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Metabolism of Wogonoside by Human Fecal Microflora and Its Anti-pruritic Effect

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Abstract − To understand the relationship between the metabolism of wogonoside from the rhizome of *Scutellaria baicalensis*, and its anti-pruritic effect, we anaerobically incubated it with human fecal microflora, identified its metabolite identified, and investigated its anti-pruritic effect in compound 48/80 or histamine-induced pruritic mice. Wogonoside was metabolized to wogonin, with metabolic activity of 6.9 ± 5.1 nmol/h/mg wet weight of fecal microflora. Orally administered wogonoside had more potent anti-scratching behavioral effect in compound 48/80 or histamine-treated mice than intraperitoneally treated one, apart from orally administered its metabolite, wogonin, which was more potent than the orally administered one. Wogonoside showed more potent anti-pruritic effects when administered at 5 h prior to the pruritic agent treatment than when administered at 1 h before. However, wogonin orally administered 1 h before the treatment with pruritic agents showed a more potent anti-pruritic effect than when treated at 5 h before. Orally administered wogonoside may be metabolized to wogonin in the intestine and its anti-scratching behavioral effect may be dependent on its metabolism by intestinal microflora.

Keywords: *Scutellaria baicalensis*, wogonoside, wogonin, pruritus, metabolism, intestinal microflora

INTRODUCTION

Most herbal medicines are orally administered to humans. Their components are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Many components may be transformed by the intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of the components by human intestinal microflora are of a great importance to an understanding of their biological effects (Kobashi and Akao, 1997; Akao *et al.,* 1998).

The rhizome of *Scutellaria baicalensis* (SB), which contains baicalin and wogonoside as the main constituents, has long been used in China, Japan and Korea as a traditional medicine and functional food for inflammation, fever, hepatitis, allergic disease, hypertension, etc (Zhu, 1998; Wu *et al.,* 2005). These constituents exhibit anti-inflammatory, antiallergic, anti-oxidant, and hepatoprotective, and antitumor effects (Chou *et al.,* 2003; Jang *et al.,* 2003; Lim, 2003; Kim *et al.,* 2005). These components are poorly absorbed from the gastrointestinal tract in its native form due to their hydrophilic properties and must be hydrolyzed by intestinal microflora in the intestine to their aglycones in human and rats (Akao *et al.,* 2000; Yim *et al.,* 2004). The absorbed metabolite, baicalein, is subsequently conjugated to baicalin in the gut mucosae by UDP-glucuronyl transferase and approximately half of the conjugate is excreted back into the gut. The absorbed baicalein are then extensively metabolized in the liver and excreted in urine and the feces via bile (Abe *et al.,* 1990; Akao *et al.,* 2000; Akao *et al.,* 2004; Xing *et al.,* 2005a; Lu *et al.,* 2007). However, studies on the metabolism of wogonoside have not been performed.

Therefore, to understand the relationship between the metabolism of wogonoside from the rhizome of SB, and its anti-pruritic effect, we anaerobically incubated it with human fecal microflora, identified its metabolite identified, and investigated its anti-pruritic effect in compound 48/80 or histamine-induced pruritic mice.

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MATERIALS AND METHODS

Materials

Azelastine, compound 48/80, and histamine were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.).

Isolation of wogonoside from the rhizome of SB

The cut and dried rhizomes of SB were purchased at KyungDong Market, Seoul, Korea (produced in Korea), and identified by one of the authors, NI Baek. The voucher specimen was deposited at the Grandual School of Biotechnology, Kyung Hee University. The cut and dried rhizomes (1 kg) were extracted with 80% MeOH (4 L×2) at room temperature for 24 h. The extracted solution was filtered through filter paper (No. 2) and concentrated by vacuum rotary evaporator. The concentrates were poured into H_2O (3 L) and extracted using EtOAc (1 L×2) and *n*-BuOH (1 L×2), successively. Each layer was evaporated to afford an EtOAc fraction (18 g), a *n*-BuOH fraction (16 g), and an aqueous fraction (SBW, 285 g). A part of fraction SBW (140 g) was dissolved in H_2O (2 L) and acidified to pH 1 by adding 2 N HCl, and stored at 10°C for one day. The fraction SBW (140 g) was dissolved with 50% MeOH (430 ml) and stored at 10°C for one day. The precipitate was filtered through a glass filter and washed with cold H_2O to yield purified wogonoside (1.5 g). These compounds were identified by comparison of their NMR data with those of the reported in literature (Takido et al., 1975; Tomimori et al., 1982; Lee et al., 2003).

