogenous compounds including hormones, growth factors, and cytokines. Metabolic activity of CYP isoenzymes in the liver was reported according to gender differences. Women have a higher standard CYP3A4 activity than men, with a greater degree of interaction on average. The menstrual cycle phases have various effects on CYP activity. Oxidation of each drug differed between male and female. Diazepam and prednisolone drug clearance rates were higher in women, but drugs such as propranolol had faster clearance rates in men. Bioavailability of orally administered drugs that are CYP3A substrates can be somewhat higher in women than in men. The total clearance rates of many CYP3A substrates are between slightly and significantly faster (mg/kg) in women than in men.

Therefore, many drugs metabolized by CYP3A4 exhibit a higher clearance in women than men, and this difference persists even after adjusting physiological factors such as body weight. CYP3A4 also promotes the hydroxylation of exogenous and endogenous steroid
hormones. The rapid elimination of prednisolone and methylprednisolone in females was reported to be caused by a significantly higher excretion of 6/3-hydroxycortisol, the major metabolite of cortisol formed by CYP3A4, in females as compared to that in males.

Differences in hepatocyte microsomal CYP450 systems contribute to gender differences. Drug metabolism has a direct effect on the therapeutic response and drug toxicity and, therefore, it is a major determinant of pharmacokinetics. Comparisons of pharmacokinetic differences between women and men after single intravenous (0.15 mg/kg) or oral (8 mg) administration of ondansetron reported that the elimination of ondansetron was faster in women than in men. Hence, CYP3A4 activity is greater in women than in men due to the faster drug metabolism in women as compared to that in men.

However, the concentration of many endogenous compounds changes as an effect of aging. Furthermore, a decrease of the drug metabolizing capacity is reportedly associated with changed physical characteristics. The decrease can be induced by changes of the body shape, body fat, muscle mass, and body water as well as a decrease of plasma albumin and blood flow, which is further affected by variations of the expression of drug metabolizing enzymes, such as CYP450s, that are associated with different age groups. Monitoring of the activity of CYP450s is important for determining drug circulation time, toxic metabolites, and drug-drug interactions. In this study, we investigated the effect of AICAR on CYP3A1 expression in cultured rat hepatocyte embryoid bodies.

**METHODS**

**Animal Care**
A male Sprague Dawley (SD) rat, 215 g, was housed in a room with controlled temperature (22±2°C), humidity (50–60%), and lighting (12/12 h light/dark cycle). The diet contained 67.5% carbohydrate, 11.7% fat, and 20.8% protein (Samtako Co., Osan, Korea).

**Experimental Design**

**Treatment with AMPK Activator AICAR**
The male SD rat was the source for hepatocyte cultures that were assigned to 4 groups receiving the following 24 h treatment: no compound, control (CTL; n=3); 1 μM AICAR (n=3); 10 μM AICAR (n=3); and 100 μM AICAR (n=3).

**RNA Extraction and Reverse Transcriptase Real-Time PCR**
RNA was isolated using the RNA-Bee regent (Tel-test, Friendswood, TX, USA). An aliquot of 1 μg RNA was added as template to a reverse transcriptase reaction for synthesizing cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, CA, USA). Aliquots of cDNA were used as the template for quantitative PCR (qPCR) with the SYBR Green PCR Master Mix (Applied Biosystems) performed in the Eppendorf Mastercycler Realplex. Each target mRNA was amplified from 50 ng cDNA using the following program: primer annealing for 30 s at 60°C and primer extension for 30 s at 72°C; performing a total of 45 cycles. Specificity was routinely assessed by melting curve analysis. Relative expression of mRNA was calculated according to the ΔΔCT method described by Livak and Schmittgen. Rat CYP3A1 mRNA was the target of this analysis, and GAPDH mRNA amplification was performed as a control. All primers used in this study are shown in Table 1.

