Water extract isolated from *Chelidonium majus* enhances nitric oxide and tumour necrosis factor-α production via nuclear factor-κB activation in mouse peritoneal macrophages

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Abstract

Chelidonium majus is used to treat several inflammatory diseases and tumours. We have examined the effect of *C. majus* on nitric oxide (NO) production using mouse peritoneal macrophages. When *C. majus* was used in combination with recombinant interferon-γ (rIFN-γ, $10 \, U \, mL^{-1}$), there was a marked cooperative induction of NO production. Treatment of rIFN-γ plus *C. majus* ($1 \, mg \, mL^{-1}$) in macrophages caused a significant increase in tumour necrosis factor-α (TNF-α) production. The increased production of NO and TNF-α from rIFN-γ plus *C. majus*-stimulated cells was almost completely inhibited by nuclear factor-κB (NF-κB) inhibitor, pyrrolidine dithiocarbamate ($100 \, \mu M$). These findings demonstrated that *C. majus* increased the production of NO and TNF-α by rIFN-γ-primed macrophages and suggested that NF-κB played a critical role in mediating the effects of *C. majus*.

Introduction

Chelidonium majus L. (*Papaveraceae*) has often contributed in the treatment of several diseases, such as stomach ache, gastric ulcer, chronic bronchitis, and pertussis (Jee & Lee 1988). The analgesic, antispasmodic and hypotensive effects are due to the therapeutic activity of some of the alkaloids from *C. majus*, such as chelidonine, chelirubine, protopine, stylopine, sangunarine and chelerythrine (Lenfeld et al 1981; Wyczolkowska et al 1996). Ukrain, a semisynthetic *C. majus* alkaloid derivative, has been reported to have antitumour and antiviral activity (Lozjuk et al 1996; Korolenko et al 1998; Nefyodov et al 2000).

Nitric oxide (NO) is a highly reactive molecule produced from the guanidino nitrogen of arginine by nitric oxide synthase enzymes (Nathan 1992). Over the past decade, NO has received increasing attention as a potent macrophage-derived effector molecule against a variety of bacteria, parasites, and tumours (Ross & Reske-Kunz 2001). NO is involved in innate immunity as a toxic agent towards infectious organisms, but can induce or regulate death and function of host immune cells, thereby regulating specific immunity. NO may induce toxic reactions against other tissues of the host. Since NO is generated at high levels in certain types of inflammation, for example in asthma, it has been implicated as a pro-inflammatory agent. Equally, it acts as an anti-inflammatory or immunosuppressive agent via its inhibitory or apoptotic effects on cells. The role of NO in contact allergen-induced skin inflammation is likewise complex. NO is pro-inflammatory at low concentrations by inducing vasodilatation and the recruitment of neutrophils, whereas at high concentrations it down-regulates adhesion molecules, suppresses activation and induces apoptosis of inflammatory cells (Gantt et al 2001).

The pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α), regulates systemic responses to microbial infection or tissue injury (Duan et al 2001). Production of TNF-α protein is enhanced by the presence of interferon-γ (IFN-γ). TNF-α acts as an autocrine signal to amplify IFN-γ-induced production of NO in macrophages (Lee et al 1998).

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Macrophages are a major source of cytokine such as TNF-α, and induction of cytokine gene expression by lipopolysaccharide (LPS) occurs primarily at transcription level and involves the action of several transcription factors, including members of the nuclear factor-κB (NF-κB)/rel, C/EBP, Ets, and AP-1 protein families (Sweet & Hume 1996). Especially, NF-κB bound to a specific consensus DNA element on the promoter of target genes initiates the transcription of TNF-α, inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 and interleukin-6 (Kuprash et al 1995).

Antitumoral, cytotoxic, anti-inflammatory and antimicrobial activity were reported for *C. majus* (Kim et al 1969; Lenfeld et al 1981; Horváth et al 1982). We hypothesize that the anti-infectious and antitumour effects of *C. majus* are due to its ability to enhance the nonspecific immunologic function by NO.

In this study, we have shown that *C. majus* synergistically induced NO and TNF-α production from peritoneal macrophages when the cells were treated by recombinant IFN-γ (rIFN-γ). To investigate the mechanism of *C. majus*-induced NO and TNF-α production, we examined the ability of the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC).

