BIOMOLECULES
& THERAPEUTICS

The Role of Intestinal Microflora in Anti-Inflammatory Effect of Baicalin in Mice

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Abstract

Baicalin, a main constituent of the rhizome of *Scutellaria baicalensis*, is metabolized to baicalein and oroxylin A in the intestine before its absorption. To understand the role of intestinal microflora in the pharmacological activities of baicalin, we investigated its anti-inflammatory effect in mice treated with and without antibiotics. Orally administered baicalin showed the anti-inflammatory effect in mice than intraperitoneally treated one, apart from intraperitoneally administered its metabolites, baicalein and oroxylin A, which potently inhibited LPS-induced inflammation. Of these metabolites, oroxylin A showed more potent anti-inflammatory effect. However, treatment with the mixture of cefadroxil, oxytetracycline and erythromycin (COE) significantly attenuated the anti-inflammatory effect of orally administered baicalin in mice. Treatment with COE also reduced intestinal bacterial fecal β-glucuronidase activity. The metabolic activity of human stools is significantly different between individuals, but neither between ages nor between male and female. Baicalin was metabolized to baicalein and oroxylin A, with metabolic activities of 1.427 ± 0.818 and 1.025 ± 0.603 pmol/min/mg wet weight, respectively. Baicalin and its metabolites also inhibited the expression of pro-inflammatory cytokines, TNF-α and IL-1β, and the activation of NF-κB in LPS-stimulated peritoneal macrophages. Of them, oroxylin A showed the most potent inhibition. Based on these findings, baicalin may be metabolized to baicalein and oroxylin A by intestinal microflora, which enhance its anti-inflammatory effect by inhibiting NF-κB activation.

Key Words: Baicalin, Baicalein, Oroxylin A, *Scutellaria baicalensis*, Metabolism, Inflammation

INTRODUCTION

Most herbal medicines are orally administered to human. Their components are inevitably contacted with intestinal microflora in gastrointestinal tract and may be metabolized by intestinal microflora, before absorption from the gastrointestinal tract to the blood (Kobashi and Akao 1997; Kim, 2002). Therefore, to express the pharmacological effects of herbal medicines, the metabolic activities of intestinal microflora for the constituents of herbal medicines may be of a great importance.

The rhizome of *Scutellaria baicalensis* (SB), which contains baicalin as a main constituent, has been used in China, Japan and Korea as a traditional medicine and functional food for inflammation, fever, hepatitis, allergic disease, hypertension, etc (Lin and Shieh, 1996; Zhu, 1998; Wu *et al.*, 2005). Baicalin exhibits anti-inflammatory, anti-allergic, anti-oxidant, hepatoprotective, and anti-tumor effects (Chou *et al.*, 2003; Jang *et al.*, 2003; Kim *et al.*, 2005; Xing *et al.*, 2005a). It is generally assumed that baicalin is poorly absorbed from the gastroin-

testinal tract in its native form 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; Trinh *et al.*, 2010). The metabolite, baicalein, is subsequently metabolized to baicalin, baicalein 6-Q-β-glucuronide-7-O-sulfate and baicalein 6,7-diglucuronide in the gut mucosae, liver and blood (Abe *et al.*, 1990; Akao *et al.*, 2004; Lu *et al.*, 2007). However, Abe *et al.* found two baicalein conjugates and one oroxylin A conjugate as main metabolites in the bile of rats orally treated with baicalin (Abe *et al.*, 1990). We also found that human fecal microflora could transform baicalin to baicalein and oroxylin A, which exhibit more potent anti-scratching behavioral effect in histamine-treated mice than a parental compound baicalin (Trinh *et al.*, 2010). Nevertheless, the role of intestinal microflora in the pharmacological effect of baicalin has not been clarified.

Therefore, to understand the role of intestinal microflora in the pharmacological effect of baicalin, we isolated baicalin from SB and its metabolites, and investigated their anti-inflammatory effects in antibiotics-treated mice and in lipopolysac-

charide (LPS)-stimulated peritoneal macrophages.

MATERIALS AND METHODS

Materials

p-Nitrophenyl-β-D-glucuronide, LPS were purchased from Sigma Chemical. Co. (St. Louis, MO, USA). Baicalin (purity, >95%), baicalein (purity, >93%) and oroxylin A (purity, >95%) was purified from the rhizome of *Scutellaria baicalensis* according to the previously reported methods of Trinh *et al.* (2010).