**Primary Hepatocyte Isolation and Culture**
Primary hepatocytes were isolated from the SD rat according to the two-step collagenase perfusion method and cultured as described by Lee et al. The rat was anesthetized by intraperitoneal injection of tribromoethanol (Avertin). Then, its liver was perfused

| Genes  | Forward         | Reverse               |
|--------|-----------------|-----------------------|
| CYP3A1 | GGAAATTCGATGTTGAGTGC | AGGTTTGCTTTTCCTTGGCC |
| GAPDH  | TGCCCAATTATGATGGACATCAAGAAG | AGGCCAGGATTGCGCTTTAGT |

TABLE 1 Primer Sets Used for qPCR
with 300–400 ml of Krebs-Ringer bicarbonate buffer and treated for 8–10 min, followed by one re-perfusion. The harvested liver was processed with 0.3 mg/mL of collagenase type IV (Sigma-Aldrich) for 10 min to gently split the tissue and release the cells. Finally, single cells were obtained by filtering the cell material using a mesh wide of 90 and 200 μm. The cell culture was initiated by seeding 6-well plates at a density of 1×10⁶ cells/cm² using Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. The hepatocytes were incubated for 3 h before adding fresh culture medium with Matrigel. After 24 h, the culture medium was changed to William’s E medium supplemented with 10 mM HEPES, 10 nM insulin, 25 nM dexamethasone, and 100 U/ml penicillin/streptomycin. The hepatocytes were cultured for 5–6 days in 5% CO₂ at 37°C.

**Statistical Analysis**

The statistical analysis was performed using SPSS Ver 21.0 (IBM Corp., Armonk, NY, USA). Descriptive statistical values are provided as the mean ± standard error, and differences between experimental groups were evaluated by applying the *t*-test for independent samples. Statistical significance was set at p<0.05.

**RESULTS**

In this study, we investigated the effect of AMPK activator AICAR on CYP3A1 mRNA expression in primary hepatocytes isolated from an SD rat. The fold change of CYP3A1 mRNA after 24 h in the CTL, the 1 μM, the 10 μM, and the 100 μM AICAR treatment group were 1.00±0.18, 1.36±0.17, 2.08±0.35, and 1.88±0.21, respectively (Figure 1).

Importantly, the treatment of hepatocytes with 1 μM, 10 μM, and 100 μM of the AMPK activator AICAR increased the expression of CYP3A1 mRNA as compared to that of CTL group hepatocytes. The fold change of CYP3A1 mRNA expression differed significantly from the CTL when the AICAR treatment was performed at 10 μM (t=1.730, p<0.05) and 100 μM (t=3.207, p<0.05). However, the fold change of CYP3A1 mRNA expression caused by 1 μM of AICAR was not statistically significant.

**FIG. 1** Effects of AICAR on CYP3A1 expression in primary cultured rat hepatocytes. Rat hepatocytes cultures were treated with media (control, CTL) (n=3), 1μM(n=3), 10μM(n=3), or 100μM(n=3) AICAR for 24 hours, Rat hepatocytes were harvested and analyzed for CYP3A1 mRNA.

Results are given as means±standard error from 6 wells each. Values are expressed relative to the CTL group. *Significantly different(p<0.05) from CTL.

**DISCUSSION**

In this study, we used AICAR to investigate the activation of AMPK in primary hepatocytes isolated from an SD rat. Specifically, we examined the effect of different concentrations of AMPK activator on the mRNA expression of CYP3A1.

We demonstrated that AICAR increased CYP3A1 mRNA expression in primary rat hepatocytes, which is consistent with the results of an earlier study, showing that AICAR increased the CYP3A1 level by entering the cell and activating AMPK. Thus, AMPK plays an important role in increasing CYP3A1 expression, and AMPK activation may be important for alleviating drug toxicity by affecting the expression of CYP3A1, which has one of the most critical functions in drug metabolism. However, the underlying mechanism of the relationship between AICAR, AMPK, and CYP450 has yet to be studied.
The CYP3A family includes the largest number of CYP450s enzymes that are involved in modifying endogenous substrates as well as metabolizing many clinical drugs. CYP3A reduces certain chemicals, metabolizes specific antibodies, and decomposes toxic materials. Therefore, CYP3A has an important function in the metabolism of endogenous substrates. Nuclear receptors are involved in the regulation of many CYPs. Regular exercise increased constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in rats. Activators of CAR and PXR significantly induced the expression of CYP3A4 and endogenous rat Cyp3A genes. The growth hormone (GH) plays an important role in the sex-dependent expression of the CYP3A4 transgene in mice. Another study has shown that CYP3A4 levels and drug metabolism are higher in women than men. 

The production and secretion of GH is an important determinant for gender-specific growth processes and metabolism. GH treatment significantly changes Cyp3A expression as demonstrated in extensive studies in mice and rats. Continuous GH treatment stimulated hepatic CYP3A4 expression in male mice, and GH is involved in the regulation of CYP3A4 expression in humans and, therefore, is potentially important as a therapeutic. GH secreted by the pituitary gland regulates the expression of the sex-specific as well as predominant P450 genes.

