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Original Article

Aquaporins 1, 3 and 8 expression and cytokines in irritable bowel syndrome rats' colon via cAMP-PKA pathway

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Abstract: Background: Irritable bowel syndrome (IBS) is one of the most common gastrointestinal (GI) disorders. The cAMP-PKA pathway plays a key role in mediating cell responses to various stimuli both of which might play an important role in IBS. Objective: Our research aimed to detect the mechanism of IBS rat models via the cAMP-PKA pathway so as to further detect the mechanisms of IBS. Methods: IBS rats were built by stress and conditioned stimulus. The study was divided into 3 groups (a control group, a model group, and a forskolin group). The expressions of AQP1, AQP3, AQP8, and CREB (Serl33) in the colons of rats were detected by immunohistochemistry. The expressions of AQP1, AQP3, AQP8, IL-1β, TGF β, and TNF α in the colon were detected by the PCR technique. Results: The IBS rats were built successfully. The expressions of AQP1, AQP3, AQP8, and CREB (Serl33) were down-regulated in the colons of IBS rats and were up-regulated with the intervention of the activator of AMP ($P<0.05$). The expressions of IL-1β, TGF β and TNF α were up-regulated in the colons of IBS rats and were down-regulated with the intervention of the activator of AMP ($P<0.05$). Conclusions: The release of inflammatory cytokines, the activation of the immune system and liquid water metabolic abnormalities are the mechanisms of IBS via the cAMP-PKA pathway.

Keywords: Irritable bowel syndrome, cAMP-PKA, pathway

Introduction

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal (GI) disorders. It is a prevalent disease which results in significant morbidity and high healthcare costs and affects about 10-20% adults in most countries [1]. During 2003-2013, the incidence rates of IBS in men and women were respectively 48.90 per 10000 person-years and 53.51 per 10000 person-years [2]. However, the pathophysiology of IBS is still not clear [3]. The mechanism of IBS includes the brain-gut axis, disorders of immune function, neurohormonal regulation, altered bile acid metabolism, alterations in the epithelial barrier, and secretory properties of the gut [1].

The cAMP-PKA pathway is a part of the receptor G protein, and it plays an important role in mediating cell responses to various stimuli [4]. Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger, and it is usually elicited by the binding of hormones and neurotransmitters to G protein-coupled receptors (GPCRs) together. The activation of the cAMP-dependent protein kinase (PKA) is exerted as the physiological effects of cAMP by phosphorylating and regulating downstream protein target functions [5]. The activation of cAMP/PKA signal transduction alters downstream effectors by disturbing the structural and functional integrity of cells in order to affect homeostasis and fundamental biological processes [6].

Recently, it was reported that aquaporins (AQPs) play important roles in the water transport system in the human body [7]. There are currently 13 types of AQPs, ranging from AQP0 to AQP12, which are expressed in various organs. Many members of the AQP family are expressed in the intestinal tract. AQP1, AQP3, AQP4, and AQP7-10, which ultimately control fecal water content, are expressed in colon tis-

sue [8-10]. In addition, it has been reported that the pathophysiology of PI-IBS involves local low-grade inflammation and immune activation, which are primarily induced and maintained by specific cytokines [11]. Our research sought to detect the mechanism of IBS rat models via the cAMP-PKA pathway.

Methods

Subjects

We started with 18 adult female SD big rats, each weighing about 200 g. The feeding environment was provided by the Experimental Animal Center of Zhejiang Chinese Medical University. The 18 SD rats were divided randomly into three groups. There were 6 rats in each group which were put into an environment with a temperature of 22-24°C, the humidity <60%, and the noise <50 db.

Experimental procedure and methods

Group

1) Control group 1: Normal rats. We observed the condition of the rats after 2 weeks of normal eating and drinking, and the rats' visceral sensitivity was evaluated by the abdominal withdrawal reaction.

2) Model group 1: The conditioned stimulus was a camphor ball with a special odor. The unconditioned stimulus was rectal distention pressure (>60 mmHg (1 mmHg = 0.133 kPa)) combined with the rats' extremities being constrained.

The process: The rats were put into a cage with a camphor ball in it, and then we fixed the extremities and trunks of the rats for 45 min. At this time inserted a catheter into their rectums. The insertion distance was about 1 cm. The catheter was fixed at the root of the tail. The balloon volume was 1.6 ml and lasted for 60 s, repeated 10 times. This was a stress process.

