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桑色素與其代謝物之免疫調節及機轉研究(1/2)

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中文摘要

桑色素是一種具有活性的黃酮類化合物，廣泛地存在各種蔬果與中藥中，具有預防心血管疾病、抗氧化、抗癌、抗發炎等作用。然而，其生物活性應決定於血清中的結合態代謝物。本研究分析比較桑色素及其桑色素結合態代謝物受到脂多醣刺激後，產生發炎介質一氧化氮(NO)，及細胞激素之影響。結果顯示 1.25 μM 桑色素結合態代謝物 與 1.25 mM 桑色素可以抑制 50%產生 NO 的能力，也同時影響細胞激素 TNF-α 與 IL-12 的產量。進一步發現長期服用桑色素的老鼠血中吞噬細胞的吞噬能力下降。因此，桑色素具有被開發為抗發炎的潛力。

關鍵詞 ： 桑色素；桑色素結合態代謝物；吞噬細胞；一氧化氮

ABSTRACT

Morin is a flavonoid present in fruits and Chinese herbs. Based on *in vitro* studies, morin has been reported to show various beneficial biological activities. Therefore, the biological effects of morin could be primarily determined by its conjugated metabolites in serum. In this study, the effects of morin and its sulfates/glucuronide metabolites on the production of nitric oxide (NO) and cytokines from lipopolysaccharide (LPS)-activated macrophages were individually investigated and compared. The results indicated that 50% NO production from both LPS-activated murine macrophage-like cell line, RAW 264.7, and peritoneal excluded cells were inhibited by 1.25 μM morin sulfates/glucuronides and 1.25 mM morin, respectively. In addition, tumor necrosis factor-α (TNF-α) and interleukin (IL)-12 productions from active macrophages were decreased significantly with IC_{50} of morin and morin sulfates/glucuronides, respectively. Furthermore, phagocyte activities in the peripheral blood were lower in mice dosed with morin for two months than those of control mice. Therefore, morin could be a promising therapeutic candidate for inflammatory disease because of the strong efficacy of its active metabolites which had never been focused by biological investigators.

Key words: morin; morin sulfates/glucuronides; macrophage; nitric oxide

INTRODUCTION

Flavonoids are ubiquitous and abundant in plants and considered very important for preventing a wide variety of diseases, including allergies, cardiovascular disease, certain forms of cancer, hepatic diseases, and inflammation [1]. Morin (3, 5, 7, 2', 4'-pentahydroxyflavone) is a flavonoid constituent of many herbs and fruits. Based on *in vitro* studies, it has been reported to show biological activities, including anti-oxidation [2-4], anti-mutagenesis [5,6] and anti-inflammation [7,8]. However, there is growing evidence that conjugation metabolism is central to the fate of flavonoids *in vivo* [9]. A previous study reported that the serum level of morin-conjugated metabolites was much higher than the parent form after oral administration of morin to rabbits at a dose of 25 mg/kg [10]. In contrast to previous *in vitro* studies of flavonoids including morin [7,8], the present investigation assessed the modulating effects of morin-conjugated metabolites in addition to the parent form morin on immune system.

Macrophages play major roles in both innate and acquired immunity. They can be stimulated by cytokines, such as interferon-γ (IFN-γ), or microbial components, such as lipopolysaccharide (LPS) [11]. Therefore, how to well control the activities of macrophages is an important strategy for the treatment of chronic inflammatory diseases. In this study, we evaluated the anti-inflammatory activities of morin and also morin sulfates/glucuronides by assessing their effects on the functions of LPS-activated macrophages *in vitro*. Moreover, *in vivo* studies were also conducted by giving mice morin orally for two months to investigate their phagocyte activities in the peripheral blood and the protective effects in the experimental septic shock.

METHODS

Mice

BALB/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University and maintained in the Animal Center of the China Medical College. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (NRC, USA).

Materials

Morin hydrate, LPS, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). RPMI-1640 medium, HBSS, antibiotics, L-glutamine and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Morin sulfates/glucuronides were prepared from serum of rabbit which had been administered with morin (provided by Chao P.D.L.,).

Cell culture

The murine macrophage cell line (RAW 264.7) was purchased from American Type Culture Collection (Manassas, VA, U.S.A.). Mouse peritoneal exudates macrophages were obtained from mice by lavage with HBSS per mouse [12]. Cells were seeded in 96-well cluster plates at a density of $2x10^6$ cells/ml.

Cell viability

Mitochondrial respiration-dependent MTT assay was then employed to determine their cytotoxicity [13].

NO determination

NO production was determined according to the Griess reaction [14].

Cytokine assay

The supernatants were collected and stored in –80ºC before analysis by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instruction, as described earlier [15].

Quantification of phagocyte activity

The blood was obtained from the retro-orbital venous plexus on day -2 (pre-feeding), and after dosing morin to mice for two months. The quantitative determination of phagocyte activity followed the manufacturer's instruction (Phagotest®).

Experimental sepsis

Mice were given ketamine (20 mg/kg) by intramuscular injection and then LPS (50 mg/kg) was induced by intraperitoneal (*i.p.*) injection into two oral doses of morin (0, 100 mg/kg) mice.

Statistical analysis

Statistical analysis was performed using Student's *t*-test, and the significant difference was set at $p < 0.05$.