Subjects

The subjects were 100 healthy Korean persons (average, 40.74 ± 13.87 years; range, 20-72 years; 54 males, 46 females). Exclusion criteria included smoking and current medication, especially regular or current use of antibiotics. The recruit of subjects and collection of their stools were approved by the Committee for the Care and Use of Clinical Study in the Medical School, Kyung Hee University.

Fecal specimen preparation

The human fecal specimens (1 g) were collected in plastic cups, and then carefully mixed with a spatula and suspended with cold 9 ml saline according to a previous method (Choi *et al.*, 2011). Fecal bacterial suspension was centrifuged at 500 × g for 5 min. The resulting supernatant was sonicated for 10 min and then centrifuged at 10,000 × g for 20 min. The resulting supernatant was used for the assay of enzyme activity.

Animals

Male ICR mice (24-28 g) were supplied by Orient Experimental Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 20-22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea) and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University Guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

Assay of fecal of β-D-glucuronidase and β-D-glucosidase activity

For the assay of β-D-glucuronidase and β-D-glucosidase activity in mouse stools, the reaction mixture (total volume of 1 ml) contained 0.4 ml of 1 mM p-nitrophenyl-β-D-glucuronide or p-nitrophenyl-β-D-glucopyranoside, 0.4 ml of 0.1 M phosphate buffer, pH 7.0, and 0.2 ml of the fecal enzyme fraction. The reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 1 ml of 0.5 N NaOH, centrifuged at 3,000 × g for 10 min and measured the absorbance at 405 nm (UV-vis spectrophotometer, JASCO V-530, Tokyo, Japan).

Assay of fecal baicalin-metabolizing activity

For the fecal baicalin-metabolizing activity, the reaction mixture (2 ml) containing 0.2 ml of the human fecal suspension 50 mg of fresh feces and 0.2 ml of 1 mM baicalin was incubated at 37°C for 6 h, and 2 ml of MeOH was added to stop the reaction. The reaction mixture was centrifuged at 3,000 × g for 10 min and the levels of baicalin and its metabolites baicalein
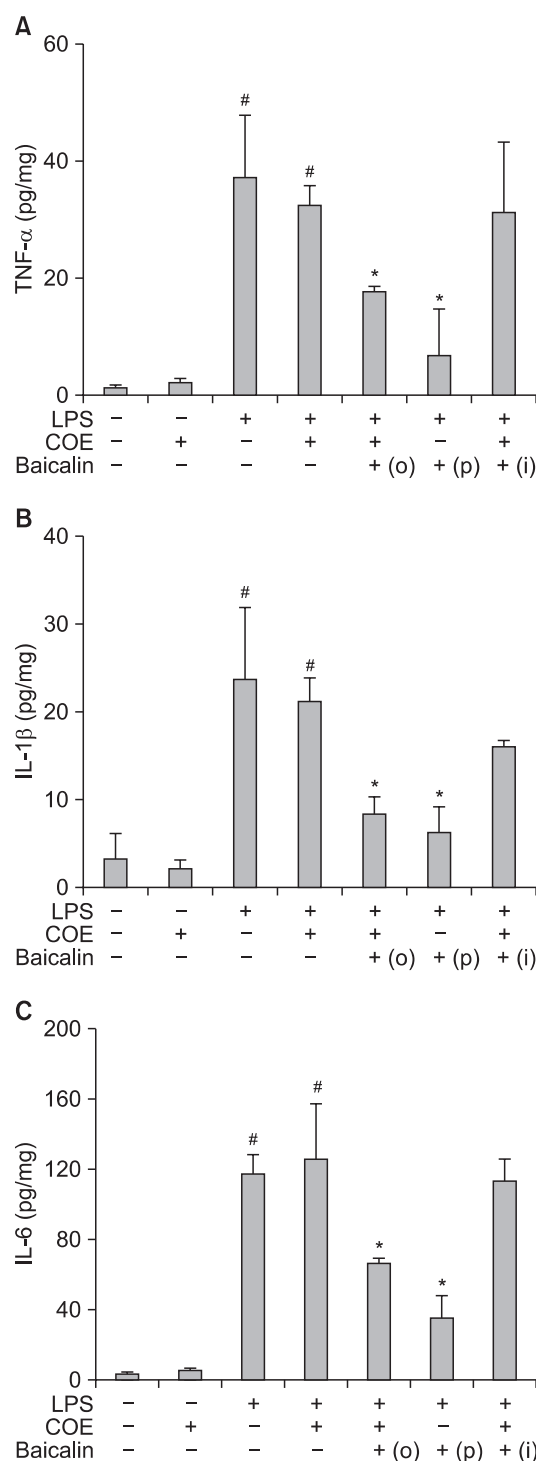