The first day we did one process, and on the second day the process was performed at the same time. On the fourth day the conditioned stimulus was done. The fifth day the process was performed again. And the conditioned stimulus was performed on the sixth day and the eighth day. Then the rats' visceral sensitivity was evaluated by abdominal withdrawal reaction.

3) Forskolin group: The IBS model rats were injected with an AMP activator (forskolin, 5 mg/kg/d) for 7 days by intraperitoneal injection.

Model authentication

Visceral sensitivity was evaluated by abdominal withdrawal reaction (AWR). An 8F urethral catheter lubricated by liquid paraffin was inserted into the anus. The distance was about 1 cm, and it was fixed at the root of the tail. The rats were put on the platform, and after they became accommodated to the environment, we gradually affused water on to their sacculi and recorded the volume of the injected water when the rats raised their abdomens and made their backs like a bow. The rectal distention lasted for 30 s and was repeated 3 times. We recorded the mean number.

Experimental sample

After giving each rat a laparotomy incision, we removed the colon and placed it in an oxygenated Tyrode's solution. The colon tissue was put in a 10 ml organ bath containing Tyrode's solution, and it was bubbled with a 95% O_2 and 5% CO_2 mixture, and the temperature was held at 37°C.

Experimental procedure (immunohistochemical technique)

1) Two footwork: We used a dropper to drop 3% hydrogen dioxide on the tissue without light, and then the tissue was incubated for 15 min. Next, the tissue was washed with distilled water, and we put each tissue into a PBS balanced solution, and soaked them three times for 5 min. We dropped 50-100 ml antibody fluid on each tissue, and incubated it for 30 min. Then we washed the tissues with PBS and soaked them in a PBS balanced solution three times for 4 min. After that we washed the tissues with PBS and soaked them in the PBS balanced solution three times for 5 min. Then we dropped 50-100 ml developer DAB fluid onto the tissues, and incubated them for 5-20 min, and after this the coloration in the tissue was washed with distilled water. The tissue was dewatered by 85%, 90%, 95%, 100%, 100%,