RESULTS

Effect of morin or morin sulfates/glucuronides on NO production by the activated murine macrophage cell line and peritoneal excluded macrophages

To investigate the anti-inflammatory effects of morin or morin sulfates/glucuronides on macrophages, the levels of NO were then examined when various concentrations of morin or morin sulfates/glucuronides were co-cultured with LPS/IFN-γ-stimulated RAW 264.7 cell line. As shown in Figure 1a&b, morin or morin sulfates/glucuronoids inhibited NO production from activated RAW 264.7 cells in a dose-dependent manner. The similar inhibitory effect on NO synthesis was observed in thioglycollate-elicited murine peritoneal macrophages stimulated with LPS and IFN-γ (Fig. 1c&d).

Effect of morin or morin sulfates/glucuronides on TNF-~= and IL-12 production by the activated murine macrophage cell line and peritoneal excluded macrophages

To further investigate the effects of morin or morin sulfates/glucuronides on the other immunological functions of macrophages, we compared the cytokine productions from activated macrophages in the presence or absence of morin or morin sulfates/glucuronides. The results indicated that morin or morin sulfates/glucuronides significantly inhibited the TNF-α (Fig. 2) and IL-12 (Fig. 3) production by activated macrophages at the concentration of IC_{50} of morin and morin sulfates/glucuronides as determined above.

In vivo effect of oral morin on phagocytic activity in the peripheral blood of mice

To understand the effect of oral morin on the activities of macrophages *in vivo*, the mice were divided into four groups: A to D, receiving 0, 50, 100, or 200 mg/kg morin once daily for two months, respectively. There were similar phagocyte activities in the peripheral blood among mice before experiment. After two-month treatment, no significant difference in body weight among four groups indicated that the treatment did not affect the normal growth of mice. The result showed significant lower activities of phagocytic cells in the peripheral blood of the higher morin dose groups-C and D than the control group-A (Fig. 4), whereas the lowest dose group B did not show significant difference.

DISCUSSION

This study is the first report on the anti-inflammatory effects of the major metabolites of morin, morin sulfates/glucuronides, although some previous *in vitro* studies had investigated the effects of morin. It also indicated that morin sulfates/glucuronides showed remarkably stronger inhibitory effect than morin In comparison to those results, we demonstrated that morin was only active at very high concentration comparable to that reported before, whereas interestingly morin sulfates/glucuronides exerted remarkably stronger activity on NO inhibition. In previous studies, overproduction of NO has been associated with oxidative stress [16,17] and with the pathophysiology of various diseases such as arthritis, septic shock, autoimmune diseases, and chronic inflammation [18,19]. In addition to many synthetic inhibitors of iNOS, natural products for inhibiting NO production have been investigated [20-22]. On the other hand, excessive amount of TNF-α has also been implicated in the pathogenesis of many chronic inflammatory diseases [23]. Because of the pivotal role in pathogenesis, a significant effort has been focused on developing therapeutic drugs that interfere with TNF-α production or action [24,25].

Recent studies emphasized a pivotal role for the antigen presenting cells in the control of the balance between Th1 and Th2 [26]. In this study, we found that morin sulfates/glucuronides reduce the TNF-α (Fig. 2) and IL-12 (Fig. 3) secretion from activated macrophage. It implies that morin sulfates/glucuronides may modulate the T cells development through regulating the cytokines produced by antigen presenting cells.

In conclusion, we have demonstrated that the morin sulfates/glucuronides are active metabolites for inhibiting the functions of macrophages. Meanwhile, we also showed that oral administration of morin for two-month significantly reduced the phagocyte activity in blood.

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FIGURE LENGENS

Fig. 1 Effects of morin or morin sulfates/glucuronides on NO synthesis. RAW 264.7 cells (a&b) and murine peritoneal macrophages (c&d) were stimulated with LPS plus IFN-γ in the presence of various concentrations of morin or morin sulfates/glucuronides indicated. Statistically significant when compared with the values in the absence of morin or morin sulfates/glucuronides, * $p<0.05$; ** $p<0.01$.

Fig. 2 Effects of morin or morin sulfates/glucuronides on TNF-α synthesis by LPS/IFN-γ-stimulated RAW 264.7 cells and murine peritoneal macrophages. RAW 264.7 cells (a&b) and murine peritoneal macrophages (c&d) were stimulated with LPS plus IFN-γ in the presence or absence of various concentrations of morin or morin sulfates/glucuronides. Statistically significant when compared with the values in the absence of morin or morin sulfates/glucuronides, * $p<0.05$.

Fig. 3 Effects of morin or morin sulfates/glucuronides on IL-12 synthesis by LPS/IFN-γ-stimulated RAW 264.7 cells and murine peritoneal macrophages. RAW 264.7 cells (a&c) and murine peritoneal macrophages (b&d) were stimulated with LPS plus IFN-γ in the presence or absence of various concentrations of morin or morin sulfates/glucuronides. Statistically significant when compared with the values in the absence of morin or morin sulfates/glucuronides, * $p<0.05$.

Fig. 4 Effect of morin on phagocyte activity of macrophages *in vivo*. The blood was obtained from groups A to D. Each group contains 8 mice. Statistically significant when compared with the values of group A, * $p<0.05$.

計畫成果自評

本年度計畫進度與原計畫書內容相同，已有完整性研究，先整理目前的研究成果，並已準備寫成論文投稿。

下一年度擬依原計畫，深入探討桑色素之作用機轉，與探討影響其他免疫細胞之機制。