Fig. 1. Anti-inflammatory effect of baicalin in LPS-stimulated mice, treated with or without antibiotics. Male ICR mice were intraperitoneally injected with LPS (4 mg/kg) in the absence or presence of test agents (20 mg/kg). Test agents were orally (o) or intraperitoneally (i) administered either 6 h before treatment with LPS in mice treated with or without antibiotics. The antibiotics-treated groups were treated with COE once a day for 3 day and then test agents were treated 48 h before the LPS injection. Mice were sacrificed 4 h after LPS injection, and whole blood was obtained by cardiac puncture. The levels of IL-6, TNF-α and IL-1β in the serum were determined using ELISA kit. *Significantly different vs. each group treated with LPS alone ($p<0.05$). #Significantly different vs. each LPS-non-treated group ($p<0.05$).

and oroxylin A in the resulting supernatant were analyzed by HPLC.

HPLC system consisted of Hewlett Packard series 1050 and UV detector (Ramsey, MN, USA): column, Hypersil ODS (4.6×150 mm i.d., 5.0 m, Agilent, Santa Clara, CA, USA); detector, 203 nm; elution solvent, linear-gradient mixture of 30% water and 70% acetonitrile for 15 min; flow rate, 1.0 ml/min; and injection volume, 20 μl. The retention times of baicalin, baicalein and oroxylin A were 6.7 min, 8.1 min, and 9.5 min, respectively.

Isolation and culture of peritoneal macrophages

Male ICR mice were intraperitoneally injected with 2 ml of 4% thioglycolate solution. Mice were sacrificed 4 days after injection and the peritoneal cavities were flushed with 10 ml of RPMI 1640. The peritoneal lavage fluids were centrifuged at 200 × g for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37°C, the cells were washed three times and nonadherent cells were

removed by aspiration. Cells were cultured in 24-well plates (0.5×10^6 cells/well) at 37°C in RPMI 1640 plus 10% FBS. The attached cells were used as peritoneal macrophages (Joh and Kim, 2011).

Assay of serum TNF-α, IL-1β and IL-6 in LPS-stimulated mice

Male ICR mice were intraperitoneally injected with LPS (4 mg/kg) in the absence or presence of test agents (20 mg/kg). Mice were sacrificed 4 h after LPS injection, and whole blood was obtained by cardiac puncture. Serum was prepared by centrifugation at 13,000 × g for 20 min at 4°C. The levels of IL-6, TNF-α and IL-1β in the serum were determined using ELISA kits (R&D Systems, MN, USA).

Test agents were orally or intraperitoneally administered 6 h before treatment with LPS in mice treated with or without antibiotics. The antibiotics-treated groups were daily treated with cefadroxyl (100 mg/kg), oxytetracycline (300 mg/kg) and erythromycin (300 mg/kg) (COE) for 3 day and then test agents were treated 48 h before the LPS injection.

Determination of cytokines and immunoblot analysis in LPS-induced peritoneal macrophage

Cytokines, TNF-α and IL-1β were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

For the immunoblot analysis of NF-κB, the cell supernatant extracts prepared from macrophages were separated by 9% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried-milk proteins in PBST, then probed with COX-2, iNOS, p65, p-p65 or β-actin antibody. After washing with PBST, proteins were detected with horseradish peroxidase-conjugated secondary antibodies for 1 h. Bands were visualized with enhanced chemiluminescence (ECL) reagent (Joh *et al.*, 2011).

