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INHIBITION OF NITRIC OXIDE (NO$^{\bullet}$) PRODUCTION IN MURINE MACROPHAGES BY FLAVONES

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Abstract—The effect of flavone (2-phenylbenzopyran-4-one) and three amino-substituted flavones on the production of nitrite by murine activated peritoneal macrophages was studied *in vitro*. Activated peritoneal macrophages obtained from mice pre-treated with concanavalin A (Con A) (*in vivo*), after exposure *in vitro* to lipopolysaccharide (LPS) at a concentration of 100 ng/ml, produced nitrite (20.3 ± 2.5 nmol/10^6 cells), as measured after 24 hr by the Griess reaction. Stimulation of production of nitrite was inhibited by N^G-monomethyl-L-arginine, suggesting that nitrite was formed via nitric oxide (NO$^{\bullet}$) as a product of metabolism of arginine. Stimulation was inhibited by flavone and the aminoflavones (20–100 μM). 3′-amino-4′-hydroxyflavone was the most potent inhibitor of nitrite production. Genistein (5,7-dihydroxy-3-(4-hydroxy-phenyl)-4H-1-benzopyran-4-one) also inhibited production of nitrite, by a mechanism that appears not to involve protein tyrosine kinases. These results suggest that the flavones can modulate the immune responses and the inflammatory reactions by controlling production of nitric oxide.

Key words: macrophages, nitric oxide, flavones, 3′-amino-4′-hydroxyflavone, NO$^{\bullet}$

Macrophages are known to play an important role in host defence mechanisms. Among a variety of mediators released by activated macrophages [1, 2], nitric oxide (NO$^{\bullet}$) has been identified as a potent molecule that may exert regulatory or cytotoxic effects depending on the concentration acting on the target cell [3, 4]. The inducible form of the arginine-dependent enzyme nitric oxide-synthase generates high, toxic amounts of nitric oxide, which enable the activated macrophages to destroy tumour cells, intracellular bacteria, parasites [5, 6, 7], and even normal tissue in situations of autoimmune reactivity [8, 9]. Earlier studies have shown that functions of activated macrophages such as killing of tumour cells, release of cytokines, and generation of oxygen radicals, can be regulated by flavones (2-phenylbenzopyran-4-ones) [10–12]. Various flavones have been shown to be good scavengers of free radicals [13–18] and to act as natural antioxidants. They have also been shown to inhibit oxido-reductases [19–21], thus preventing the formation of free radicals resulting from the reduction of oxygen. These two mechanisms can account for the role of many flavonoids in protecting cells from oxidative damage.

The present study was designed to investigate the role of flavones in the regulation of NO$^{\bullet}$ release from activated murine macrophages. Nitrite and nitrate are formed as end products of the metabolism of reactive nitrogen intermediates, with the measurement of nitrite using the Griess reagent (with or without the reduction of nitrate) being generally employed as a marker of formation of nitric oxide.

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Abbreviations: Con A, concanavalin A from *Canavalia ensiformis;* NMR, nuclear magnetic resonance spectrum; PBS, phosphate-buffered saline solution; FCS, heat-inactivated foetal calf serum; LPS, lipopolysaccharide from *E. coli* (serotype O127:B8); N^GMMA, N^G-monomethyl-L-arginine monoacetate salt.

MATERIALS AND METHODS

Mice

Specific pathogen-free 8-to-10 week old BALB/c male mice were purchased from the Institute of Oncology, Gliwice, Poland.

Reagents

Flavone (2-phenyl-4H-1-benzopyran-4-one, sample 1), 4′-aminoflavone (sample 2), and 3′-amino-4′-hydroxyflavone (sample 3) were synthesised as previously described [22]. 3′-amino-4′-methoxyflavone (sample 4) was prepared by reduction of 4′-methoxy-3′-nitroflavone with tin(II) chloride by the general method [22] (^{1}H NMR δ 6.83 (1 H, s, 3-H), 7.22 (1 H, d, J = 8.4 Hz, 5′-H), 7.50 (1 H, dd, J = 8.4, 7.0 Hz, 6-H), 7.69 (1 H, d, J = 8.1 Hz, 8-H), 7.86 (1 H, ddd, J = 8.4, 7.0, 1.8 Hz, 7-H), 7.94 (1 H, dd, J = 8.4, 2.2 Hz, 6′-H), 8.04 (1 H, dd, J = 8.4, 1.8 Hz, 5-H), 8.06 (1 H, d, J = 2.2 Hz, 2′-H), 9.7 (2 H, br, NH_2), 11.7 (1 H, s, OH)). The structures of the flavones used in this work are shown in Fig. 3. Phosphate-buffered saline solution (PBS), RPMI 1640 medium without phenol red, and heat-inactivated foetal calf serum (FCS, low in endotoxin) were purchased from GIBCO Life Technologies Ltd. (Paisley, U.K.). Thioglycollate broth, phenol red solution (0.5% aqueous solution of the sodium salt), N-(1-naphthyl)ethylenediamine dihydrochloride, sulphanilamide, and heparin sodium (pyrogen-free) were purchased from Serva Chemicals (Heidelberg, Germany). Lipopolysaccharide (LPS) from *E. coli* (serotype O127:B8), Concanavalin A (Con A) from *Canavalia ensiformis*, N^G-monomethyl-