Materials and Methods

Reagents

Murine rIFN-γ (1×10^6 U mL^{-1}) was purchased from PharMingen (San Diego, CA). Dulbecco's Modified Eagle's Medium (DMEM), *N*-(1-naphtyl)-ethylenediamine dihydrochloride, LPS, sodium nitrite and PDTC were purchased from Sigma (St Louis, MO). Rabbit polyclonal antisera to iNOS was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Recombinant TNF-α, biotinylated TNF-α and anti-murine TNF-α were purchased from R & D System Inc, USA. N^G-Monomethyl-L-arginine (N^GMMA) was purchased from Calbiochem (San Diego, CA). Thioglycollate was purchased from Difco Laboratories (Detroit, MI). A 0.2-μm syringe filter and 96-well and 4-well tissue culture plates, and 100-mm diameter dishes were obtained from Nunc (Naperville, IL). DMEM containing L-arginine (84 mg L^{-1}), Hank's balanced salt solution (HBSS), foetal bovine serum (FBS) and other tissue culture reagents were from Life Technologies (Grand Island, NY). Male C57BL/6 mice (25–30 g) were purchased from Da Mul Science (Daejeon, Republic of Korea).

Peritoneal macrophage cultures

All studies were performed in accordance with international regulations for the handling and use of laboratory animals. The institutional Animal Care and Use Committee approved the protocols.

Mice were injected intraperitoneally with 2.5 mL thioglycollate, and three to four days later thioglycollate-elicited macrophages were harvested and isolated (Narumi et al 1990). Peritoneal lavage was performed using 8 mL HBSS containing 10 U mL^{-1} heparin. The cells were then distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) and incubated for 3 h at 37 °C in an atmosphere of 5% CO_2. This was then washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM, containing 10% FBS, before treatment.

Preparation of *C. majus*

The plant sample was obtained from the Oriental drug store, College Oriental Pharmacy (Iksan, Republic of Korea). The voucher specimen (Number 03-02-179) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. An extract of *C. majus* was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was approximately 3 h. The decoction was filtered, lyophilized and kept at 4 °C. The yield of dried extract from starting materials was approximately 9%. Dilutions were made in saline then filtered through a 0.2-μm syringe filter.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ (10 U mL^{-1}) for 6 h. The cells were then stimulated with various concentrations of *C. majus*. NO synthesis in cell cultures was measured by a microplate assay method (Xie et al 1992). To measure nitrite, 100-μL samples were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5–8 μM NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Assay of TNF-α release

Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (10 U mL^{-1}), *C. majus*, rIFN-γ plus LPS (10 μg mL^{-1}) and rIFN-γ plus various concentrations of *C. majus* for 24 h. The amount of TNF-α secreted by the cells was measured by a modified ELISA (Jeong et al 2002). The ELISA was devised by coating 96-well plates of murine monoclonal antibody with specificity for TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. rTNF-α was diluted and used as a standard. Serial dilutions starting from 5 ng mL^{-1} were used to establish the standard curve. Assay plates were exposed sequentially to alkaline-phosphatase-conjugated goat anti-rabbit IgG. Optical density readings were made within 10 min of the substrate on a Titertek Multiskan with a 405-nm filter. Appropriate specificity controls were included.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ ($10\,\mathrm{U\,mL^{-1}}$). The cells were then stimulated with *C. majus* ($1\,\mathrm{mg\,mL^{-1}}$) or LPS ($10\,\mu\mathrm{g\,mL^{-1}}$) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and then incubated with anti-iNOS antibodies. After washing in PBS-Tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Assay for endotoxin determination

C. majus extracts used in this experiment were found to be free of endotoxin as determined within the limits of an assay E-TOXATE kit (Sigma), performed according to the manufacturer's protocol.

Statistical analysis

Results were expressed as the mean ± s.e.m. of independent experiments. Kruskal–Wallis non-parametric test and post hoc tests were used to compare values measured for the different experimental groups. $P < 0.05$ was considered to be statistically significant.

Results

Effects of *C. majus* on NO production in activated peritoneal macrophages

To determine the effect of *C. majus* on the production of NO by mouse peritoneal macrophages, we treated non-primed (resting) or rIFN-γ-primed cells with *C. majus*. The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48-h treatment. As shown in Figure 1, *C. majus* had no effect on NO production in resting mouse peritoneal macrophages. However, when mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with *C. majus*, NO production was significantly increased compared with non-primed conditions (Figure 1).