A recent study demonstrated that the levels of bile acid and testosterone are higher in CYP3A knockout mice than in normal mice. Although there are differences between the mouse and the human CYP3A, using the CYP3A1 from the mouse or other related animal species is a critical method for examining the CYP3A-dependent metabolism of chemical compounds. In basic research, the CYP3A1/2 enzymes of the rat model represent an important research tool for studying the regulation of the metabolism of drugs involving CYP3A and their pharmacokinetics.

Future studies should include an in-depth analysis of CYP3A4, which is predominantly expressed in the human liver. However, based on this study, there should also be an investigation of SD rats that differ in age for examining potential changes of CYP3A1 expression.

CONCLUSION

In summary, we demonstrated that primary hepatocytes, which were isolated from a male SD rat, increased their CYP3A1 mRNA expression level in response to a treatment with AICAR, an activator of AMPK. In future studies, we will utilize our approach for directly testing the efficacy of AICAR using an extensive concentration series of the compound as well as various treatment periods and methods.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Merrill GF, Kurth EJ, Hardie DG, et al. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273(6 Pt 1):1107–12.
2. Chambers MA, Moylan JS, Smith JD, et al. Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. J Physiol 2009;587(13):3363–73.
3. Witczak CA, Sharoff CG, Goodyear LJ. AMP-activated protein kinase in skeletal muscle: from structure and localization to its role as a master regulator of cellular metabolism. Cell Molec Life Scie 2008;65(23):3737–55.
4. Gibala MJ, McGee SL, Garnham AP, et al. Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. J Appl Physiol 1985;106(3):929–34.
5. Williamson DL, Bolster DR, Kimball SR, et al. Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. American journal of physiology. Endocrin Metab 2006;291(1):80–89.
6. Guengerich FP, Shimada T, Yun CH, et al. Interactions of ingested food, beverage, and tobacco components involving human cytochrome P450I2A, 2A6, 2E1, and 3A4 enzymes. Environ Health Perspect 1994;9:49–53.
7. Stupans I, Murray M, Kirlich A, et al. Inactivation of cytochrome P450 by the food-derived complex phenol oleuropein. Food Cheml Toxicol 2001;39(11):1119–24.
8. Martignoni M, Groothuis G, de Kanter R. Comparison of mouse and rat cytochrome P450-mediated
metabolism in liver and intestine. Drug Metabol Disposit 2006;34(6):1047–54.
9. Furge LL, Guengerich FP. Cytochrome P450 enzymes in drug metabolism and chemical toxicology: An introduction. Biochem Molec Biol Educat 2006;34(2):66–74.
10. Smith DA, Abel SM, Hyland R, et al. Human cytochrome P450s: selectivity and measurement in vivo. Xenobiotica 1998;28(12):1095–28.
11. Wang P, Mason PS, Guengerich FP. Purification of human liver cytochrome P-450 and comparison to the enzyme isolated from rat liver. Arch Biochem Biophys 1980;199(1):206–19.
12. Frenkl R, Szeberenyi S. Enzyme inducing effect of muscular exertion in the rat. Acta Medica Academ Scient Hungaricae 1976;33(1):95–100.
13. Ware WR. Nutrition and the prevention and treatment of cancer: association of cytochrome P450 CYP1B1 with the role of fruit and fruit extracts. Integrat Cancer Ther 2009;8(1):22–28.
14. Guengerich FP. Cytochrome P-450 3A4: regulation and role in drug metabolism. Ann Rev Pharmacol Toxicol 1999;39:1–17.
15. Watkins PB, Wrighton SA, Schuetz EG, et al. Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man. J Clin Invest 1987;80(4):1029–36.
16. Lown KS, Bailey DG, Fontana RJ, et al. Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression. J Clin Investig 1997;99(10):2545–53.
17. Kolars JC, Lown KS, Schmiedlin-Ren P, et al. CYP3A gene expression in human gut epithelium. Pharmacogenetics 1994;4(5):247–59.
18. Huang Z, Fasco MJ, Figge HL, et al. Expression of cytochromes P450 in human breast tissue and tumors. Drug Metab Disposit 1996;24(8):899–905.
