Icariin Reverses the Elevated Expression of the Hepatic Transporters Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2 and Mrp2 in Spontaneously Hypertensive Rats

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Abstract

The hepatic transporters organic anion transporting polypeptides (Oatps/OATPs) and multidrug resistance-associated proteins (Mrps) transport endogenous or exogenous substances such as bile acids. The aforementioned transporters also regulate blood pressure. Oatps/OATPs and Mrps were aberrantly expressed in spontaneously hypertensive rats (SHRs). Icariin (ICA), one of the main active components of *Herba Epimedii*, delays hypertensive heart disease in SHRs. However, researchers have not yet determined whether ICA affects the aforementioned liver transporters in SHRs. The present study was designed to investigate the effects of icariin on the hepatic expressions of OATPs and Mrp2 in SHRs. The main methods used were real-time PCR and Western blot analysis. The results showed that ICA significantly reduced the abnormally elevated levels of the Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2, and Mrp2 mRNAs and/or proteins in the livers of SHRs. In conclusion, ICA reverses the elevated expression of the hepatic transporters Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2 and Mrp2 in SHRs.

Keywords

icariin, spontaneously hypertensive rats, organic anion transporting polypeptides, multidrug resistance-associated protein 2, liver

1. Introduction

The liver transporters organic anion transporting polypeptides (Oatps/OATPs) and multidrug resistance-associated proteins (Mrps) play important roles in transporting endogenous or exogenous substances, as well as in drug-drug interactions. Oatps/OATPs, the uptake transporters, belong to the superfamily of solute carriers and are very important intake proteins located in the cell membrane in animals and humans. These proteins have important functions in the absorption, distribution and elimination of endogenous and exogenous substances, including drugs [1, 2]. Oatps/OATPs also affect the transport of bile acids [3] and may contribute to the mechanism regulating changes in blood pressure [4, 5]. Among these proteins, Oatp1a1, Oatp1a4, and Oatp1b2 have been shown to mediate the uptake of bile acids in vitro [6-8]. Oatp1a1 and Oatp1a4 are responsible for the hepatic uptake of anti-hypertensive drugs in rats [9]. Mrps are the major efflux transporters that mediate the efflux of drugs and metabolites in the liver and kidneys, and Mrp2 mainly mediates the efflux of bile [1].

Hypertension is a multifaceted disease that disrupts the physiological balance in the body and influences a large number of organs, including the heart, kidney, and liver, among others [10]. The spontaneously hypertensive rats (SHRs) is the most widely used phenotypic experimental model in studies of the diseases associated with hypertension and its complications [11]. Few reports have described the changes in liver transporters in animals or human with hypertension; however, compared to Wistar-Kyoto (WKY) rats, the expression of transporter mRNAs (Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2 and Mrp2) in the livers of SHRs was recently shown to be significantly increased [12].

Icariin (ICA) is one of the main active components of *Herba Epimedii*, and this herb has been used in traditional Chinese medicine for thousands of years to treat sexual dysfunction, osteoporosis, some cardiovascular diseases, and other conditions [13, 14]. In addition, we have confirmed that ICA is a beneficial treatment for hypertension and its cardiovascular complications, such as hypertensive heart disease, in SHRs [15-17]. The liver is also one of the target sites of icariin [18]. As shown in the study by Li Z *et al.*, ICA inhibits OATP1B3 and affects Oatp-mediated cellular uptake of specific substrates as a basis of herb-drug interactions, and ICA also modifies the transporter activity of OATP1B1 [19, 20]. However, researchers have not yet determined whether ICA alters the expression of liver transporters such as Oatps and Mrp2 in SHRs. Therefore, the study was designed to explore the effects of ICA on the expression of Oatps (Oatp1a1, Oatp1a4, Oatp1a5, and Oatp1b2) and Mrp2 in the liver and to analyze the significance of the changes in Oatps and Mrp2 levels in SHRs.