Wogonoside (2) Yellow powder (H_2O); mp 194-196°C; [α]D -8.1°; FABMS 461[M+1]+.

Metabolism of wogonoside by human fecal microflora

The human fecal specimens (about 30 g) prepared according to a previous method (Lee *et al.,* 2003) were collected in plastic cups 9 h after fasting, and then carefully mixed with a spatula and suspended with cold 270 ml saline. The fecal suspension was centrifuged at 100×g for 5 min. The supernatant was then centrifuged at 10,000×g for 20 min. The resulting precipitates (about 3 g) were used as a metabolic enzyme source for the assay of enzyme activity. The preparation and assay of the enzyme source were performed within 24 h at 4°C.

To identify the metabolites of wogonoside by human intestinal bacteria, the reaction mixture contained 100 mg/ml of wogonoside and 2.5 g fresh human feces in a final volume of 500 ml of anaerobic dilution medium. The mixture was incubated at 37°C for 20 h. An aliquot (1 ml) of the reaction mixture was periodically extracted with ethyl acetate, concentrated, dissolved in MeOH, and their metabolites were analyzed by LC-MS/MS (Agilent 1,200 series LC-MS/MS system consisting of a quaternary pump, a vacuum degasser, an autosampler, a thermostat column compartment, a diode array detector, and an Agilent G6410 triple quadrupole mass spectrometer with an electro spray ionization (ESI) source): column, ZORBAX Extend C18 (100×2.1 mm i.d., 1.8 μm, Agilent); elution solvent, a linear-gradient applied by 5% in solvent A to 95% B for 13 min (solvent A - 0.1% aqueous formic acid and solvent B - acetonitrile); and elution rate, 0.3 ml/min. Mass spectra was acquired in ESI mode using nitrogen gas at a temperature of 350°C, flow rate of 10 L/min, nebulizer pressure of 45 psi, quadrupole temperature of 30°C, and capillary voltage of 4,000 V. The mass spectrometer was operated in positive mode with multiple reaction monitoring (MRM). The instrument was controlled and data were processed by Agilent MassHunter workstation software (Rev. B.01.00).

Each odd reaction mixture was evaporated to afford an EtOAc fraction (38 mg). The ethyl acetate fraction was subjected to a MPLC equipped with a slica gel 100 C18 column (3×50 cm) - reversed phase eluted with 30% MeOH (5 L). The EtOAc fraction of wogonoside gave the metabolite M1 (12 mg, wogonin). The metabolite was verified by comparison of its NMR data with those of the reported in literatures (Takido *et al.,* 1975; Tomimori *et al.,* 1982).

M1 (Wogonin) Pale yellow needles. mp 198-199oC, EI-Mass (*m/z*): 284 [M^+]. ^{1}H-NMR (400 MHz, C_5D_5N, δ): 12.35 (1H, s), 8.73 (1H, s), 8.07 (2H, d, *J*=6.8 Hz), 7.61 (3H, m), 7.04 (1H, s), 6.94 (1H, s), 3.88 (3H, s). ^{13}C-NMR (100 MHz, C_5D_5N, δ): 182.43 (C-4), 163.61 (C-2), 151.38 (C-7), 156.08 (C-5), 149.25 (C-9), 132.42 (C-4'), 130.84 (C-1'), 129.43 (C-8, 3', 5'), 126.54 (C-2', 6'), 105.40 (C-3, 10), 98.98 (C-6), 61.37 (OCH_3).

Animals

The male ICR and BALB/c mice (18-22 g) were supplied from Charles River Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20-22°C, a relative humidity of 50 ± 10% humidity, a frequency of air ventilation of 15-20 times/h, and 12 h illumination (0.7:00-19:00; intensity, 150-300 Lux), fed standard laboratory chow (Charles River Orient Experimental Animal Breeding Center, Seoul Korea) and allowed water ad libitum. All procedures relating to the animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23, revised 1985).