Effects of *C. majus* on rIFN-γ-primed iNOS expression

Figure 2A shows the effects of rIFN-γ plus *C. majus* treatments on the expression of iNOS protein in mouse peritoneal macrophages. rIFN-γ plus *C. majus* synergistically increased the expression of iNOS protein in mouse
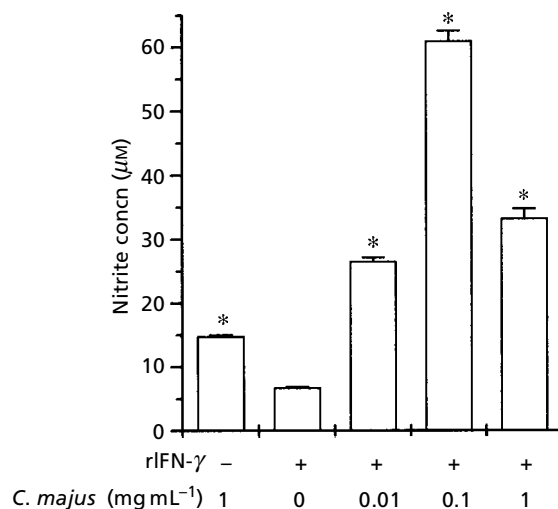
Figure 1 NO production effects of *C. majus* in rIFN-γ-treated peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were cultured with rIFN-γ ($10\,\mathrm{U\,mL^{-1}}$). The peritoneal macrophages were then stimulated with various concentrations of *C. majus* for 6 h after incubation. After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± s.e.m. of three independent experiments duplicated in each run. *$P < 0.05$ compared with rIFN-γ alone.

peritoneal macrophages. Figure 2B shows the iNOS synthesis in Figure 2A, normalized to control value using an Image Master program (Pharmacia Biotech).

Inhibition of *C. majus*-induced NO production by N^GMMA

N^GMMA is the specific inhibitor of NO production in the L-arginine-dependent pathway (Takao et al 1997). To determine if the signalling mechanism in *C. majus*-induced NO production participated in the L-arginine-dependent pathway in mouse peritoneal macrophages, the cells were incubated for 6 h in the presence of rIFN-γ plus N^GMMA. The production of nitrite by rIFN-γ plus *C. majus* in mouse peritoneal macrophages was progressively inhibited with increasing amounts of N^GMMA (Figure 3).

Inhibition of *C. majus*-induced NO production by PDTC

Schreck et al (1992) reported that an antioxidant compound, PDTC, inhibited NF-κB activation. To define the signalling mechanism of *C. majus* on NO production, we examined the influence of NF-κB inhibitor, PDTC, in rIFN-γ plus *C. majus* ($1\,\mathrm{mg\,mL^{-1}}$)-treated mouse peritoneal macrophages. Adding PDTC ($100\,\mu\mathrm{M}$) to the rIFN-γ plus *C. majus*-treated mouse peritoneal macrophages decreased the synergistic effects of *C. majus* on NO production (Table 1).

A

B
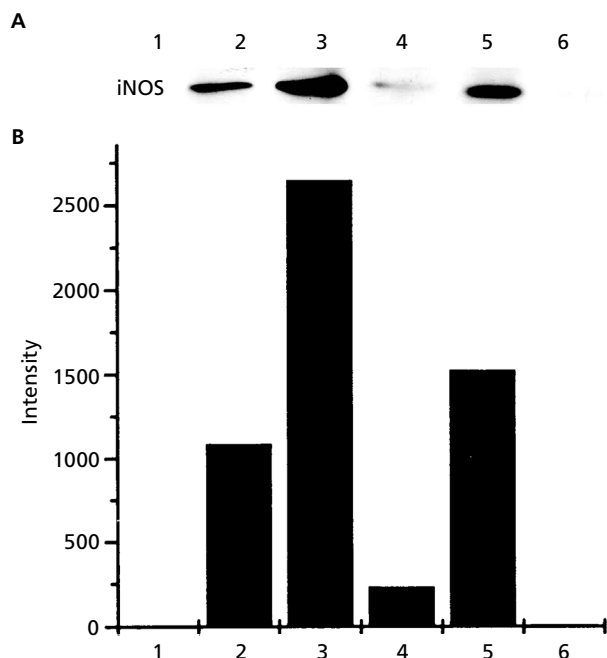
Figure 2   Effects of *C. majus* on the expression of iNOS by rIFN-γ plus *C. majus*-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (10 U mL^{-1}). The peritoneal macrophages were then stimulated with *C. majus* (1 mg mL^{-1}) or LPS (10 μg mL^{-1}) for 12 h. The protein extracts were prepared, and then samples were analysed for iNOS expression by Western blotting as described in the methods section (A). The iNOS levels were quantitated by densitometry (B). 1, Control; 2, rIFN-γ; 3, rIFN-γ + LPS; 4, *C. majus* (1 mg mL^{-1}); 5, rIFN-γ + *C. majus* (1 mg mL^{-1}); 6, rIFN-γ + *C. majus* (1 mg mL^{-1}) + PDTC (100 μM).