2. Materials and methods

2.1 Animals and groups

Fourteen-week-old male SHRs and homologous WKY rats (260-300 g) were purchased from Vital River Experimental Animal Technology Co., Ltd. (Certificate No. SCXK 2012-0001; Beijing, China). The rats were housed in SPF-grade animal facilities (Certificate No. SYXK 2011-004) with a controlled environment (22 ± 1 °C, $50 \pm 2\%$ humidity and a standard 12 h light schedule) and free access to purified water. All rats were acclimated for one week. SHRs were randomly divided into the following groups: an untreated group (SHR, n=7), a low-dose ICA-treated group (SHR-ICA10, 10 mg/kg, n=7), a medium-dose ICA-treated group (SHR-ICA20, 20 mg/kg, n=7), and a high-dose ICA-treated group (SHR-ICA40, 40 mg/kg, n=7) [17, 21, 22]. Wistar-Kyoto rats were used as the normal control group (WKY-Control, n=7). ICA (purity $\geq 98\%$ by high-performance liquid chromatography; Zelang Medical Technology Co. Ltd., Nanjing, China) was dissolved in double distilled water as a suspension and the SHR-ICA10, SHR-ICA20 and SHR-ICA40 groups were intragastrically administered ICA 10, 20 and 40 mg/kg twice a day for 12 weeks. The rats in the SHR group and WKY-Control group were administered the same amount of double distilled water. After 12 weeks of administration [17, 21, 22], the livers were collected, snap frozen, and stored at -80 °C for future analysis. The study was approved by Zunyi Medical University Committee on Animal Resources.

2.2 Determination of body weight and blood pressure

The body weight of the animals were recorded every two weeks using an electronic scale (Beijing Sartorius Electronics Co., Ltd.). A tail-cuff method was used to record the blood pressure of rats at the using a Kent Scientific CODA (Kent Scientific, Connecticut, USA).

2.3 Total RNA isolation and real-time PCR analysis

Total RNA was isolated from rat liver tissues using RNAiso Plus (TaKaRa Biotechnology, Dalian, China). Total RNA in the rat livers was reverse-transcribed into cDNAs using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were designed with Primer 3 software and are listed in Table 1. The 15 μ l PCR reaction mix contained 3 μ l of cDNA (10 ng/ μ l), 7.5 μ l of iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 μ l of primer mix (10 μ M each), and 4 μ l of DEPC H₂O. After 5 min denature at 95 °C, 40 cycles will be performed: annealing and extension at 60 °C for 45 s and denature at 95 °C for 10 s. Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. Five rats in each group were randomly selected for the measurement of mRNA levels, and each sample was measured three times. The relative expression of genes was calculated by the $2^{-\Delta \Delta C_t}$ method and normalized to the housekeeping gene β -actin. The relative transcript levels were calculated as a percentage of β -actin expression [23].

Table 1. Primer pairs used in Real time PCR

Gene	GenBank accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
β -actin	NM-031144	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
Oatp1a1	NM_013797	ATCCAGTGTGTGGGGACAAT	GCAGCTGCAATTTTGAAACA
Oatp1a4	NM_030687	GGAAGATTGGACACGCATCT	GGCATTGTGACTGAAGCAGA
Oatp1a5	AF041105	GCAGGATGATGTGGATGGAACAAAC	GCATGTAAATCGGATTGCAGGAG
Oatp1b2	NM_031650	AACATGCTTCGTGGGATAGG	CATGGAAGTGTGCCCTTCTT
Mrp2	NM_012833	TCTCTTGCCTCACAGAAGA	GAAACTGGAATACGCCGCA

Oatp1a1, organic anion transporting polypeptides 1a1; Oatp1a4, organic anion transporting polypeptides 1a4; Oatp1a5, organic anion transporting polypeptides 1a5; Oatp1b2, organic anion transporting polypeptides 1b2; Mrp2, multidrug resistance-associated proteins 2.

2.4 Western blot analysis

Three rats were randomly selected from each group to measure protein levels in liver tissues, and each sample was measured three times. The liver tissues from rats in each group weighing approximately 100 mg were homogenized in 1 ml of RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride and proteinase inhibitors. Homogenates were centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatants were collected. Protein concentrations were quantified using the BCA assay (Beyotime, Shanghai, China). Aliquots of proteins were denatured with the loading buffer (Beyotime, Shanghai, China) at 90 °C for 10 min, and proteins (30 μ g/10 μ l) in each lane were separated on 8% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk in TBST, followed by an overnight incubation at 4 °C with 1:200 dilutions of rabbit polyclonal antibodies against Mrp2 (sc-20766; Santa Cruz, CA), mouse monoclonal antibodies against Oatp1a4 (sc-376424; Santa Cruz, CA) and Oatp1b2 (sc-376904; Santa Cruz, CA), and mouse antibodies against β -actin (Cat. N.: 60008-1-Ig; Proteintech, Shanghai, China) in 1% BSA. After three washes with TBST, membranes were incubated with a 1: 5000 dilution of HRP-conjugated goat anti-rabbit (D110058; Sangon Biotech, Shanghai, China) or goat anti-mouse IgG (D110087; Sangon Biotech, Shanghai, China) for 1 hour at room temperature. Protein-antibody complexes were visualized using enhanced chemiluminescence reagents (ECL-Plus) (7sea biotech, Shanghai, China), and exposed to a ChemiDoc XRS Gel Imager (Bio-Rad, USA). The intensity of each band was semiquantitatively analyzed using Quantity® One software.

