Protective effects of catalpol and rhein in murine experimental autoimmune encephalomyelitis via regulation of T helper (Th) 1, Th2, Th17, and regulatory T cell responses

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Abstract

OBJECTIVE: To examine the effects of catalpol and rhein on pro- and anti-inflammatory responses in C57BL/6 mice with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis.

METHODS: Female C57BL/6 mice were randomly divided into four groups \((n = 30)\): (a) normal saline control, (b) EAE control, (c) EAE + prednisone acetate \((PA, 6 \text{ mg/kg})\), and (d) EAE + catalpol \((40 \text{ mg/kg})\) and rhein \((5 \text{ mg/kg})\). EAE was induced by injection of myelin oligodendrocyte glycoprotein 35-55 plus pertussis toxin. Treatments were orally administered daily for 40 d. Disease progression and neurological function were assessed using a semi-quantitative scale of tail and limb paralysis. Brains and spinal cords were collected on Days 6, 20, and 40 and assessed for histopathological changes by hematoxylin and eosin staining. Production of interleukin \((\text{IL})\)-2, IL-4, IL-10, and IL-17A protein was measured by enzyme-linked immunosorbent assay. Expression of the T helper \((\text{Th})\)1-, Th2-, Th17-, and regulatory T cell \((\text{Treg})\)-specific transcription factors T-bet, GATA3, ROR-\(\gamma\)-t, and Foxp3, respectively, were analyzed by quantitative reverse-transcription polymerase chain reaction and western blot analysis.

RESULTS: Combination treatment with catalpol and rhein significantly alleviated the clinical disability and neurological dysfunction of mice with EAE. Catalpol and rhein treatment also reduced the infiltration of pro-inflammatory T cells into pathological lesions; significantly increased the expression of the anti-inflammatory factors GATA3, Foxp3, IL-4, and IL-10; and significantly decreased the expression of the pro-inflammatory factors T-bet, ROR-\(\gamma\)-t, IL-2, and IL-17A.

CONCLUSION: Catalpol and rhein reduced the neurological disabilities of mice with EAE, at least in part by rebalancing the pro- and anti-inflammatory environment in the brains and spinal cords.
Keywords: Multiple sclerosis; Encephalomyelitis; Autoimmunity; Catalpol; Rhein; Th1-Th2 balance; Th17 cells

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that manifests mainly as a relapsing-remitting disease, with fewer patients experiencing primary-progressive disease in which the damage steadily increases without periods of remission. Experimental autoimmune encephalomyelitis (EAE) is the most common animal model of MS. Mice injected with myelin-derived peptides and adjuvant develop inflammatory cell infiltration around small blood vessels in the CNS, followed by demyelination of neurons, and muscle and limb weakness similar to that experienced by MS patients.

Previous studies have demonstrated that the balance between CD4+ T cell subsets play a crucial role in the pathogenesis of MS/EAE. Antigen-specific activation of naïve CD4+ T cells in the presence of interferon-γ (IFN-γ), interleukin (IL)-4, or IL-17 induces their differentiation into the three major T cell subsets; T helper (Th) 1, Th2, or Th17, respectively. In addition, regulatory T (Treg) cells can differentiate under certain conditions in the periphery. These subsets have distinct functions and characteristic patterns of cytokine production. The predominantly pro-inflammatory Th1 and Th17 cells produce cytokines such as IFN-γ and tumor necrosis factor (TNF-α) (Th1) and IL-17 and IL-22 (Th17). Anti-inflammatory Th2 cells produce IL-4 and IL-5, and Treg cells produce immunosuppressive transforming growth factor β (TGF-β) and IL-10. Early work suggested that MS was largely due to an imbalance between Th1 and Th2 cell responses. However, more recent studies have shown that multiple T cell subsets act in concert to promote or suppress neuroinflammation.