L-arginine monoacetate salt (N^GMMA), daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), and genistein (5,7-dihydroxy-3-(4-hydroxy-phenyl)-4H-1-benzopyran-4-one) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Monoclonal antibody anti-mouse Thy-1,2 fluorescein conjugate was purchased from Becton Dickinson Immunocytochemistry Systems (Mountain View, CA, U.S.A.). Flavones (1–4), isoflavones (daidzein and genistein), LPS, and N^GMMA were dissolved in phenol red-free RPMI 1640 medium, and diluted immediately before use.

Collection and cultivation of mouse peritoneal exudate macrophages

Macrophages were obtained from mice given an i.p. injection of sterile thioglycolate broth (1 ml) 4 d prior to harvest and the solution of Con A (100 μg/ml) in PBS (1 ml) 18 hr prior to harvest. Mice were killed by cervical dislocation, and cells were collected by washing the peritoneum with PBS (5 ml). The macrophage populations were enriched by adherence to plastic in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) with calculated 10^6 macrophages per well. Non-adherent cells were removed after 120 min of incubation, and the macrophages were cultured with or without tested agent in RPMI 1640 medium without phenol red, supplemented with 10% heat inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. The adherent population contained >96% macrophages as assessed by May-Grünwald/Giemsa staining and biochemical criteria (nonspecific esterase staining), and <2% T-lymphocytes by fluorescence microscopy after fluorescent antibody labelling with anti Thy-1,2 monoclonal antibody. More than 98% of the cells were viable as determined by exclusion of neutral red [23], also after incubation with all reagents.

The macrophage monolayers were then covered by culture medium (2 ml) with flavones, N^GMMA, daidzein, or genistein (concentration range 1–300 μM) and LPS (100 ng/ml), and incubated for further 6, 18, 24, 48, and 72 hr at the same temperature and atmosphere as above. In all experiments, each concentration of flavone was tested in triplicate. Control macrophages were prepared and cultured in culture medium without added flavones.

Nitrite assay

Nitrite concentrations in the culture medium were measured by a microplate assay method, based on the Griess reaction [24]. Equal volumes of culture medium supernatant and Griess reagent (0.5% sulfanilamide, 0.05% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) were added to Eppendorf tubes and incubated at 20°C for 10 min. The tubes were then centrifuged for 5 min at 8500 × g. The absorbance of culture medium and Griess reagent at 550 nm was determined with SPECOL. Nitrite content was determined by using sodium nitrite as standard. Data were expressed as nmol nitrite per 10^6 cells originally plated. In all experiments, the nitrite content in wells containing medium without cells was measured and subtracted.

Statistical analysis

For statistical evaluation of the data, the Student's *t*-test was used.

RESULTS

In the presence of LPS, production of nitric oxide was found to be induced in Con A-stimulated macrophages in a time- and dose-dependent manner (Figs. 1 and 2). Con A-stimulated macrophages were obtained from mice injected with thioglycolate broth 4 d previously and with the solution of Con A 18 hr before being killed. Thioglycolate-elicited macrophages released small amounts of nitrite (1.3 ± 0.3 nmol/10^6 cells/24 hr). Elicited macrophages were prepared from mice injected 4 d previously with thioglycolate broth. Con A-stimulated macrophages also released small amounts of nitrite in the absence of LPS (Fig. 1).