Statistics

All data are expressed as the mean ± standard deviation, with statistical significance analyzed using one-way ANOVA followed by a Student *t*-test.
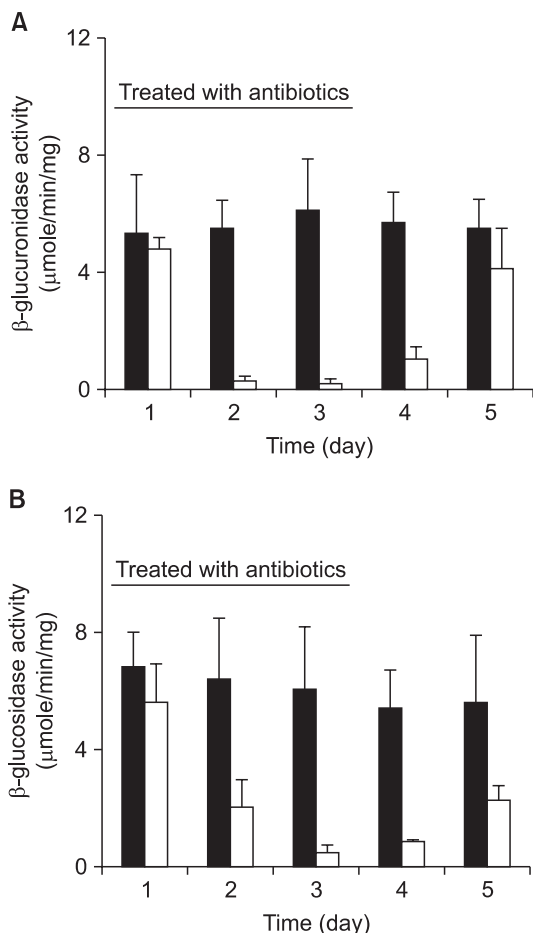

Fig. 2. Fecal β-glucuronidase and β-glucosidase activity of the stools of mice treated with or without COE. The mice were orally treated with (white bar) or without antibiotics COE (black bar). β-Glucosidase and β-glucosidase activities were measured 48 h after the final antibiotics treatement using p-nitrophenyl-β-D-glucuronide and p-nitrophenyl-β-D-glucopyranoside as a subtrate (white bar, treated with COE; (black bar, treated without COE).
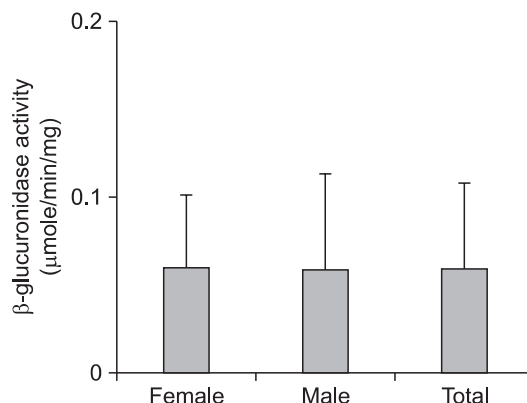

Fig. 3. Fecal β-glucuronidase activity of the stools of 100 Koreans.

RESULTS

In the preliminary study, water extract of SB, of which main constituent is baicalin, inhibited blood levels of proinflammatory cytokines in mice intraperitoneally injected with LPS. Therefore, we isolated a main constituent, baicalin, from SB and investigated its anti-inflammatory effect in mice intraperitoneally injected with LPS (Fig. 1). Intraperitoneal injection of LPS induced the blood levels of TNF-α, IL-1β and IL-6. Treatment with baicalin significantly reduced the blood levels of these proinflammatory cytokines in mice treated with LPS. To understand the role of intestinal microflora in the anti-inflammatory effect of baicalin, the anti-inflammatory effect of baicalin was investigated in mice treated with antibiotics. LPS also increased the blood levels of TNF-α, IL-1β and IL-6 in mice treated with antibiotics. Treatment with baicalin weakly reduced the blood levels of TNF-α, IL-1β and IL-6 in mice treated with antibiotics, compared to those in mice treated without antibiotics.

Next we measured the activities of intestinal bacterial β-glucuronidase, which metabolizes baicalin to baicalein in the intestine, and β-glucosidase in feces of mice treated with and without antibiotics by using p-nitrophenyl-β-D-glucuronide

and p-nitrophenyl-β-D-glucopyranoside, respectively (Fig. 2). These enzyme activities in the feces of mice treated with COE were significantly decreased compared to those in the feces of mice treated without antibiotics. Treatment with antibiotics could not reverse these enzyme activities 48 h after oral administration of antibiotics. The inhibition of the enzyme activity by treatment with and without antibiotics in mice was proportional to its anti-inflammatory effect in mice with and without antibiotics, respectively.