Pruritic behavioral experiments

Before the experiment, the BALB/c (for compound 48/80 as a pruritic inducer) and ICR (for histamine) mice were put into acrylic cages (22×22×24 cm) for about 10 min for acclimation. The behavioral experiments were performed according to the method of Sugimoto *et al.* (1998). The rostral part of the skin on the back of mice was clipped, and 50 μg/50 μl of compound 48/80 or 300 μg/50 μl of histamine for each mouse was intradermally injected with the use of a 29 gauge needle. The pruritic agents were dissolved in saline prior to injection. Control mice received a saline injection in the place of the pruritic agent. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage and, for the observation of pruritus; their behaviors recorded using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea) under unmanned conditions. The pruritus of the injected site by the hind paws was counted and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed a pruritic frequency of several scratches per second, and a series of these behaviors were counted as one incident of the pruritus for 60 min. Test agents were orally or intraperitoneally administered either 1 h or 5 h before treatment with the pruritic agent, histamine, in mice

The inhibition (%) against pruritic reaction was calculated as follows: 100×[(the pruritic behavioral frequency of control group−that of normal group)−(that of test agent-treated group−normal group)]/(the pruritic frequency of control group−that of normal group).

Statistics

All the data were expressed as the mean ± standard deviation, and statistical significance was analyzed by one way ANOVA followed by Student-Newman-Keuls test.

RESULTS

Metabolism of wogonoside by human intestinal microflora

To investigate the metabolite of wogonoside produced by human intestinal microflora, wogonoside was anaerobically incubated with human fecal microflora for 20 h and analyzed by LC-MS/MS. In the reaction mixture of wogonoside, one metabolite was observed. The metabolite possessed an MS peak at *m/z*=285 $[M+1]^+$. The metabolite of wogonoside was wogonin.

When the metabolic activity of wogonoside in five human fecal specimens was preliminarily assayed, its hydrolysis to its aglycone occurred in all specimens. The average of the hydrolyzing activities of wogonoside to wogonin was 6.9 ± 5.1 nmol/h/mg wet weight of fecal bacteria (Fig. 1).

Effect of SB extract, wogonoside and wogonin on pruritic behaviors induced by compound 48/80 or histamine in mice

SB extract inhibited compound 48/80-induced pruritic behavior in our preliminarly experiments. Therefore, the antipruritic effect of wogonoside, another main constituent of SB except baicalin, and its metabolite, wogonin, was in-

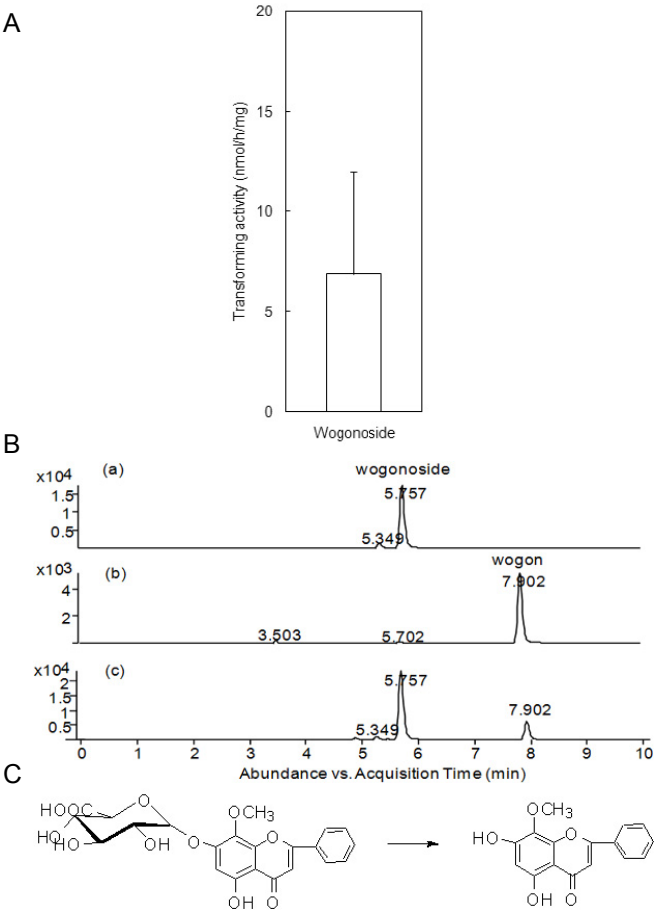

Fig. 1. Metabolic activity of wogonoside by human fecal microflora (A), its metabolic profile by LC- MS/MS analysis (B) and its proposed pathway (B). LC-MS/MS chromatogram of the reaction mixture incubated wogonoisde with human fecal suspension: (a) wogonoside standard (7.75 min), (b) wogonin standard (7.90 min), (c) 5 h after incubation after incubation of wogonoside with human fecal suspension. LC- MS/MS system (Agilent 1200 series): column, ZORBAX Extend C18 (100×2.1 mm i.d., 1.8 μm, Agilent); elution solvent, a linear- gradient applied by 5% in solvent A to 95% B for 13 min (solvent A - 0.1% aqueous formic acid and solvent B - acetonitrile); and elution rate, 0.3 ml/min. →, main pathway; ⇢, minor pathway.