Con A-stimulated macrophages produced L-arginine-dependent nitric oxide in the presence of LPS. N^GMMA (300 μM) inhibited nitrite production by 95 ± 3%. N^GMMA (400 μM) had no significant effect on the viability of macrophages exposed to LPS (100 ng/ml). Genistein, an isoflavone that inhibits tyrosine-specific protein kinases, and daidzein, an isoflavone analogue that is inactive against these tyrosine kinases, also significantly decreased the production of nitrite (Table 1).

In the next experiment, the flavones were investigated for their involvement in release of nitric oxide from Con A- and LPS-activated macrophages. Activated macrophages were found to produce detectable nitrite, which ranged in individual experiments from 17.8 to 22.8 nmol/10^6 cells/24 hr. Concentrations of the four synthetic flavones in the range 20–100 μM reduced the production of nitrite (Fig. 3). 3′-amino-4′-hydroxyflavone (3) was the most potent inhibitor, with significant inhibition at 1 μM and 84 ± 2% inhibition at 20 μM. Similar results were obtained following culture for 48 hr in the presence of the agents. Thomsen *et al.* [25] have pointed out that nitric oxide is converted both to nitrite and to nitrate. However, in representative control experiments in which the nitrate was reduced to nitrite with *Aspergillus* nitrate reductase and NADPH prior to assay, nitrate was found present in only trace amounts (results not shown). Thus, in our system, production of nitrite is a good measure of production of nitric oxide.

The viability of the macrophages was >92% following

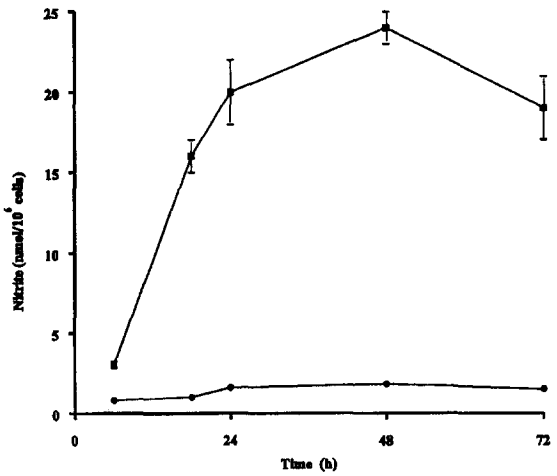

Fig. 1. Amounts of nitrite produced by macrophages cultured for 6, 18, 24, 48, or 72 hr without LPS (●) and with LPS (100 ng/ml) (■). Results are representative for three experiments.

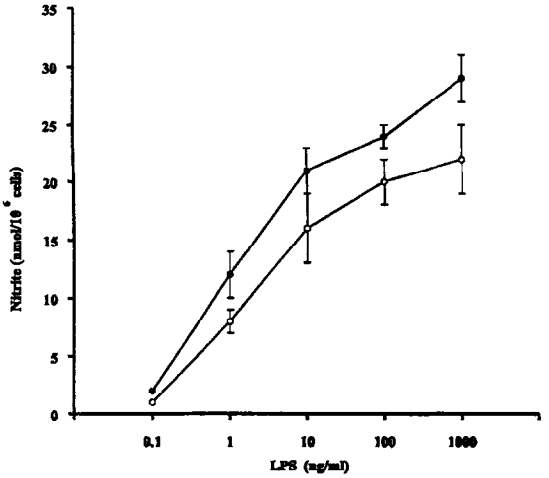

Fig. 2. Dose-dependent effects of LPS *in vitro* or production of nitrite by macrophages. Macrophages were incubated for 24 hr (○) or 48 h (●) with increasing concentrations of LPS. Each value is the mean ± *SD* of three experiments.

Table 1. Inhibitory effects of the isoflavones, genistein, and daidzein, and of N^GMMA on the nitrite produced by macrophages

Compound	Inhibition [%]
Genistein (50 μM)	48 ± 3
Daidzein (50 μM)	94 ± 2
N^GMMA (300 μM)	95 ± 3

Macrophages were incubated for 24 hr with LPS (100 ng/ml) in the presence of the inhibitors. Data show average ± *SD* from three independent experiments performed in triplicate.

treatment with agents (flavones and isoflavones) at concentrations up to 100 μM and >94% with no treatment. Hence, inhibition of production of nitrite is unlikely to be due to toxicity of the compounds. Additionally, the flavones and isoflavones had no quenching effect on the Griess reagent at the concentration used.