TNF-α and IFN-γ are classic pro-inflammatory cytokines and have been shown to promote tissue damage in MS. Th17 cells have also been shown to play a critical role in the pathogenesis of CNS demyelinating diseases through mechanisms distinct from Th1 cells. In mice, depletion of Th17 cells prevented or alleviated the symptoms of EAE and other autoimmune diseases; similarly, the production of TGF-β and IL-23 by Th17 cells is known to contribute to MS/EAE. Conversely, Th2 and Treg cells prevent tissue damage by suppressing the immune response and inhibiting excessive production of inflammatory factors. Tregs act as negative regulators of the T cell response by suppressing activation of T cells and maintaining immune tolerance.

In MS/EAE, Tregs reduce injury to the blood – brain barrier, prevent the infiltration of T cells and macrophages, and inhibit Th17 cell proliferation via secretion of IL-10. Thus, interventions that seek to rebalance the activities of Th1, Th2, Th17, and Treg cells could be effective treatments for MS/EAE.

Our previous studies showed that Bushen Yisui capsules could reduce the neurological dysfunction in patients with MS and mice with EAE. Catalpol, an iridoid glycoside and active ingredient of Bushen Yisui capsules, has been shown to have a protective effect similar to that induced by the capsules. Catalpol increases the expression of oligodendrocyte transcription factors 1 and 2 (Olig1/2), thus promoting the proliferation, migration, and differentiation of oligodendrocyte precursor cells into mature oligodendrocytes. Other studies have shown that rhein, an anthraquinone of plant origin, has significant anti-inflammatory effects, including the inhibition of IL-2 secretion by Th1 cells. We previously showed that combination treatment with catalpol and rhein can significantly reduce the neurological deficits of mice with EAE. In the present study, we investigated the immune mechanisms underlying the beneficial effects of catalpol and rhein in mice with EAE, thereby laying the foundation for the possible development of this combination as a therapy for MS.

MATERIALS AND METHODS

Experimental animals

Female C57BL/6 mice (aged 6–8 weeks; weight 18.0–22.0 g) were fed in the Center of National Standard Laboratory Animals at Capital Medical University (certification No. SCXK, Beijing, 2012-0001). The mice were maintained in a pathogen-free environment on a 12-h light/dark cycle. The experiments were approved by the Ethics Committee of Capital Medical University (No. AEEI-2014-018).

Chemicals and reagents

Catalpol and rhein were purchased from China Food and Drug Testing Institute (Beijing, China), and prednisone acetate (PA) was from Tianjin Pacific Pharmaceutical Co., Ltd. (Tianjin, China). Myelin oligodendrocyte glycoprotein (MOG) peptide (MEVGWYRRSPFSRVVHLYNRK, purity > 95%) was synthesized by Beijing Kangwei Century Biotechnology Co., Ltd. (Beijing, China). Complete Freund’s adjuvant and pertussis toxin were from Abcam (Cambridge, UK); Mycobacterium tuberculosis was from Difco (San Diego, CA, USA); and enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-2, IL-4, IL-10, and IL-17A were obtained from Abcam. Mouse anti-mouse T-bet, rabbit anti-mouse ROR-γt, rabbit anti-mouse GATA3, and rabbit anti-mouse Foxp3 were purchased from Abcam, and mouse anti-β-actin was from Immunoway (Shanghai, China). Horseradish peroxidase-conjugated sheep anti-rabbit IgG and sheep anti-mouse IgG were from Shanghai Abmart Biological Medicine Co., Ltd.
Western blot kits were from Beijing Cybernaut Biotechnology Center (Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) kits and reverse transcription kits were from Tiangen Biotech Co., Ltd. (Dalian, China), and polymerase chain reaction (PCR) primers were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China).

**Model establishment and experimental treatment**

The mice were randomly divided into four groups \((n = 30)\): normal control, EAE model control, PA-treated (EAE+PA), and catalpol and rhein-treated (EAE+CR).

To induce EAE, mice were injected subcutaneously with 200 μL of antigen-containing emulsion \((100 \mu L \text{ complete Freund’s adjuvant}, 100 \mu L \text{ normal saline (NS)}, \text{ and } 50 \mu g \text{ MOG}_{35-55} \text{ on Day } 0\), followed by intraperitoneal injection of 500 ng of pertussis toxin on Days 0 and 2. Starting on Day 0, the CR-treated EAE group was orally administered 40 mg/kg catalpol and 5 mg/kg rhein daily until Day 40. The PA-treated EAE group was orally administered PA \((6 \text{ mg/kg})\) daily starting at disease onset (-day 16-17) until Day 40. Normal and EAE groups were administered NS. All compounds were administered between 9:00 a.m. and 11:00 a.m.