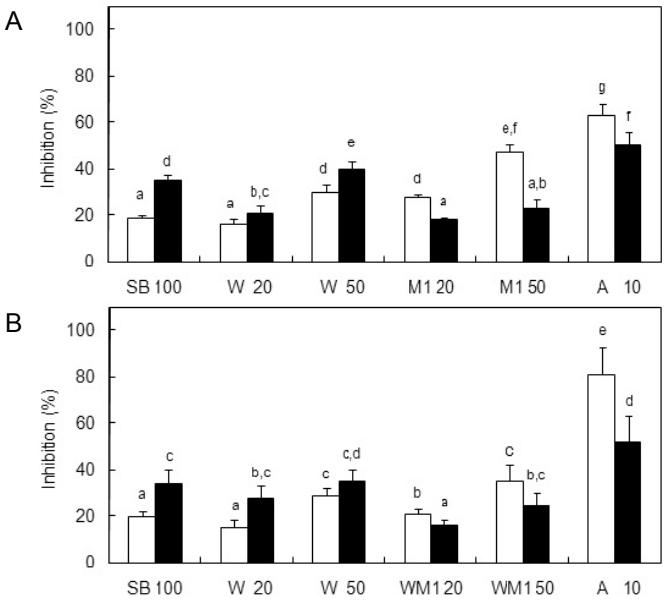

Fig. 2. Inhibitory effects of orally administered Scutellariae radix extract, wogonoside, and its metabolite, and azelastine, on compound 48/80 (A)- and histamine (B)-induced pruritus in mice. The pruritus was induced by compound 48/80 in BALB/c mice or histamine in ICR mice. Mice were treated with or without oral administration of test agents (10, 20, 50 and/or 100 mg/kg) 1 h (white bar) or 5 h (black bar) before the intradermal injection of 50 μg/50 μl of compound 48/80 or 300 μg/50 μl of histamine into the shaved back skin of mice: SB100, the radix extract of Scutellaria baicalensis (SB, 100 m/kg); W 20, 20 mg/kg wogonoside; W 50, 50 mg/kg wogonoside; M1 20, 20 mg/kg wogonin; M1 50, 50 mg/kg wogonin; A 10, 10 mg/kg azelasitine. Numbers of pruritic behavior frequency of normal group (treated with saline alone) and control groups (treated with compound 48/80 or histamine) for 1 h were 3 ± 1, 222 ± 21, and 89 ± 5, respectively. The values indicate mean ± S.D. (n=6). [a, b, c, d, e, f, g] Items with the same letter are not significantly different ($p > 0.05$).

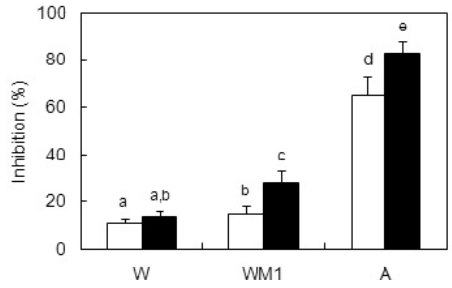

Fig. 3. Inhibitory effects of intraperitoneally administered wogonoside, its metabolite, and azelastine, on histamine-induced pruritus in mice. The pururitus was induced by histamine in ICR mice. Mice were treated with or without intraperitoneal administration of test agents [5 (white bar) and 10 mg/kg (black bar)] 1 h before the intradermal injection of 300 μg/50 μl of histamine into the shaved back skin of mice. W: wogonoside, M1: wogonin, A: azelasitine. Numbers of pruritic behavior frequency of normal group (treated with histamine) and control group (treated with saline alone) for 1 h were 85 ± 4, and 2 ± 1, respectively. The values indicate mean ± S.D. (n=6). [a,b,c,d,e] Items with the same letter are not significantly different ($p > 0.05$).

vestigated on compound 48/80-induced experimental pruritic mice (Fig. 2A). When compound 48/80 was injected into mice, prurirtic behavior was significantly induced. When wogonoside was orally administered 1 h or 5 h before the treatment with a pruritic agent compound 48/80, it inhibited the pruritic behavior. Wogonoside orally administered 5 h before the treatment with compound 48/80 more potently inhibited the pruritus than one administered 1 h before. Wogonoside at a dose of 50 mg/kg showed the anti-pruritic effect in compound 48/80-treated mice, with inhibitions of 31% and 40%, respectively. However, wogonin orally administered 1 h before the treatment with compound 48/80 more potently showed the anti-pruritic effect than one treated 5 h before. Wogonin orally administered 1 h after pruritic agent, inhibited the pruritic behaviors by 47%.