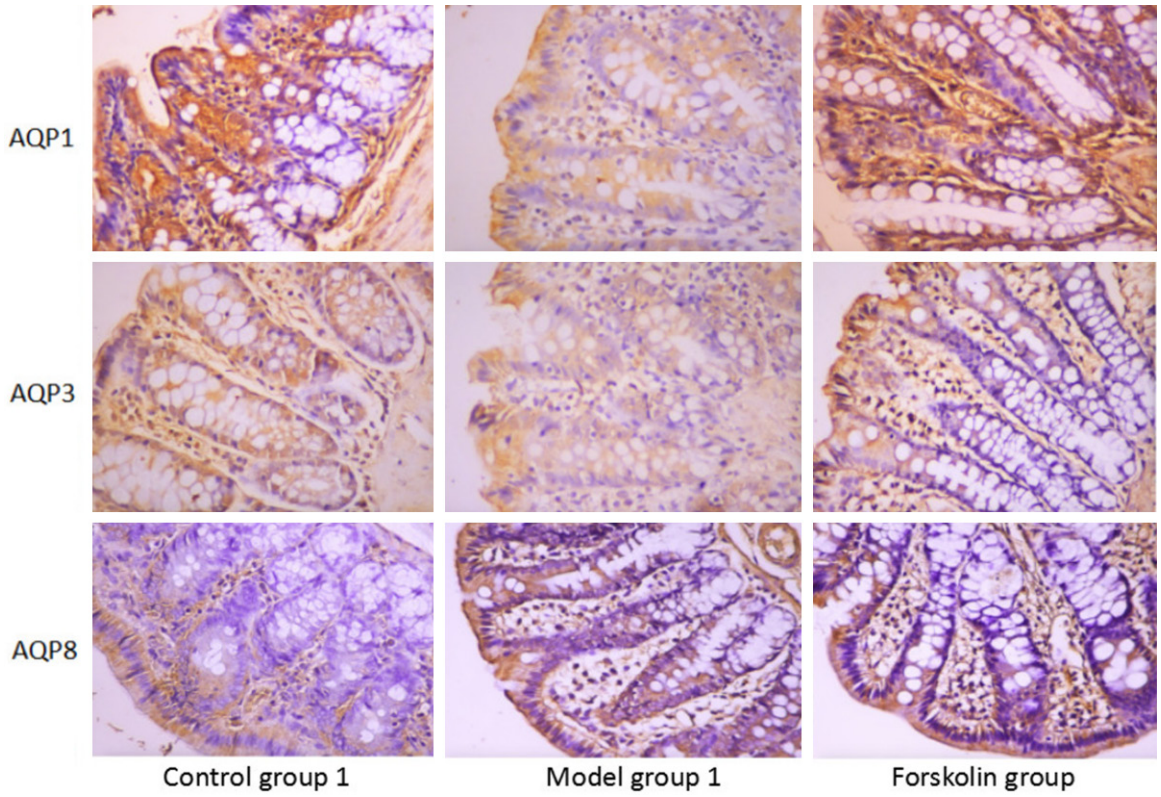

Figure 1. The expression of AQP1, AQP3, AQP8 in the colon by immunohistochemical technique of experiment 1 (*400) AQP1, AQP3, AQP8 positive reaction material presents brown, the cell membrane and cytoplasm were dyed.

100% alcohol. Then we put the tissue into xylene for three times for 5 min.

2) Negative control: We replaced one antibody with PBS, and the consequence was negative.

3) Analytical method: We used the computer image analysis software (the Carl Zeiss of the Imaging Systems of the Carl Zeiss company) to analyze the images. We searched the typical places, and took 10 high power campus visualis (×400) successively. Then we analyzed the masculine expression by quantitative analysis and calculated the photodensity.

Experimental procedure (RT-PCR technique)

After preparing the total RNA, a primer test and a sample assay were performed. Then reverse transcription was performed. A PCR reaction plate was prepared, and after that we ran the PCR reaction plate, and then we analyzed the result. The main procedure was the high-throughput sequencing that we conducted. Total RNA was extracted from the tissues using the one-step method. After purification, the RNA concentration was analyzed and quality testing was conducted using BioAnalyze. A small amount of RNA was purified from the total RNA to enrich molecules in the range of 16-30 nt, and then 3' and 5' linker sequences were attached, and then SuperScript II reverse transcriptase was used to synthesize cDNA.

Statistical analysis

The data was demonstrated as' $x \pm s$. We used the SPSS package to perform the statistical analysis. The two samples' mean numbers were compared by a T test. The significance level was $P<0.05$.

Results

Changes of the model

After completing the models, we found that there was no change of behavior. The rats in the IBS model group (including the model group and the group before injection) expressed an

Table 1. The expression of AQP1, AQP3, AQP8, NF-κB p65 and CREB (Serl33) in the colon by immunohistochemical technique of experiment 1

	Control group 1	Model group 1	Forskolin group
AQP1	1.786 ± 0.089	0.892 ± 0.129*	1.712 ± 0.157#
AQP3	1.056 ± 0.159	0.380 ± 0.092*	0.668 ± 0.045#
AQP8	2.042 ± 0.195	0.567 ± 0.102*	1.941 ± 0.172#
CREB (Serl33)	1.930 ± 0.266	0.831 ± 0.137*	1.427 ± 0.168#

**P<0.05 compared with control group; #P<0.05 compared with model group.*

excessive reaction when they were frightened or the intragastric administration was done on them. There was also no change of their stools.

Model authentication

The rectum effusion amount of the model rats (0.833 ± 0.143 ml) were lower than the control group (1.467 ± 0.054 ml), and the difference had statistical significance (P<0.01), so the sensitivity of the model rats was higher, and it suggested that the model building method was successful.

Expression of AQP1 in the colon by immunohistochemical technique

1) AQP1 cell immunochemical staining: The AQP1 positive reaction material presents as brown, the cell membranes and cytoplasms were dyed, but the negative control was not dyed (**Figure 1**).

2) The expression of AQP1 compared among the rats in the different group: The positive cell numbers of the model group was less than the control group (P<0.05), and the positive cell numbers of the forskolin group was more than model group (P<0.05). However, there was no difference between the control group and the forskolin group (P>0.05) (**Table 1**).