DISCUSSION

The adherent populations from mouse peritoneal cavities were used as macrophage models in this study. Thioglycollate-elicited peritoneal macrophages from BALB/c mice released significant amounts of nitrite upon stimulation with Con A (*in vivo*) and LPS (*in vitro*). This model for activation of macrophages has been reported [26, 27]. These activated macrophages produce a variety of biologically active molecules both in their normal role and in pathological immune responses and inflammatory reactions. Thioglycollate-elicited and Con A + LPS-primed macrophages express cytostatic activity against P815 tumour cells, and release interleukin-6 and nitrite [26]. Similarly activated macrophages secrete reactive oxygen intermediates (Król W, Czuba ZP and Threadgill MD, unpublished results).

We have shown here that flavone and three amino derivatives inhibit production of nitrite, a chemical product of nitric oxide. The cellular mechanisms of the effect are not clear, but may be related to known biological effects of the flavones, such as antioxidant properties and inhibition of cellular enzymes involved in signal transduction. Some phenolic compounds are known to have radical scavenging activities, and many isoflavonoids and related compounds reportedly possess strong antioxidative activities [28, 29]. However, direct reaction of nitric oxide or nitrite with the flavones, leading to apparent inhibition of production of NO$^{\bullet}$, is rendered highly unlikely by consideration of the stoichiometry of the experiments involving the most potent flavone (3). At 10 μM, the experimental volume (2 ml) contains 20 nmol of 3, yet the loss of production of nitrite compared to control is 16 nmol; at 1 μM, the experimental volume contains 2 nmol of 3, yet the loss of production of nitrite is approximately 4 nmol. These data preclude stoichiometric reaction of 3 with nitric oxide or nitrite from being any more than a very minor contribution to the inhibition observed.

Prior to this work, the only true flavone to have been shown to influence production of nitric oxide by macrophages was flavone-8-acetic acid [25]. This 8-substituted flavone has stimulatory activity, in contrast to the inhibitory activity demonstrated here for the unsubstituted and 3′,4′-substituted flavones 1–4. Indeed, it has recently been suggested that decarboxylation of flavone-8-acetic acid by the radical nitrogen dioxide may contribute to its apparent antitumour activity [30].

Three specific protein tyrosine kinase inhibitors (genistein, tyrphostin, and herbimycin A) have been reported to block production of nitrite in both C3H/HeN and C3H/HeJ macrophages [31]. Flavone itself and several aminoflavones are inhibitors of protein tyrosine kinase activity *in vitro* [22, 32, 33], with a variety of potencies. Cushman *et al.* reported that a series of 4′-aminoflavones inhibit the activity of the protein tyrosine kinase $p56^{lck}$; the most potent, 4′-amino-6-hydroxyflavone, inhibits this activity with IC_{50} = 1.2 μM [33]. In our previous study on the activity of a series of synthetic flavones against protein tyrosine kinases [22], we identified 3′-amino-4′-methoxy-flavone (4) as the most potent inhibitor of the EGF receptor tyrosine kinase activity derived from A431 cells (42% inhibition at 50 μM). This compound was inactive against the corresponding activity of p*tabl*50 at 500 μM, whereas quercetin (3,3′,4′,5,7-pentahydroxyflavone) had K_i = 3.7 μM. Interestingly, compound 4 showed the greatest selective cytotoxicity towards ANN-1 cells, which are Abelson-transformed 3T3 cells. In the present study, the isoflavone genistein was also found to inhibit the production of nitrite by macrophages. However, the analogous isoflavone daidzein, which does not inhibit protein tyrosine kinases, was found to be a more potent inhibitor of production of nitric oxide by these macrophages than is genistein. This suggests that inhibition of tyrosine kinases may not be involved in the mechanism of inhibition of release of nitric oxide in these macrophages.

In a subsequent study, a related series of flavones were evaluated for inhibition of the generation of reactive oxygen species by macrophages that had been primed by LPS-polyinosinic-polycytidylic acid, as shown by inhibition of luminol-dependent chemiluminescence [12]. Of these flavones, 3′-amino-4′-hydroxyflavone (3) was the most potent inhibitor, causing 93% inhibition at 100 μM, 52% inhibition at 10 μM, and 36% inhibition at 1.0 μM, corresponding to IC_{50} = 3.3 μM.