Effects of *C. majus* on rIFN-γ-induced TNF-α production

The synergistic co-operative effect of *C. majus* on rIFN-γ-induced TNF-α production was examined. Mouse peritoneal macrophages secreted very low levels of TNF-α after 24-h incubation with medium alone or rIFN-γ alone. *C. majus* alone increased TNF-α production by approximately three times compared with rIFN-γ alone. However, *C. majus* in combination with rIFN-γ markedly increased TNF-α production in a dose-dependent manner (Table 2).

We investigated the effect of *C. majus* on the signal transduction pathway of TNF-α production. As shown in Table 3, adding the NF-κB inhibitor, PDTC (100 μM), to the rIFN-γ plus *C. majus* (1 mg mL^{-1})-treated mouse peritoneal macrophages decreased significantly the synergistic effects of *C. majus* on TNF-α production.

Discussion

We have demonstrated that NO production by *C. majus* could be highly stimulated in combination with rIFN-γ in
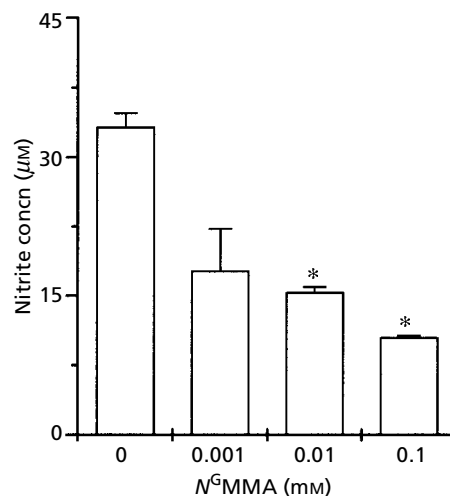
Figure 3   Effects of N^GMMA on *C. majus*-induced nitrite accumulation in the cultured medium of peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were incubated for 6 h with rIFN-γ (10 U mL^{-1}) plus various concentrations of N^GMMA. The peritoneal macrophages were then treated with *C. majus* (1 mg mL^{-1}) and cultured for 48 h. NO release was measured by the Griess method (nitirite). NO (nitrite) released into the medium is presented as the mean $\pm$ s.e.m. of three independent experiments duplicated in each run. *$P < 0.05$ compared with control (absence of N^GMMA).

Table 1   Effects of PDTC on rIFN-γ plus *C. majus*-induced NO production in mouse peritoneal macrophages.

Addition[a]			Nitrite concn (μM)[b]
rIFN-γ (10 U mL^{-1})	*C. majus* (1 mg mL^{-1})	PDTC (100 μM)	
−	−	−	<5
+	−	−	6.3 $\pm$ 0.1
+	+	−	33.2 $\pm$ 1.5
+	+	+	6.0 $\pm$ 0.5*

[a]Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with rIFN-γ, or *C. majus* + rIFN-γ, or rIFN-γ + *C. majus* + PDTC. [b]The amount of nitrite released by peritoneal macrophages was measured after 48 h by the Griess method. Values are the mean $\pm$ s.e.m. of three independent experiments duplicated in each run. *$P < 0.01$ compared with rIFN-γ plus *C. majus*.

mouse peritoneal macrophages. The results of this study suggested that *C. majus* might provide a second signal for synergistic induction of NO production in mouse peritoneal macrophages. N^GMMA, an analogue of L-arginine, inhibited rIFN-γ plus *C. majus*-induced NO production in peritoneal macrophages. The strong inhibition of NO production by N^GMMA indicated that it was likely to depend upon nitric oxide synthase.

At present, the precise physiological significance of NO production by *C. majus* is unknown. However, an important role for the synthesis of NO in host defense against

Table 2 Effects of *C. majus* on the production of TNF-α by rIFN-γ plus *C. majus* in peritoneal macrophages.