Expression of AQP3 in the colon by immunohistochemistry

1) AQP3 cell immunochemical staining: AQP3 positive reaction material presents as brown, the cell membranes and cytoplasms were dyed, but the negative control was not dyed (**Figure 1**).

2) The expression of AQP3 compared among the different group: The positive cell numbers of model group was less than the control group (P<0.05), and the positive cell numbers of forskolin group was more than model group (P<0.05). However, there was no difference between control group and forskolin group (P>0.05) (**Table 1**).

Expression of AQP8 in the colon by immunohistochemical technique

1) AQP8 cell immunochemical staining: The AQP8 positive reaction material presents brown, the cell membranes and cytoplasms were dyed, but the negative control was not dyed (**Figure 1**).

2) The expression of AQP8 compared among the different groups: The positive cell numbers of the model group were less than the control group (P<0.05), and the positive cell numbers of forskolin group were more than model group (P<0.05). However, there was no difference between the control group and the forskolin group (P>0.05) (**Table 1**).

Expression of CREB (Serl33) in the colon by immunohistochemistry

1) CREB (Serl33) cell immunochemical staining: CREB (Serl33) positive reaction material presents brown, the cell membrane and cytoplasm were dyed, but the negative control was not dyed (**Figure 2**).

2) The expression of CREB (Serl33) compared among the rats in the different group: The positive cell numbers of the model group was less than the control group (P<0.05), and the positive cell numbers of forskolin group was more than model group (P<0.05). However, there was no difference between the control group and the forskolin group (P>0.05) (**Table 1**).

Expression of AQP1, AQP3, AQP8,IL-1β,TGF β and TNF α in the colon by PCR technique

The expressions of AQP1, AQP3. and AQP8 mRNA of the model group were less than the control group (P<0.05), and the expressions of the forskolin group were more than model group (P<0.05), and the expressions of IL-1β, TGF β, and TNF α mRNA of the model group were more than the control group (P<0.05), and the expressions of forskolin group were less than the model group (P<0.05), but there was

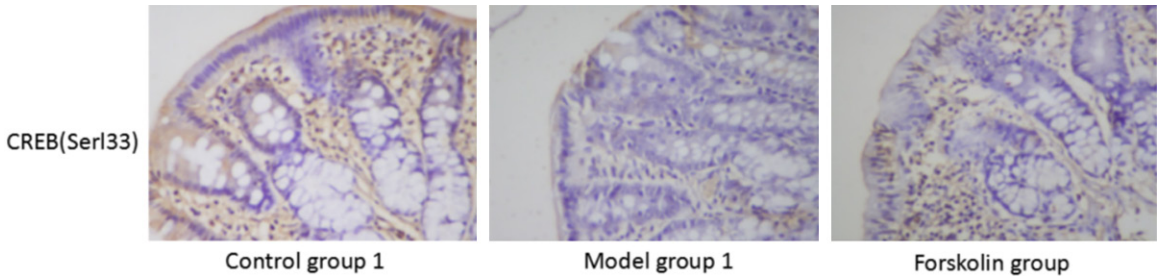

Figure 2. The expression of CREB (Ser133) in the colon by immunohistochemical technique of experiment 1 (*400) CREB (Ser133) positive reaction material presents brown, the cell membrane and cytoplasm were dyed.

Table 2. The expressions of AQP1, AQP3, AQP8,IL-1β,TGF β and TNF α in the colon by PCR of experiment 1

	Control group 1	Model group 1	Foskolin group
AQP1	0.899 ± 0.058	0.661 ± 0.061*	0.858 ± 0.040#
AQP3	1.125 ± 0.095	0.507 ± 0.103*	0.852 ± 0.060#
AQP8	1.148 ± 0.114	0.600 ± 0.099*	0.851 ± 0.044#
IL-1β	0.471 ± 0.072	1.002 ± 0.068*	0.805 ± 0.046#
TGF β	0.513 ± 0.032	1.466 ± 0.138*	0.807 ± 0.054#
TNF α	0.524 ± 0.084	1.216 ± 0.098*	0.791 ± 0.063#

$^{*}P<0.05$ compare with control group; $^{\#}P<0.05$ compared with model group.

no difference between the control group and the forskolin group (P>0.05) (**Table 2**).