Next, we measured β-glucuronidase activity of 100 human fecal specimens by using p-nitrophenyl-β-D-glucuronide (Fig. 3). β-Glucuronidase activity was 0.003-0.285 μmol/min/mg. The average activities (mean ± S.D.) of total, female and male specimens were 0.059 ± 0.049, 0.060 ± 0.042, and 0.058 ± 0.055 μmol/min/mg. The enzyme activity was not different between males and females, or between ages. We also measured baicalin-metabolizing activity of 100 human fecal specimens (Fig. 4). Baicalin-metabolizing activity was 0-6.452 pmol/min/mg. The average activities (mean ± S.D.) of total, female and male specimens were 4.471 ± 1.129, 4.580 ± 1.209, and 4.377 ± 1.058 μmol/min/mg. Transforming activities of baicalin to baicalein were 1.427 ± 0.818, 1.185 ± 0.854 and 1.643 ± 0.727 pmol/min/mg, respectively. Production activities
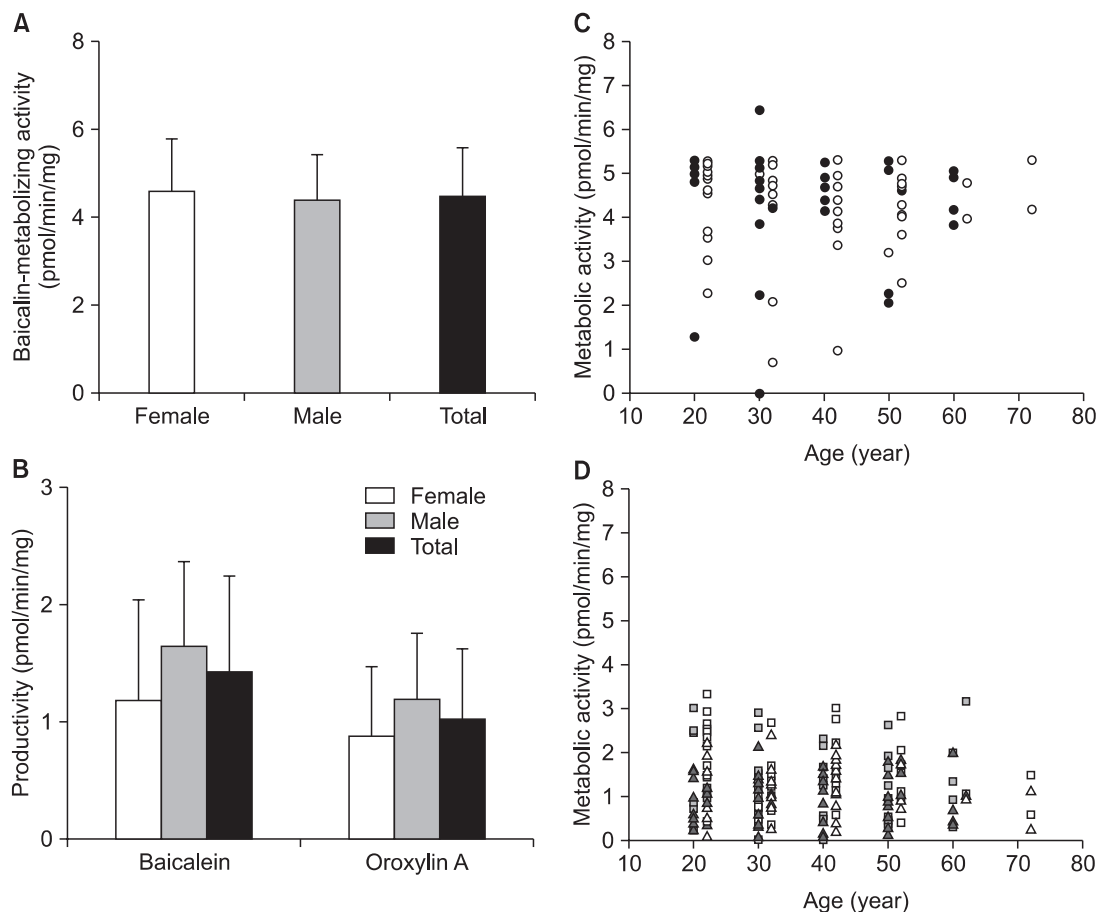