2.5 Statistical analysis

Statistical analysis was performed using SPSS18.0 software. All data are presented as the means $\pm$ standard errors. The comparisons among groups were statistically analyzed using one-way ANOVA. Post hoc comparisons were performed using the LSD test when equal variances were observed or using Dunnett's T3 test when unequal variances were observed. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1 Effects of ICA on the body weight of SHRs

Figure 1 shows the body weight changes in each group, which increased with age, but statistically significant differences in body weight at the same time points were not observed among the WKY-Control, SHR, SHR-ICA10 (10 mg/kg), SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups ($P > 0.05$).

3.2 Effects of ICA on the systolic blood pressure of SHRs

Figure 2 shows the changes in the systolic blood pressure of each group. Compared with the WKY-Control group, the systolic blood pressure of the SHRs group increased 1.69-fold ($P < 0.05$, $F=136.455$; Figure 2) at the twenty-sixth week. However, ICA (20 mg/kg, 40 mg/kg) treatments decreased the systolic blood pressure by 11.4% and 17.0%, respectively ($P < 0.05$, $F=136.455$; Figure 2).

3.3 Effects of ICA on the levels of the Oatp1a1 mRNA in the livers of SHRs

Figure 3 shows the expression of the Oatp1a1 mRNA in the livers of each group. In SHRs, the hepatic expression of the Oatp1a1 mRNA increased 4-fold (10.07% versus 2.37%) compared with WKY rats ($P < 0.05$, $F=61.980$; Figure 3). The ICA (10 mg/kg, 20 mg/kg, and 40 mg/kg) treatments decreased the expression of the Oatp1a1 mRNA by 19.9%, 42.5% and 57.3%, respectively ($P < 0.05$, $F=61.980$; Figure 3).

Figure 1. The body weight of rats in each group. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors.

Figure 2. Effect of ICA on the systolic blood pressure of SHRs. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

Figure 3. Effect of ICA on the expression of the Oatp1a1 mRNA in the livers of SHRs. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

3.4 Effects of ICA on the levels of the Oatp1a4 mRNA and protein in the livers of SHR

Figure 4 shows the levels of the Oatp1a4 mRNA and protein in liver tissues from each group. In SHRs, the expression of the Oatp1a4 mRNA increased 3.17-fold (8.93% versus 2.82%) compared with WKY rats ($P < 0.05$, $F = 28.469$; Figure 4C). However, in the SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups, the expression of the Oatp1a4 mRNA decreased by 42.1% and 60.7%, respectively ($P < 0.05$, $F = 28.469$; Figure 4C). Compared with the WKY-Control group, the protein levels were increased 1.18-fold in SHRs ($P < 0.05$, $F = 12.331$; Figure 4A and B). The ICA treatments (20 mg/kg and 40 mg/kg) decreased the levels of the Oatp1a4 protein by 20.2% and 26.1%, respectively ($P < 0.05$, $F = 12.331$; Figure 4A and B).

Figure 4. Effects of ICA on the levels of the Oatp1a4 mRNA and protein in the livers of SHRs. (A) Levels of the Oatp1a4 protein were determined using western blotting; protein levels are plotted for the WKY-Control, SHR, SHR-ICA10, SHR-ICA20, and SHR-ICA40 groups. (B) Quantitation of relative levels of the Oatp1a4 protein. (C) Quantitation of relative levels of the Oatp1a4 mRNA. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

3.5 Effect of ICA on the levels of the Oatp1a5 mRNA in the livers of SHRs

Figure 5 shows the hepatic expression of the Oatp1a5 mRNA in each group. In SHRs, the expression of the Oatp1a5 mRNA was increased compared with WKY rats ($P < 0.05$, $F = 39.962$; Figure 5). In the SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups, levels of the Oatp1a5 mRNA were decreased by 23.1% and 58.3%, respectively ($P < 0.05$, $F = 39.962$; Figure 5).