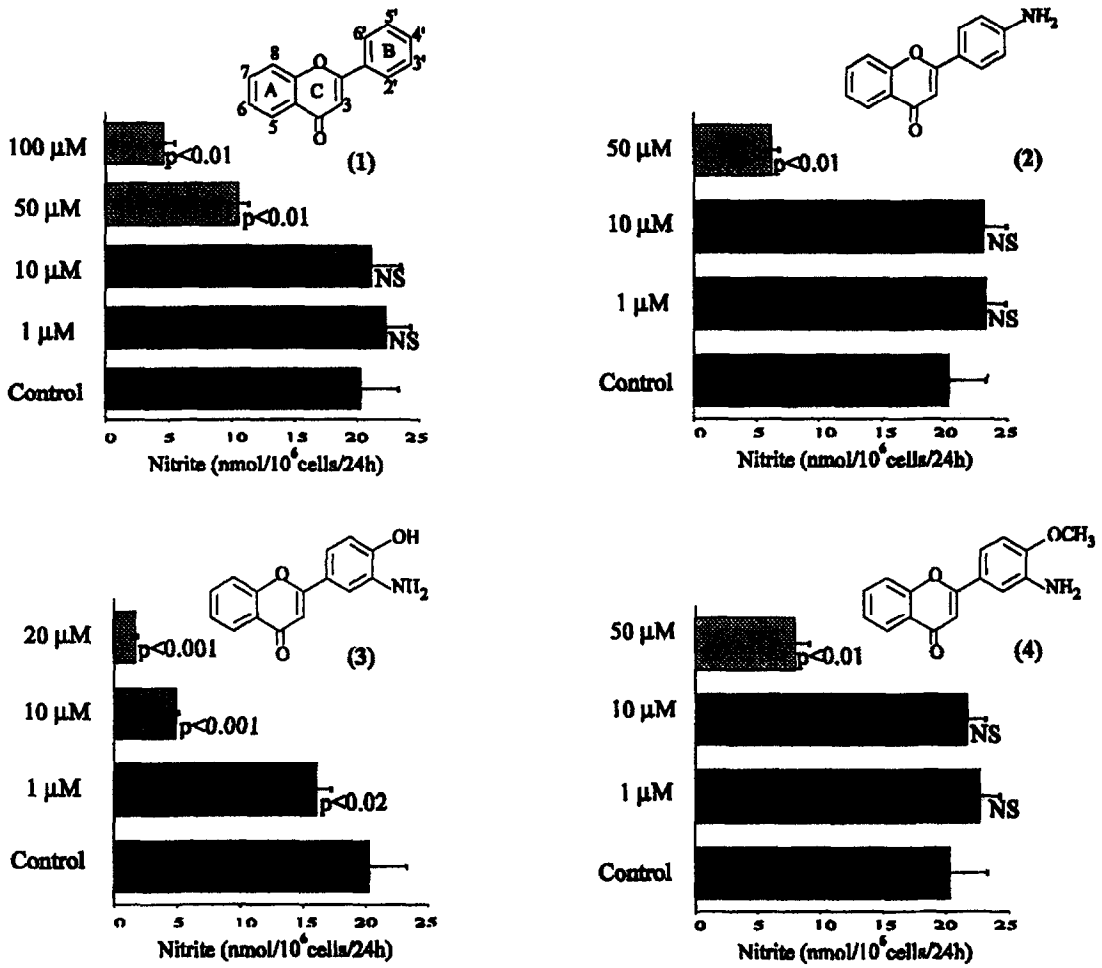

Fig. 3. Effects of flavone (1), 4′-aminoflavone (2), 3′-amino-4′-hydroxyflavone (3), and 3′-amino-4′-methoxyflavone (4) on production of nitrite by macrophages. Macrophages were incubated with LPS (100 ng/ml), without flavones (control) or with flavones, for 24 hr. Concentrations of nitrite (nmol/10^6 cells, mean ± *SD* of three wells from three independent experiments) produced by control macrophages were 20.3 ± 2.5.

Flavone (1), 4′-aminoflavone (2), and 3′-amino-4′-methoxyflavone (4) were less potent, causing 55%, 70%, and 65% inhibition, respectively, at 100 μM, and insignificant inhibition at 10 μM and at 1.0 μM. Interestingly, the concentrations of these four flavones required for inhibition of release of nitric oxide by the macrophages closely match these values (Fig. 3). These data strongly suggest that production of nitric oxide is a major controlling factor in generation of reactive oxygen species upon activation of the macrophages. Thus, flavones may have a role to play in modulating inflammatory and immune responses by affecting production of nitric oxide. Studies as to whether the flavones act as direct inhibitors of nitric oxide synthase or act upon a control system are in progress, and will be reported separately.

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