Fig. 4. Fecal baicalin-metabolizng activity of the stools of 100 Koreans. (A) Average metabolic activities of baicalin-metabolic activities baicalin (black bar, total specimens; white bar, female; gray bar, male). (B) Average baicalein or oroxylin A producing activities from baicalin (black bar, total specimens; white bar, female; gray bar, male). (C) Comparison of baicalin-metabolizing activity between male (black circle) and female (white circle), or between ages. (D) Comparison of baicalin-metabolizing activity to baicalein (square) or oroxylin A (triangle) between male (gray) and female (white), or between ages.
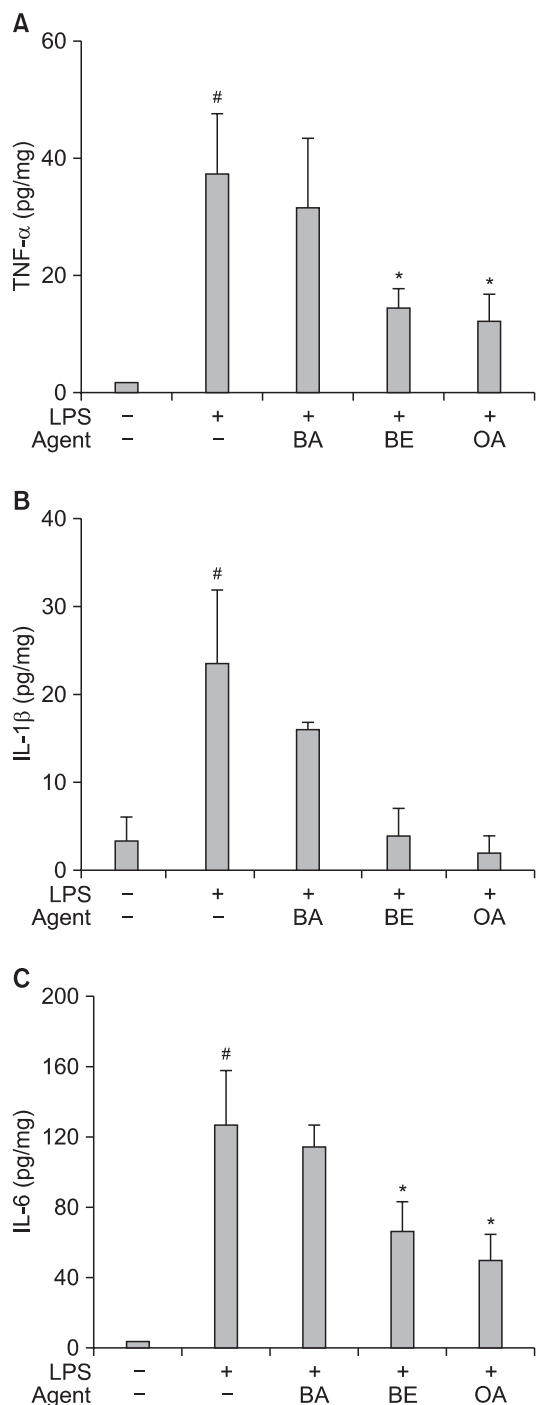
Fig. 5. Effect of intraperitoneally administered baicalin and its metabolites on LPS-stimulated inflammation in mice. ICR mice were intraperitoneally injected with LPS (4 mg/kg) in the absence or presence of test agents (20 mg/kg). Test agents were orally or intraperitoneally administered either 6 h before treatment with LPS in mice treated with or without antibiotics. Mice were sacrificed 4 h after LPS injection, and whole blood was obtained by cardiac puncture. The levels of IL-6, TNF-α and IL-1β in the serum were determined using ELISA kit. *Significantly different vs. group treated with LPS alone ($p<0.05$). #Significantly different vs. LPS-non-treated group ($p<0.05$).

of baicalin to oroxylin A were 1.025 ± 0.603, 0.879 ± 0.592, and 1.193 ± 0.564 pmol/min/mg, respectively. The enzyme activity was not different between males and females, or between ages.

Next we measured anti-inflammatory effects of intraperitoneally administered baicalin and its metabolites, baicalein and oroxylin A, in mice intraperitoneally injected with LPS (Fig. 5). These compounds all showed anti-inflammatory effects in LPS-treated mice. Of them, oroxylin A inhibited the LPS-induced inflammation most potently. Oroxylin A (20 mg/kg) inhibited the TNF-α, IL-1β and IL-6 expressions by 67.9%, 92.6% and 60.6%, respectively.