Addition[a]			TNF-α secretion (ng mL^{-1})[b]
rIFN-γ (10 U mL^{-1})	*C. majus* (mg mL^{-1})	LPS (10 μg mL^{-1})	
–	–	–	0.073 ± 0.003
+	–	–	0.085 ± 0.002
–	1	–	1.230 ± 0.309*
+	–	+	6.546 ± 0.371**
+	0.01	–	0.662 ± 0.229**
+	0.1	–	1.375 ± 0.207**
+	1	–	3.121 ± 0.869**

[a]Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with rIFN-γ or *C. majus* + rIFN-γ. [b]The amount of TNF-α secreted by peritoneal macrophages was measured by an ELISA method after 24-h incubation. Values are the mean $\pm$ s.e.m. of three independent experiments duplicated in each run. *$P < 0.05$ compared with negative control. **$P < 0.05$ compared with rIFN-γ alone.

Table 3 Effect of PDTC on rIFN-γ plus *C. majus*-induced TNF-α production in peritoneal macrophages.

Addition[a]			TNF-α secretion (ng mL^{-1})[b]
rIFN-γ (10 U mL^{-1})	*C. majus* (1 mg mL^{-1})	PDTC (100 μM)	
–	–	–	0.073 ± 0.003
+	–	–	0.085 ± 0.002
+	+	–	3.121 ± 0.869
+	+	+	0.116 ± 0.013*

[a]Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with rIFN-γ, or rIFN-γ + *C. majus*, or rIFN-γ + *C. majus* + PDTC. [b]The amount of TNF-α secreted by peritoneal macrophages was measured by an ELISA method after 24-h incubation. Values are the mean $\pm$ s.e.m. of three independent experiments duplicated in each run. *$P < 0.05$ compared with rIFN-γ + *C. majus*.

pathogens and tumour cells has been recognized (Ross & Reske-Kunz 2001). NO contributes to almost every stage of inflammation by impacting leucocyte migration, adherence, antimicrobial activities and phagocytic ability, and in fact, can act to restrict the development of inflammation (Bogdan 2001). An increase in NO levels at the site of infection could lead to increased bacterial killing and facilitated inflammation recovery. Therefore, we hypothesized that *C. majus* would alter development and resolution of the inflammatory process following a live, bacterial challenge and this effect may be due, in part, to elevations in NO. In addition, since NO has emerged as an important intracellular and intercellular regulatory molecule having functions as diverse as vasodilation, neural communication, cell growth regulation and host defense (Kim & Moon 1996), it was tempting to hypothesize that this molecule was involved in the local control of the various

fundamental processes. Kim et al (1995) reported that LPS stimulation of rIFN-γ-primed macrophages induced NF-κB activation. NF-κB is now known to be ubiquitously expressed and to play a major role in controlling the expression of protein involved in immune, inflammatory and acute phase responses (Baeuerle & Henkel 1994). Expression of iNOS and TNF-α genes is dependent on the activation of NF-κB (Baldwin 1996). We found that the addition of the NF-κB inhibitor PDTC inhibited the synergistic effect of *C. majus* with rIFN-γ on NO and TNF-α production. These results suggested that *C. majus* increased NO and TNF-α production through NF-κB activation. The NF-κB system might provide a future target in cures for tumours. However, as shown in Figure 1, 0.1 mg mL^{-1} *C. majus* induced maximal nitrite production whilst the same concentration of *C. majus* had very little activity on TNF-α production (Table 2). NO can induce p53 expression and cell death in RAW 264.7 macrophages (Messmer et al 1995). In this study, high production of NO induced by *C. majus* was assumed to evoke p53 expression on macrophages, which may have caused death of macrophages. This could be a reason why TNF-α production was low at 0.1 mg mL^{-1} *C. majus*, but a more precise study on this inconsistency is needed.

Conclusion

Our results demonstrated that *C. majus* acted as an accelerator of peritoneal macrophage activation by rIFN-γ via a process involving L-arginine-dependent NO production and that it increased the production of TNF-α significantly via NF-κB activation. Using a neutralizing antibody specific for TNF-α, NO synthesis was greatly diminished (Kim et al 1999). Thus, we supposed that the synergism between IFN-γ and *C. majus* in increasing NO synthesis by stimulated macrophages could be due to enhanced TNF-α secretion triggered by *C. majus* in IFN-γ-primed macrophages. Although the precise mechanism of *C. majus* to promote NO and TNF-α production induced by rIFN-γ remains to be elucidated, NO and TNF-α production by *C. majus* might explain its beneficial effect in the treatment of inflammation.

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