Discussion

In our study, we found that AQP1, AQP3, and AQP8 were downregulated in the colons of IBS rats, and IL-1β, TGF β and TNF α were up-regulated in the colons of IBS rats. It is understood that aquaporins (AQPs) play a critical role in the transport of water and many other solutes across cell membranes [12]. Several AQPs were reported to be also detected in the human colon (AQP1, 3, 4, 7-9), and immunohistochemistry localized AQP1 to the apical plasma membrane of epithelial cells in the bottom of the crypts, whereas AQP3 (rat, human) and AQP4 (mice, human) were localized predominantly in the basolateral plasma membrane, and AQP8 was localized intracellularly and at the apical plasma membrane of epithelial cells, which suggested that these AQPs may be involved in the pathogenesis of bile acid-induced diarrhea [13]. As AQPs play important roles in the water transport system in the human body, AQP3 is reported to be one of the most important functional molecules in water transport in the colon, the expression level of which, in the colon, plays an important role in the laxative effects by osmotic laxatives and stimulant laxatives [14]. And AQP3 was reported to be expressed in the human small intestine via Northern blotting [15]. The mRNA expression and protein expressions of AQP3 and AQP8 are downregulated in the ileum and the colon, suggesting that AQP3 and AQP8 may play significant roles in the regulation of intestinal fluid homeostasis and disorders in rats [16]. Our study showed that the regulation of AQP1, AQP3 and AQP8 was involved in the mechanism of IBS which means liquid water metabolic abnormalities could be one of the mechanisms of IBS. That IL-1β increased in the systemic circulation in IBS was reported [17]; however, the expression level of IL-1β was not significantly different between the control group and the IBS group [18]. The plasma concentration of TGF-β was higher in patients with IBS, which means IBS has a state of altered immune regulation [19]. One the other hand, the higher serum level of IL-6, IL-8 and TNF-α in IBS was reported to suggest the important role of cytokines as immune mediators in the pathogenesis of functional GI disorder [20]. In our study, the result of TGF β and TNF α was in line with other studies, but the result of IL-1β was in doubt, so it needs further study to be resolved. The upregulation of IL-1β, TGF β, and TNF α suggests that the mechanism of IBS is involved in immune reaction.

The cAMP-PKA pathway is a part of the receptor G protein and the cAMP-PKA signaling pathway, and it plays an important role in mediating cell responses to various stimuli [21]. That the cellular effects of cAMP are mediated by PKA was confirmed [22]. The cAMP/PKA signaling pathway is considered to be involved in the metabolism, proliferation and development,

and moreover, some researchers have further claimed that the stimulation of the cAMP/PKA signal transduction pathway represents a novel mechanism for the regulation of cell death [23]. CREB is a cellular transcription factor [24], and it is phosphorylated subsequent to the cAMP activation of PKA. And then CREB translocates into the nucleus, where it activates the transcription of target genes [25, 26]. Now no study has reported that IBS is connected with the cAMP-PKA pathway, but our study revealed that the cAMP-PKA pathway might be an important signaling pathway in the mechanism of IBS.

In our study, it was found that the expressions of AQP1, AQP3, and AQP8 were upregulated, and IL-1β, TGF β, and TNF α were downregulated with the activator of AMP, which suggests that the cAMP-PKA pathway is involved in the mechanism of IBS by the regulation of AQPs, IL-1β, TGF β, and TNF α. On the other hand, we found that the expression of CREB (Serl33) was down-regulated in the colon of IBS rats and was up-regulated with the intervention of the activator of AMP. It has been reported that the cAMP-PKA-NF-kB pathway might play a key role in noradrenergic-mediated immune dysfunction and the immune system might be inhibited after a stroke, since the pro-inflammatory effect of NF-kB slacked off [27]. As a result, from the conclusion of our study we speculate that the cAMP-PKA pathway was inhibited for decreasing the expression of CREB (Serl33). As a result of the regulation of AQPs, IL-1β, TGF β, and TNF α, we speculate that the cAMP-PKA pathway plays an important role in IBS by the regulation of the release of inflammatory cytokines, the activation of the immune system. and liquid water metabolic abnormalities.

Conclusion

The cAMP-PKA signaling pathway is involved in the mechanism of IBS. The release of inflammatory cytokines, the activation of the immune system, and liquid water metabolic abnormalities are the mechanisms of IBS which are related to the cAMP-PKA pathway.

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Disclosure of conflict of interest

None.

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