Neurological function (clinical score) was assessed as:

- 0: no paralysis;
- 1: flaccid tail;
- 2: moderate hind-limb paralysis or gait instability;
- 3: complete hind-limb paralysis;
- 4: partial or complete fore-limb paralysis; and
- 5: death. The average score per group was reported.

**Sample collection**

Groups of mice were sacrificed on Day 6 (early stage; no neurological symptoms), Day 20 (acute stage; neurological function scores at or near the peak), and Day 40 (remission stage; no further progression of EAE signs). The brains and spinal cords were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

**Histopathology**

Brain tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (thickness, 3 μm), and stained with hematoxylin and eosin (HE). Sections were observed using a light microscope (Nikon Eclipse 80i, Tokyo, Japan).

**Cytokine measurement by ELISA**

The brains and spinal cords were weighed, homogenized with cold NS \((1:9, \text{ wt/vol})\), and centrifuged at 3000 rpm for 20 min at 4 °C. The supernatants were removed and analyzed for cytokine content using the recommended protocols of the ELISA kit manufacturers. IL-2, IL-4, IL-10, and IL-17A concentrations were determined using standard curves.

**Western blot analysis**

Protein extraction and quantification were performed according to the procedures specified by the manufacturers. Western blot analysis was performed according to standard protocols. In brief, samples containing 20 μg protein were resolved by 5% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrotransferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked and then incubated with primary antibodies: (a) anti-T-bet \((1: 500 \text{ dilution})\), (b) anti-GATA3 \((1: 1000)\), (c) anti-ROR-γt \((1: 1000)\), (d) anti-Foxp3 \((1: 5000)\), and anti-β-actin \((1: 5000)\) in blocking solution at 4 °C overnight. After washing, the membranes were incubated with secondary goat anti-mouse or goat anti-rabbit IgG \((1: 10000 \text{ dilution})\) for 60 min, and then incubated in the electrochemiluminescence reagent for 1 min. Finally, the membranes were exposed to film (Tokyo, Japan), and the bands were quantified using ImageQuant TL 2005 image analysis software (Amersham, Biosciences, Piscataway, NJ, USA).

**mRNA analysis by qRT-PCR**

Total RNA was extracted from approximately 30 mg brain tissue according to the manufacturer’s instructions. The concentration and integrity of the RNA samples were measured by spectrophotometry and agarose gel electrophoresis, respectively. cDNA was synthesized from total RNA using a reverse transcription kit. Quantitative PCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA, USA) with the following amplification conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 10 s, 52 °C for 30 s, and 72 °C for 30 s. Relative quantification of mRNA levels was performed using the 2^{ΔΔCt} method, with normalization to β-actin mRNA levels. PCR primers were designed using Primer Premier 5.0 software and were based on the Genbank sequences as follows: T-bet Forward (F), 5’-CCTGGAACCCACTGTAACCTGCTT-3’, T-bet Reverse (R), 5’-CAGCTC- GGAAATCCTCC-GCTTCATAAC-3’; ROR-γt F, 5’-GGTCCAGA-CAGCCACTGATTCA-3’, ROR-γt R, 5’-GCTCC-GCTGCCGTTAGAGGT-3’; GATA-3 F, 5’-CTGAGGAGGAGACGCTAACTG-3’, GATA-3 R, 5’-GGTCCAGA-CAGCCACTGATTCA-3’, and β-actin F, 5’-GGTCCAGA-CAGCCACTGATTCA-3’, and β-actin R, 5’-GGTCCAGA-CAGCCACTGATTCA-3’. The amplified fragments were 179, 153, 131, 165, and 159 bases.