Figure 5. Effect of ICA on the expression of the Oatp1a5 mRNA in the livers of SHRs. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

3.6 Effects of ICA on the levels of the Oatp1b2 mRNA and protein in the livers of SHRs

Figure 6 shows the hepatic levels of the Oatp1b2 mRNA and protein each group. In SHRs, the expression of the Oatp1b2 mRNA was increased 3.1-fold (1.67% versus 0.539%) compared with WKY rats ($P < 0.05$, $F=35.569$; Figure 6C). In the SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups, the expression of the Oatp1b2 mRNA was decreased by 20.1% and 31.7%, respectively ($P < 0.05$, $F=35.569$; Figure 6C). Compared with the WKY-Control group, the protein levels were increased 1.57-fold in SHRs ($P < 0.05$, $F=18.158$; Figure 6A and B). In the SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups, levels of the Oatp1b2 protein were decreased by 28.6% and 37.8%, respectively ($P < 0.05$, $F=18.158$; Figure 6A and B).

3.7 Effects of ICA on the levels of the Mrp2 mRNA and protein in the livers of SHRs

Figure 7 shows the hepatic levels of the Mrp2 mRNA and protein in each group. In SHRs, the expression of the Mrp2 mRNA was increased 2.7-fold (61.95% versus 22.79%) compared with WKY rats ($P < 0.05$, $F=32.002$; Figure 7C). However, the ICA (10, 20, and 40 mg/kg) treatments decreased the expression of the Mrp2 mRNA in SHRs by 32.3%, 39.2% and 44.5%, respectively ($P < 0.05$, $F=32.002$; Figure 7C). Compared with the WKY-Control group, the protein levels were increased 1.5-fold in SHRs ($P < 0.05$, $F=20.534$; Figure 7A and B). In the SHR-ICA10 (10 mg/kg), SHR-ICA20 (20 mg/kg) and SHR-ICA40 (40 mg/kg) groups, levels of the Mrp2 protein were decreased by 23.8%, 52.5% and 69.8%, respectively ($P < 0.05$, $F=20.534$; Figure 7A and B).

Figure 6. Effects of ICA on the levels of the Oatp1b2 mRNA and protein in the livers of SHRs. (A) Levels of the Oatp1b2 protein were determined by western blotting; protein levels in the WKY-Control, SHR, SHR-ICA10, SHR-ICA20, and SHR-ICA40 groups are plotted. (B) Quantitation of relative levels of the Oatp1b2 protein. (C) Quantitation of relative levels of the Oatp1b2 mRNA. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means ± standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

4. Discussion

OATP/Oatps, the uptake transporters, exert significant effects on the absorption, distribution and elimination of endogenous and exogenous substances, including bile acids [2]. Oatp1a1 is mainly expressed in the liver and kidney and increases the sensitivity of extrahepatic bile deposition in rats [1, 24]. Oatp1a4 is mainly expressed in the liver, lung, retina and brain, and in particular, it is expressed at high levels in the liver [1, 25]. The hepatic functions of rat Oatp1a4 are similar to human OATP1A2. Additionally, Oatp1a4 shares similar substrates with human OATP1A2, which are responsible for the hepatic uptake of anti-hypertensive drugs [1, 9]. Several studies have reported increased expression of the Oatp1a1 and Oatp1a4 mRNAs in rodents with nonalcoholic steatohepatitis [10]. Oatp1a5 is mainly expressed in the lung, liver, intestine and brain [26]. In addition, it has a wide range of substrates with different properties in the liver, is closely related to the low substrate of Oatps, and plays an important role in the liver [27]. In the present study, ICA can decrease the expression of Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1b2 mRNA, and can decrease the expression of Oatp1a4 and Oatp1b2 proteins. Generally, an examination of the levels of the Oatp1a1 and Oatp1a5 proteins would provide more insights into the effects of ICA on

these Oatps. However, due to the lack of the corresponding commercial antibodies, we were unable to assess the levels of the Oatp1a1 and Oatp1a5 proteins in the present studies, and further investigations will be performed under the same conditions in the future.