To confirm the anti-inflammatory effect of baicalin and its metabolites, we investigated their inhibitory effects in LPS-stimulated peritoneal macrophages (Fig. 6). LPS significantly increased IL-1β and TNF-α expressions. Treatment with baicalin and its metabolites baicalein and oroxylin A (10 μM) in the presence of LPS potently inhibited 9.7%, 44.2% and 76.0% for TNF-α expression, respectively, 7.8%, 38.5% and 78.9% for IL-1β expression, respectively. These agents also inhibited iNOS and COX-2 expressions. Particularly, baicalein and oroxylin A LPS-induced activation of the transcription factors, NF-κB, which regulate TNF-α, IL-1β, iNOS and COX-2 expressions.

DISCUSSION

Most herbal medicines are orally administered to humans, their hydrophilic constituents glycosides may be in contact with intestinal microflora, transformed to hydrophobic compounds, absorbed into the blood, and their pharmacological effects may be expressed (Takido *et al.*, 1995; Kim, 2002; Trinh *et al.*, 2010). For example, when ginsenoside Rb1, which is a major component of ginseng, is administered to humans or rats, it is transformed to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (compound K) by intestinal microflora and absorbed to the blood (Wakabayashi *et al.*, 1997; Akao *et al.*, 1998a; Akao *et al.*, 1998b). However, ginsenoside Rb1 was not detected in blood. Compound K exhibits the potent antitumor and anti-allergic actions more than ginsenoside Rb1 (Odashima *et al.*, 1985; Shin *et al.*, 2005). When baicalin is orally administered to rats, the sulfate and glucuronic acid conjugates, baicalein 6-O-glucuronide-7-O-sulfate, baicalein 6,7-diglucuronide and oroxylin A-7-O-β-glucuronide, of its metabolites baicalein and oroxylin A were detected in bile and/or the blood (Abe *et al.*, 1990; Akao *et al.*, 2004; Lu *et al.*, 2007). Furthermore, when baicalin was anaerobically incubated with human fecal suspension, it was metabolized to its aglycone baicalein, and then produced oroxylin A. Therefore, intestinal bacteria may play an important role in expressing the pharmacological activities of baicalin, such as ginsenoside Rb1.

In the present study, orally administered baicalin, isolated from SB, inhibited mouse inflammation induced by intraperitoneal injection of LPS. However, treatment with antibiotics (COE) attenuated anti-inflammatory effect of baicalin. Intraperitoneally administered baicalin inhibited LPS-induced systemic inflammation in mice barely, but its metabolites, baicalein and oroxylin A, showed the potent inhibition. Furthermore, the oral administration of baicalin in mice treated without antibiotics was metabolized to baicalein and oroxylin A more potently than in mice treated with antibiotics. Further-

more, the baicalin-metabolizing activity of the stools of mice was potently reduced by the oral administration of antibiotics. These results were supported by the previous report that

A (a)

(b)

B

Fig. 6. Effect of baicalin and its metabolites on the expression of TNF-α, IL-1β, COX-2 and iNOS and the activation of NF-κB in LPS-stimulated peritoenal macrophages. Peritoneal macrophages (0.5×10^6 cells) were treated with 50 ng/ml LPS in the absence (LPS) or presence of test agents (5, 10 μM) for 20 h. The normal control group (NOR) was treated with vehicle alone. (A) Effect on TNF-α (a) and IL-1β (b) expressions. The levels of these cytokines in culture supernatants were measured by ELISA. (B) Effect on COX-2 and iNOS expressions and on NF-κB activation. The expression levels of these proteins were determined by immunoblotting. *Significantly different vs. group treated with LPS alone (p<0.05). #Significantly different vs. LPS-non-treated group (p<0.05).

antibiotics inhibited the metabolism and absorption of baicalin in the intestine of rats (Xing *et al.*, 2005b). Based on these findings, baicalin orally administered to mice is metabolized to baicalein and oroxylin A by intestinal microflora, its metabolites are absorbed into the blood and then expressed their anti-inflammatory effect.

Above all, β-D-glucuronidase, which metabolizes baicalin to baicalein, produced by intestinal microflora may play an important role in the pharmacological effects of the root of *Scutellaria baicalensis* and its main component, baicalin. When we anaerobically incubated baicalin with human fecal suspension, it was metabolized to its aglycone baicalein, and then produced oroxylin A, like the previously reported (Trinh *et al.*, 2010). The β-glucuronidase activity between individuals significantly varied. However, the activity between males and females, or between ages was not different. These results suggest that, if SB or baicalin is orally administered to humans for inflammatory diseases, its efficacy between individuals may be so different and their pharmacological effects may be dependent on its metabolic activities of intestinal microflora.