19. Shimada T, Yamazaki H, Mimura M, et al. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Experiment Ther 1994;270(1):414–23.
20. Takizawa D, Kakizaki S, Horiguchi N, et al. Constitutive active/androstane receptor promotes hepatocarcinogenesis in a mouse model of non-alcoholic steatohepatitis. Carcinogenesis 2011;32(4):576–83.
35. Pritchard JF, Bryson JC, Kernodle AE, et al. Age and gender effects on ondansetron pharmacokinetics: evaluation of healthy aged volunteers. Clin Pharmacol Ther 1992;51(1):51–55.
36. Kobayashi K, Abe C, Endo M, et al. Gender Difference of hepatic and intestinal CYP3A4 in CYP3A humanized mice generated by a human chromosome-engineering technique. Drug Metab Lett 2017;11(1):60–67.
37. Staskin DR. Overactive bladder in the elderly: a guide to pharmacological management. Drugs Aging 2005;22(12):1013–28.
38. De Stefano F, Zambon S, Giacometti L, et al. Obesity, muscular strength, muscle composition and physical performance in an elderly population. J Nutrition Health Aging 2015;19(7):785–91.
39. Wauthier V, Verbeeck RK, Calderon PB. The effect of ageing on cytochrome p450 enzymes: consequences for drug biotransformation in the elderly. Curr Med Chem 2007;14:745–57.
40. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) method. Methods 2001;25(4):402–8.
41. Lee CM, Pohl J, Morgan ET. Dual mechanisms of CYP3A protein regulation by proinflammatory cytokine stimulation in primary hepatocyte cultures. Drug Metabol Disposit 2009;37(4):865–72.
42. Hu N, Hu M, Duan R, et al. Increased levels of fatty acids contributed to induction of hepatic CYP3A4 activity induced by diabetes - in vitro evidence from HepG2 cell and Fa2N-4 cell lines. J Pharmacol Sci 2014;124(4):433–44.
43. Vignati L, Turlizzi E, Monaci S, et al. An in vitro approach to detect metabolite toxicity due to CYP3A4 dependent bioactivation of xenobiotics. Toxicology 2005;216(2-3):154–67.
44. van Herwaarden AE, Wagenaar E, van der Kruisjen CM, et al. Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. J Clin Investig 2007;117(11):3583–92.
45. Lee BS, So WY, Kim SH, et al. Expression profiles of cytochrome P450s following swimming exercise in aging rats. J Men's Health 2017;13(2):e25–e33.
46. Lee BS, So WY, Chung WY. Suppression of expression levels of constitutive androstane receptor by moderate exercise in BALB/c nude mice with breast cancer. Iran J Pub Health 2017;46(8):1154–55.
47. Jaffe CA, Ocampo-Lim B, Guo W, et al. Regulatory mechanisms of growth hormone secretion are sexually dimorphic. J Clin Investig 1998;102(1):153–64.
48. Park SH, Liu X, Hennighausen L, et al. Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression: impact of STAT5a gene disruption. J Biol Chem 1999;274(11):7421–30.
49. Waxman DJ, LeBlanc GA, Morrissey JJ, et al. Adult male-specific and neonatally programmed rat hepatic P-450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression. J Biol Chem 1988;263(23):11396–406.
50. Robertson GR, Farrell GC, Liddle C. Sexually dimorphic expression of rat CYP3A9 and CYP3A18 genes is regulated by growth hormone. Biochem Biophys Res Commun 1998;242(1):57–60.
51. Kawai M, Bandiera SM, Chang TK, et al. Growth hormone regulation and developmental expression of rat hepatic CYP3A18, CYP3A9 and CYP3A2. Biochem Pharmacol 2000;59(10):1277–87.
52. Cheung C, Yu AM, Chen CS, et al. Growth hormone determines sexual dimorphism of hepatic cytochrome P450 3A4 expression in transgenic mice. J Pharmacol Exp Ther 2006;316(3):1328–34.
53. van den Berg G, Veldhuis JD, Frolich M, et al. An amplitude-specific divergence in the pulsatile mode of growth hormone (GH) secretion underlies the gender difference in mean GH concentrations in men and premenopausal women. J Clin Endocrinol Metab 1996;81(7):2460–67.
54. Hashimoto M, Kobayashi K, Watanabe M, et al. Knockout of mouse Cyp3a gene enhances synthesis of cholesterol and bile acid in the liver. J Lipid Res 2013;54(8):2060–68.