Orally administered wogonoside and wogonin inhibited histamine-induced pruritus (Fig. 2B). Wogonoside orally administered 5 h before the treatment with histamine, more potently inhibited the scratching behaviors than one treated 1 h before. Wogonoside at a dose of 50 mg/kg inhibited the pruritus by 29% and 35%, respectively. Its metabolite, wogonin orally administered 1 h before the treatment with histamine, more potently inhibited the pruritus than one administered 5 h before.

The inhibitory effect of intraperitoneally administered wogonoside and its metabolite, wogonin, in histamine-induced pruritic mice was measured (Fig. 3). Although all these agents, wogonoside and wogonin, inhibited the pruriritic behavior, wogonin was more effective than baicalin.

DISCUSSION

Pruritus, an unpleasant cutaneous sensation which provokes the desire to scratch, can be local or widespread and is associated with atopic dermatitis, uriticaria, cholestasis, uraemia, etc. Many endogenous amines, proteases, growth factors, neuropeptides, opioids, ecosanoids and cytokines can act as pruritogens (Lerner, 1944; Hagemark, 1995; Schmeiz *et al.,* 1997). Pruritus can cause skin lesions and contribute to severe psychological disturbances (Raiford, 1995). Therefore, inhibition of this response is beneficial for improving the quality of life. However, there is no specific remedy available for this common symptom. As part of a screening program aimed at discovering anti-pruritic agents from natural products, we found that SB potently inhibit compound 48/80-induced pruritus. Many re-

searchers also reported the inhibitory effects of SB and baicalin against allergic diseases, asthma and inflammation (Koda *et al.,* 1970; Liaw *et al.,* 1999; Taniguchi *et al.,* 2000). However, anti-allergic or anti-pruritic effect of wogonoside, a main constituent of SB, was not studied, whereas its anti-inflammatory effects were reported. In the present study, we found its metabolite, wogonin, by human fecal microflora. Wogonoside was metabolized to wogonin, which was not transformed any further during a 24 h incubation period. The metabolite may be absorbed from the intestine into the blood. This suggestion is supported by the reports of Xing *et al.* (2005a, 2005b) that baicalein and wogonin conjugates were detected in the blood of rats orally treated with baicalin and wogonoside, respectively.

Next we evaluated the anti-pruritic effects of wogonoside and its metabolite in compound 48/80 or histamine-induced pruritic mice. Compound 48/80 or histamine were intradermally injected into the rostral part of the back skin of BALB/c and ICR mice, and the pruritic behaviors evaluated for 60 min. The scratching frequencies for the 60 min period for each inducer increased in a dose?dependent manner. These scratching agents more potently induced pruritic behaviors in ICR mice than in BALB/c mice, which are in agreement with a previous report (Inagaki *et al.,* 2001). Particularly, compound 48/80 caused vigorous scratching behavior in ICR mice. The accurate counting of scratching behavior frequencies proved too difficult. However, BALB/c mice were dull for histamine. Therefore, in the present study, BALB/c mice were used in our compound 48/80-induced pruritic mouse model and ICR were use in our histamine-induced pruritic mouse model. SB and its constituent, wogonoside, showed anti-pruritic effects in compound 48/80 or histamine-stimulated mice. Orally administered wogonoside at 5 h before the treatment with scratching agents showed the potent anti-pruritic effects than one treated at 1 h before. However, wogonin showed more potent inhibition, when treated at 1 h before the treatment with scratching agents than when treated at 5 before. These results suggest that, to express the anti-pruritic effect of wognoside, it should be metabolized by intestinal microflora, but that of wogonin may be independent on its metabolism by intestinal microflora. This suggestion support that the anti-pruritic effect of orally administered wogonoside was more potent than that of intraperitoneally administered one, whereas intraperitoneally administered wogonin showed more potent anti-pruritic effect than orally treated one. These results suggest that orally administered wogonoside may be metabolized to wogonin by intestinal microflora. Following absorption by intestinal microflora, its metabolite may express pharmacological effects, such as anti-pruritic, anti-inflammatory and antioxidant effects.

Finally, these findings suggest that SB and its constituent, baicalin, can attenuate the pruritus and, and that its anti-pruritic effects may be dependent on its metabolism by intestinal microflora.

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