**Statistical analysis**

The data were analyzed with Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and are expressed as the mean ± standard error of mean (SEM). All data were first checked for normality. Normally distributed data were analyzed using one-way analysis of variance with
RESULTS

Neurological function and weight change
Mice with EAE developed neurological disease starting on about Day 16. Sequentially appearing symptoms included flaccid tail, staggering gait, hind-limb paralysis, four-limb paralysis, and death. Neurological function scores in the EAE group reached a peak on Day 19 (1.5 ± 0.6) and declined thereafter. From Day 35 onwards, the scores for the catalpol and rhein-treated mice were significantly lower than the scores for the EAE group (Figure 1A). Similarly, the body weight of mice with EAE decreased dramatically at disease onset, but mice treated with catalpol and rhein showed less severe weight loss (Figure 1B).

Pathological changes in the brain tissues of EAE mice
To assess the effects of catalpol and rhein on the brain pathology of mice with EAE, we examined HE-stained brain sections from mice sacrificed on Days 20 and 40 after disease induction (Figure 2). The brains of normal mice showed intact and orderly neuronal structure, with distributed glial cells and very few lymphocytes around small venous vessels (Figure 2, A1, B1). In contrast, analysis of the EAE group revealed a large number of inflammatory cells aggregated around the small blood vessels, forming "sleeve-like" structures typical of the acute stage (Day 20, Figure 2, A2), and this was still present at a reduced level in the remission stage (Day 40; Figure 2, B2). Consistent with the effects of catalpol and rhein on the neurological symptoms, the brains of catalpol and rhein-treated mice showed a striking reduction in the inflammatory response in both the acute and remission phases of disease (Figure 2, A3-4, B3-4).

IL-2, IL-4, IL-10, and IL-17A protein levels in the brains and spinal cords of mice with EAE
To assess cytokine production in the CNS, brain and spinal cord extracts were prepared from mice sacrificed on Days 6, 20, and 40 post-induction, and cytokines were quantified by ELISA. Compared with the levels in normal mice, the brains and spinal cords of mice with EAE contained significantly higher concentrations of IL-2 on Days 6, 20, and 40, and significantly higher concentrations of IL-17A on Days 6 and 20. In contrast, mice treated with catalpol and rhein or PA showed significantly reduced production of IL-2 in the brains and spinal cords on Days 20 and 40 compared with the EAE group. PA treatment significantly reduced IL-17A levels in the brain and spinal cord on Day 20, and catalpol and rhein treatment significantly reduced IL-17A in the brain on Day 20 and in the spinal cord on Day 40. Finally, Catalpol and Rhein-treated mice showed reduced IL-17A expression in the spinal cord on Day 20 compared with the PA-treated mice (Figure 3).

Anti-inflammatory factors such as IL-4 and IL-10 are key players in determining the outcome of EAE. Compared with tissues from normal mice, the brains and spinal cords of mice with EAE contained significantly different levels of IL-4 and IL-10 at various times during disease development and remission (Figure 3). Thus, on Day 6, IL-4 and IL-10 levels were reduced in the brains of EAE mice compared with normal mice; on Day 20, IL-4 and IL-10 levels were reduced in both the brains and spinal cords, and on Day 40, IL-4 levels in the brains and IL-10 levels in the spinal cords were reduced. However, catalpol and rhein or PA treatment significantly increased IL-4 and IL-10 levels. In the PA-treated mice, IL-10 levels in the brains and spinal cords were elevated on Day 20. In the catalpol and rhein-treated mice, IL-10 was elevated in the brains on Days 20 and 40 and in the spinal cords on Day 20, whereas IL-4 was increased in the brains and spinal cords only on Day 20 (Figure 3). Thus, catalpol and rhein treatment effectively reduced the production of pro-inflammatory cytokines and increased the production of anti-inflammatory cytokines in the CNS of mice with EAE.