Inflammation can be mediated by inflammatory mediators, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-12, IL-18, nitric oxide, and prostaglandins (Fairweather and Rose, 2005). Among these inflammatory mediators, pro-inflammatory cytokines such as IL-1β and TNF-α are activated through nuclear factor-kappaB (NF-κB) but they also activate NF-κB (Collins *et al.*, 1995; Baldwin, 2001). Regulating expression of these inflammatory mediators can therefore be beneficial in decreasing inflammatory diseases. Thus, to prevent chronic diseases associated with inflammation (O'keefe *et al.*, 2008), the application of dietary ingredients has recently become a focus of interest (Paradkar *et al.*, 2004; Davis *et al.*, 2006). In the present study, baicalin and its metabolites baicalein and oroxylin A inhibited LPS-stimulated inflammation in vitro and in vivo, like the previously reported (Chen *et al.*, 2000; Chou *et al.*, 2003; Yang *et al.*, 2008). Of them oroxylin A most potently inhibited the expressions of proinflammatory cytokines TNF-α and IL-1β in LPS-stimulated mice and peritoneal macrophages. Oroxylin A also inhibited NF-κB activation and iNOS and COX-2 expressions in LPS-stimulated peritoneal macrophages, like the previously reported (Chen *et al.*, 2000). It is suggested that baicalin and its metabolites may inhibit TNF-α and IL-1β expressions by regulating the activations of their transcription factor, NF-κB.

Based on these findings, orally administered baicalin is metabolized to baicalein and oroxylin A in the intestine, which are absorbed to the blood and then express their anti-inflammatory effect by inhibiting NF-κB activation. The anti-inflammatory effect of baicalin may be significantly variant between individuals due to the difference of intestinal bacterial metabolic activity for baicalin between individuals.
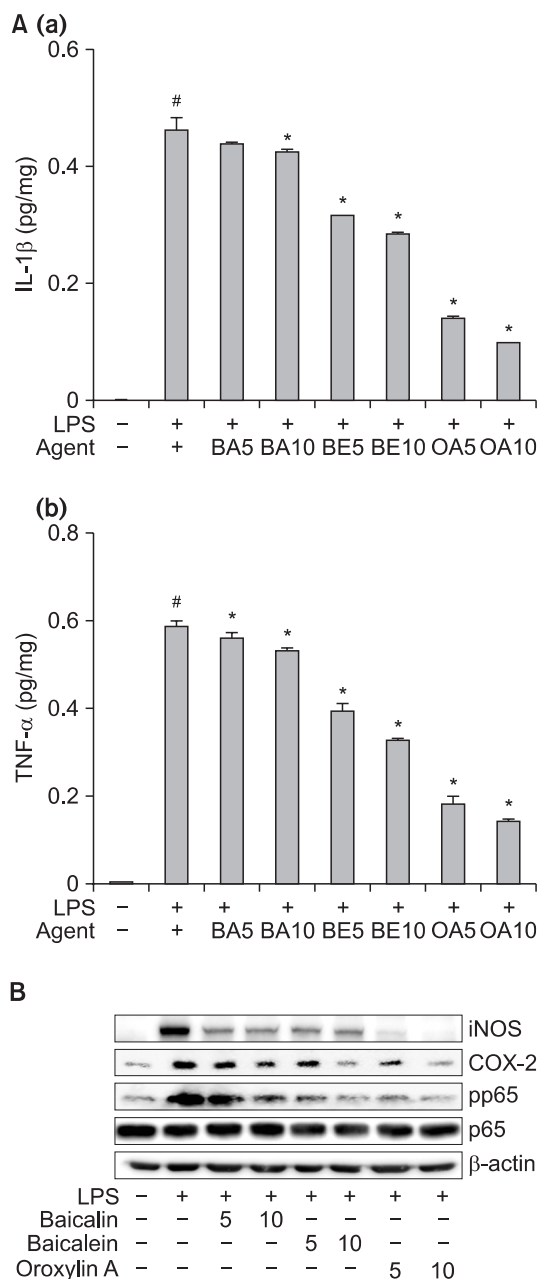
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