

### 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-β-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.<sup>1-4</sup> 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.<sup>5</sup>

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.<sup>6-8</sup> 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.<sup>9</sup> Some CYP450s regulate the oxidation of xenobiotics such as drugs or environmental pollutants.<sup>10,11</sup> They need NADPH-P450 reductase or interact with cytochrome b5 and electron donors to metabolize endogenous substrates and xenobiotics.<sup>12</sup>

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.<sup>13</sup> 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.<sup>14-19</sup> Grapefruit, a tropical fruit, suppresses CYP3A4 expression, affecting the metabolism of 60~80% of drugs during clinical

trials.<sup>20,21</sup> 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.<sup>22</sup> For instance, aging can reduce the clearance of antipyrine by increasing its half-life in the plasma.<sup>23</sup> However, regular physical behaviours can reduce aging-related drug toxicity by effectively minimizing the risk of age-related illness and improving drug clearance.<sup>24</sup>

The expression of CYP450s is affected by endogenous compounds including hormones, growth factors, and cytokines.<sup>25</sup> Metabolic activity of CYP isoenzymes in the liver was reported according to gender differences.<sup>26</sup> 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.<sup>27,28</sup> 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.<sup>29</sup>

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.<sup>30,31</sup> 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.<sup>32,33</sup>

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.<sup>34</sup> 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.<sup>35</sup> Hence, CYP3A4 activity is greater in women than in men due to the faster drug metabolism in women as compared to that in men.<sup>26,36</sup>

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.<sup>37–39</sup> 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 reagent (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  $\Delta\Delta C_T$  method described by Livak and Schmittgen.<sup>40</sup> 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.<sup>41</sup> The rat was anesthetized by intraperitoneal injection of tribromoethanol (Avertin). Then, its liver was perfused

**TABLE 1** Primer Sets Used for qPCR

Genes	Forward	Reverse
CYP3A1	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC
GAPDH	TGCCAAGTATGATGACATCAAGAAG	AGCCCAGGATGCCCTTTAGT

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  $\mu\text{m}$ . The cell culture was initiated by seeding 6-well plates at a density of  $1 \times 10^6$  cells/cm<sup>2</sup> 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% CO<sub>2</sub> 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  $\pm$  standard error, and differences between experimental groups were evaluated by applying the *t*-test for independent samples. Statistical significance was set at  $p < 0.05$ .

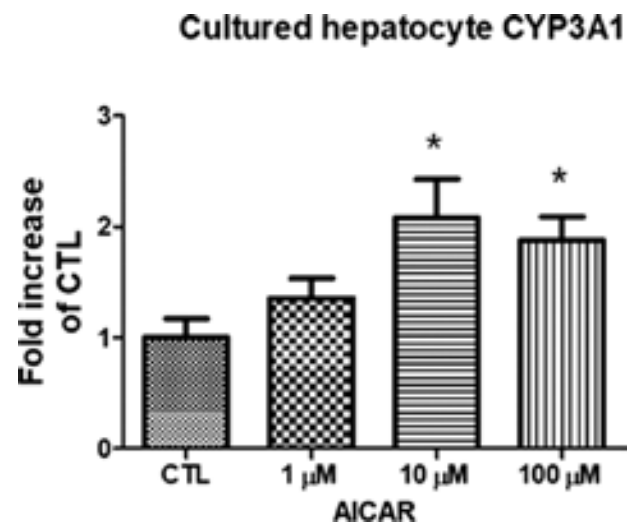
### RESULTS

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  $\mu\text{M}$ , the 10  $\mu\text{M}$ , and the 100  $\mu\text{M}$  AICAR treatment group were  $1.00 \pm 0.18$ ,  $1.36 \pm 0.17$ ,  $2.08 \pm 0.35$ , and  $1.88 \pm 0.21$ , respectively (Figure 1).

Importantly, the treatment of hepatocytes with 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$  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  $\mu\text{M}$  ( $t = 1.730$ ,  $p < 0.05$ ) and 100  $\mu\text{M}$  ( $t = 3.207$ ,  $p < 0.05$ ). However, the fold change of CYP3A1 mRNA expression caused by 1  $\mu\text{M}$  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) ( $n = 3$ ), 1  $\mu\text{M}$  ( $n = 3$ ), 10  $\mu\text{M}$  ( $n = 3$ ), or 100  $\mu\text{M}$  ( $n = 3$ ) AICAR for 24 hours. Rat hepatocytes were harvested and analyzed for CYP3A1 mRNA.

Results are given as means  $\pm$  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.<sup>5</sup> Thus, AMPK plays an important role in increasing CYP3A1 expression,<sup>42</sup> 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.<sup>43</sup> Therefore, CYP3A has an important function in the metabolism of endogenous substrates.<sup>44</sup> Nuclear receptors are involved in the regulation of many CYPs. Regular exercise increased constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in rats.<sup>45,46</sup> Activators of CAR and PXR significantly induced the expression of CYP3A4 and endogenous rat Cyp3A genes.<sup>47</sup> The growth hormone (GH) plays an important role in the sex-dependent expression of the CYP3A4 transgene in mice.<sup>48</sup> Another study has shown that CYP3A4 levels and drug metabolism are higher in women than men.<sup>26</sup>

The production and secretion of GH is an important determinant for gender-specific growth processes and metabolism.<sup>47</sup> GH treatment significantly changes Cyp3A expression as demonstrated in extensive studies in mice and rats.<sup>49–52</sup> 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.<sup>53</sup> GH secreted by the pituitary gland regulates the expression of the sex-specific as well as predominant P450 genes.<sup>47,54</sup>

A recent study demonstrated that the levels of bile acid and testosterone are higher in CYP3A knockout mice than in normal mice.<sup>55</sup> 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.

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