Figure 1 Clinical score and body weight of mice with EAE
A: clinical score; B: body weight. Normal and EAE groups were administered normal saline (2 mL each). EAE + PA and EAE + CR groups were treated daily with PA (6 mg/kg) or CR (40 and 5 mg/kg, respectively) for 40 d. Normal: normal control; EAE: EAE model control; EAE + PA: PA-treated; EAE + CR: catalpol and rhein-treated. EAE: experimental autoimmune encephalomyelitis; PA: prednisone acetate; CR: catalpol + rhein. Data are the means of 30 mice/group.
**T-bet, GATA3, ROR-γt, and Foxp3 mRNA expression in the brains of mice with EAE**

To assess expression of the four pivotal T cell transcription factors in mice with EAE, we performed qRT-PCR analysis of brain tissue on Days 6, 20, and 40 post-induction of disease (Figure 4). Compared with the normal mice, the brains of mice with EAE contained significantly higher levels of T-bet mRNA on Days 6 and 20, but not on Day 40. However, the increase in T-bet transcription was significantly reduced in catalpol and rhein-treated mice on Days 6, 20, and 40, whereas T-bet mRNA levels were significantly reduced in PA-treated mice only on Days 20 and 40 (Figure 4).

GATA3 mRNA levels were significantly lower in the brains of mice with EAE compared with normal mice on Days 20 and 40, but the reduction was significantly smaller in the brains of PA-treated and, even more so, in catalpol and rhein-treated mice (Figure 4).

ROR-γt mRNA levels were significantly higher in the brains of mice with EAE compared with normal mice on Days 20 and 40, but not on Day 6. Compared with mice with EAE, catalpol and rhein-treated mice showed significantly lower brain expression of ROR-γt on Days 20 and 40 (Figure 4).

Foxp3 mRNA levels were lower on Day 20 in the brains of mice with EAE compared with normal mice; however, catalpol and rhein treatment reversed this effect and Foxp3 express levels were increased on Days 20 and 40. The effects of catalpol + rhein and PA on ROR-γt expression differed significantly on Day 20 after disease induction (Figure 4).

**Western blot analysis of T-bet, GATA3, ROR-γt, and Foxp3 protein expression in the brains of mice with EAE**

To verify the qRT-PCR analysis results, we performed western blotting of brain tissues from the untreated and treated mice. We found that T-bet protein expression was significantly increased in mice with EAE compared with normal mice on Days 6, 20, and 40. Catalpol and rhein, but not PA, reduced the disease-associated rise in T-bet protein expression on Day 6, whereas both treatments reduced the expression on Day 20, and neither treatment had an effect on Day 40 (Figure 5).

GATA3 protein expression in the brain was decreased in mice with EAE compared with normal mice on Days 20 and 40. Compared with mice with EAE, catalpol and rhein treatment increased GATA3 protein expression on Days 20 and 40, but PA treatment had a significant effect only on Day 20 (Figure 5).

Brain expression levels of ROR-γt protein were significantly higher in mice with EAE compared with normal mice on Days 20 and 40; however, the levels were reduced on both days by treatment with either PA or catalpol and rhein (Figure 5).

Finally, Foxp3 protein expression in the brains of mice with EAE was significantly lower than that in normal mice on Day 20. Notably, catalpol and rhein treatment significantly increased the expression of Foxp3 compared with the untreated mice with EAE on both Day 20 and Day 40 (Figure 5).
A balance between T cell activation and self-tolerance is essential for the maintenance of immune homeostasis. In autoimmune and inflammatory diseases such as MS, this balance is perturbed, leading to aberrant activation of self-reactive T cells and uncontrolled production of inflammatory mediators. The absence of