Figure 7. Effects of ICA on the levels of the Mrp2 mRNA and protein in the livers of SHRs. (A) Levels of the Mrp2 protein were determined by western blotting; protein contents in the WKY-Control, SHR, SHR-ICA10, SHR-ICA20, and SHR-ICA40 groups are plotted. (B) Quantitation of relative levels of the Mrp2 protein. (C) Quantitation of relative levels of the Mrp2 mRNA. WKY-Control: control group; SHR: SHR model group; SHR-ICA10: SHRs treated with 10 mg/kg ICA; SHR-ICA20: SHRs treated with 20 mg/kg ICA; SHR-ICA40: SHRs treated with 40 mg/kg ICA. The data are presented as means $\pm$ standard errors. * $P < 0.05$ compared with the WKY group; # $P < 0.05$ compared with the SHR group.

Oatp1b2 is a liver-specific transporter and the most highly expressed isoform among the known Oatps in rats; this protein may play a key role in the selective uptake of xenobiotics in the liver [25]. Rat Oatp1b2 is homologous with mouse Oatp1b2, human OATP1B1 and OATP1B3 [1], which affect the hepatic uptake of drugs, poisons and environmental chemicals [28]. In particular, it is also a transporter of sodium and bile acids [6-8, 27, 29]. Rat Oatp1b2 is an enzyme-resistant inducer, which plays an important role in the maintenance of levels of endogenous substances [30]. Several studies have reported increased expression of the Oatp1b2 gene in rodents with nonalcoholic steatohepatitis [10]. ICA has recently been shown to inhibit OATP1B3 and affect Oatp-mediated cellular uptake of specific substrates as a basis of herb-drug interactions [19], as well as to modify the transport activity of OATP1B1 [20]. In the present study, ICA significantly decreased the abnormally increased levels of the Oatp1b2 mRNA and protein in SHRs.

Additionally, levels of the Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1b2 mRNAs and/or proteins were increased in SHRs. However, ICA reduced the expression of the aforementioned Oatps. Moreover, the increased blood pressure observed in SHRs was also reduced by ICA to some extent in the present study. Interestingly, most Oatps were thought to have important functions in bile acid transport [3], and bile acids have been shown to regulate blood pressure in rats [4, 5]. Rat Oatp1a1, Oatp1a4 and Oatp1b2 have been shown to mediate bile acid uptake in vitro [6-8]. Therefore, we speculated that one of mechanisms by which ICA lowers blood pressure might be through regulating the expression of Oatp1a1, Oatp1a4, Oatp1a5 or Oatp1b2 and subsequently affecting the transport of bile acids and other substances. However, further investigations are needed to verify this hypothesis.

Mrps are the major efflux transporters that mediate the efflux of drugs and metabolites in the liver [1]. Mrp2 mainly mediates the efflux of bile and the detoxification of endogenous and xenobiotic organic anions, particularly the unidirectional efflux of substances conjugated with glutathione, sulfate or glucuronate [30]. Mrp2 deficiency is responsible for Dubin-Johnson syndrome with conjugated hyperbilirubinemia [31]. The anomalous expressions of Mrp2 in subjects with liver diseases, such as cholestasis, fibrosis, hepatitis, cirrhosis, and liver cancer, has been extensively documented [32-36]. Meanwhile, Mrp2 is involved in drug disposition and drug-drug interactions [37]. In addition, bilirubin is ingested through OATPs and excreted into canaliculi via Mrp2 [38]. According to Sun P *et al.*, the efflux clearance of anti-hypertensive drugs in rats is mainly mediated by Mrp2 [9]. In the present study, ICA reduced the expression of the hepatic transporter Mrp2 in SHRs. Accordingly, we speculated

that when combined with ICA, the blood pressure-lowering effects of antihypertensive drugs might be enhanced. Further in-depth studies are needed to confirm the hypothesis.

In summary, ICA reverses the elevated expression of the hepatic transporters Oatp1a1, Oatp1a4, Oatp1a5, Oatp1b2 and Mrp2 in SHR.

Acknowledgments

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Abbreviations

Oatps/OATPs, organic anion transporting polypeptides; Mrps, multidrug resistance-associated proteins; SHRs, spontaneously hypertensive rats; ICA, Icariin; WKY, Wistar-Kyoto.

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