DISCUSSION

Figure 3 ELISA analysis of IL-2, IL-4, IL-10, and IL-17A protein concentrations in the brains and spinal cords of mice with EAE A1, A2: IL-2; B1, B2: IL-4; C1, C2: IL-10; D1, D2: IL-17A. A1, B1, C1, D1: brain; A2, B2, C2, D2: spinal cord. Normal and EAE groups were administered normal saline (2 mL each). EAE+PA and EAE+CR groups were treated daily with PA (6 mg/kg) or CR (40 and 5 mg/kg, respectively) for 40 d. Normal: normal control; EAE: EAE model control; EAE+PA: PA-treated; EAE + CR: catalpol and rhein-treated. Data are the mean ± standard error of mean of 30 mice/group. EAE: experimental autoimmune encephalomyelitis; PA: prednisone acetate; CR: catalpol + rhein; IL: interleukin; ELISA: enzyme-linked immunosorbent assay. "P < 0.05, "P < 0.01, "P < 0.001, compared with the normal group; "P < 0.05, "P < 0.01, "P < 0.001, compared with the EAE group; "P < 0.05, compared with the EAE + PA group.
proper negative regulation and immune tolerance results in excessive levels of inflammation, which eventually leads to tissue damage and destruction. In this study, we employed the EAE mouse model to identify the immune mechanisms underlying the previously established beneficial effects of catalpol and rhein in humans with MS and mice with EAE. To this end, we compared the pattern of T cell subset-specific cytokine and transcription factor expression; namely, T-bet and IL-2 (Th1), GATA3 and IL-4 (Th2), ROR-γt and IL17A (Th17), and Foxp3 and IL-10 (Tregs), in the brains and spinal cords of mice with EAE, using PA as a positive control treatment. Our results suggest that the combination of catalpol and rhein can reduce differentiation of naïve CD4+ T cells into Th1 cells and promote Th2 cell differentiation by decreasing and increasing expression of T-bet and GATA3, respectively. Uregulated T-bet expression not only induces Th0 to Th1 differentiation but also redirects differentiated Th2 cells to acquire Th1 properties, thus reducing the abundance of Th2 effector cells. Conversely, increased expression of GATA3 inhibits the differentiation of Th1 cells and INF-γ secretion while maintaining the phenotype of terminally differentiated Th2 cells.

Consistent with the analyses of transcription factors, we detected increased and reduced concentrations of IL-2 and IL-4, respectively, in the lesions of catalpol and rhein-treated compared with untreated mice with EAE. IL-2 is mainly involved in cellular immune responses, particularly the function of phagocytes and the activation of effector T cells, whereas IL-4 plays a major role in the development and maintenance of humoral immunity. In the pathogenesis of EAE, Th1 cytokines have been shown to aggravate inflammatory tissue damage, while Th2 cytokines have a more immunosuppressive regulatory role.

We also investigated the expression of Th17- and Treg-associated transcription factors (ROR-γt and Foxp3, respectively) and cytokines (IL-17A and IL-10, respectively) in the mice with EAE. We found that catalpol and rhein decreased the expression of ROR-γt and increased that of Foxp3, thus reducing Th17 cell differentiation and promoting Treg cell differentiation. ROR-γt directly promotes the transcription of IL-17A, suggesting that it could be a potential therapeutic target for chronic autoimmune and inflammatory diseases. As a characteristic marker of Treg cells, Foxp3 plays a vital
role in regulating the expression of immunosuppressive molecules, and is indispensable in maintaining self-immune tolerance and homeostasis. As expected, we found that the effects of catalpol and rhein on IL-17A and IL-10 expression closely mirrored the patterns observed for ROR-γt and Foxp3, respectively, in the brains and spinal cords of mice with EAE. These data suggested that catalpol and rhein decreased and increased the differentiation of Th17 and Treg cells, respectively. A recent clinical study demonstrated that IL-17A levels correlated significantly with the incidence of MS. IL-17A is the main functional member of the IL-17 family of cytokines, and dysregulation of its inflammatory functions can lead to autoimmune disease. IL-10 is a multifunctional cytokine that regulates cell growth and differentiation in addition to other functions in the immune response. IL-10 is secreted by almost all immune cells and plays a key role in suppressing activation of the innate and adaptive immune response and maintaining immunologic homeostasis. As such, IL-10 is an important inhibitory factor in the regulation of inflammation and immunity.

In conclusion, we showed here that combination therapy with catalpol and rhein inhibits pro-inflammatory transcription factor and cytokine expression and promotes anti-inflammatory factor and cytokine expression in mice with EAE. The neuroprotective effects of catalpol and rhein in alleviating inflammatory CNS injury and the subsequent neurofunctional deficit suggests that this combination therapy may have utility as a treatment for MS.
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