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MODULATION OF THE CYTOTOXIC ACTIVITY OF MURINE MACROPHAGES BY FLAVONES

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The effect of flavonoids (13 flavones and 2 isoflavones) on the production of nitrite and on luminol - dependent chemiluminescence by murine activated peritoneal macrophages (F4/80 positive) was studied *in vitro*. The accumulated nitrite as a stable final product of nitric oxide (NO) was determined by the Griess reaction. Nitrite production was inhibited by flavonoids. 3'-Amino-4'-hydroxyflavone and 3-hydroxyflavone were the most potent inhibitors of nitrite production. These compounds also inhibited chemiluminescence. Chemiluminescence was used in this study as an indicator for production of reactive oxygen species (ROIs) by macrophages. These data suggested a causative connection between NO and ROIs production in macrophages. Also these results show that the flavones can modulate the immune responses and the inflammatory reactions by controlling production of NO.

INTRODUCTION

The production of reactive oxygen (ROIs) and reactive oxynitrogen (RONIs) intermediates by macrophages is critical to host defence. Upon stimulation with both soluble and particulate matter, oxidative metabolism is stimulated in the macrophage resulting in the respiratory burst which is accompanied by activation of an NADPH-oxidising enzyme. This enzyme catalyses the reduction of molecular oxygen to superoxide anion ($O_2^{\cdot-}$) and the burst is paralleled by consumption of oxygen. $O_2^{\cdot-}$ is the precursor of other ROIs, including hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). Oxidation of cellular molecules by ROIs contributes to killing of phagocytized microbes and can cause severe toxicity to cancer cells (Fridovich, 1986). Until 1987, the only inducible biochemical mechanism that could explain activated macrophage cytotoxicity was the synthesis of ROIs by NADPH oxidase. Subsequently, the production of nitric oxide ($^{\cdot}NO$) from L-arginine by an immune/ inflammatory isoform nitric oxide synthase (iNOS) was described by Hibbs, Vavrin and Taintor (1987a, 1987b). Biosynthesis of NO may lead to the production of other RONIs, including nitrosonium (NO^+) and nitroxyl (NO^-) ions, nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and S-nitrosothiols (Stamler, Singel & Loscalzo, 1992). RONIs kill some cancer cells and are the ultimate effector molecules in the host defence against many intracellular pathogens, such as *Toxoplasma gondii*, *Leishmania*, and *Mycobacterium tuberculosis* and extracellular pathogens, such as *Cryptococcus neoformans* and *Schistosoma mansoni*.

However, both ROIs and RONIs are potentially toxic to the host making regulation of the production of these highly reactive molecules of utmost importance to host survival. NO, $O_2^{\cdot-}$ and its intermediates produced by activated phagocytes may play an important role in the multistage carcinogenesis process, triggered by chronic infection and inflammation. Inoue and Kawanishki (1995) suggest that NO reacts with $O_2^{\cdot-}$ to form $ONOO^-$ and the $ONOO^-$ induces oxidative DNA damage through an active intermediate of the reactivity which is similar to $HO^{\cdot}$. Our earlier studies have shown that functions of activated macrophages can be regulated by flavones (2-phenyl-4*H*-1-benzopyran-4-ones) (Middleton & Kandaswami, 1992). Flavones are a group of naturally occurring compounds, widely distributed as secondary metabolites in the plant kingdom. They have been ac-

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knowledged as having interesting medicinal properties, such as antiinflammatory, antiallergic, antiviral, antibacterial, and antitumour activities (Havesteen, 1983). Flavones have also been the scavengers of free radicals and act as natural antioxidants. They have also been shown to inhibit oxido-reductase, thus preventing the formation of free radicals resulting from the reduction of oxygen. These two mechanisms can account for the role of many flavones in protecting cells from oxidative damage (Król, Czuba & Scheller, 1986).

The present study was aimed to investigation of the role of flavones in the regulation of RONIs and RONIs release from activated murine macrophages. Chemiluminescence was used in this study as an indicator for the productions of ROIs by macrophages stimulated by PMA as stimulant for oxygen metabolism through the activation of protein kinase C (PKC). RONIs release from activated macrophages was determined by nitrite as a stable end product of NO.

MATERIAL 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, 4'-aminoflavone, 3'-amino-4'-hydroxyflavone, and 3'-amino-4'-methoxyflavone were synthesised as previously described (Cunningham, Threadgill, Groundwater, Dale & Hickman, 1992; Król, Czuba, Threadgill, Cunningham & Shani, 1995a). Flavone-8-acetic acid (FAA) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA, through the courtesy of Dr. K. Paull. 4'-amino-6-hydroxyflavone, daidzein, genistein, lipopolysaccharide (LPS) from *E. coli* (serotype 0127 : B8), Concanavalin A (Con A), N^G-monomethyl-L-arginine monoacetate salt (N^GMMA), superoxide dismutase (SOD) from human placental (4750 U/mg) were purchased from Calbiochem (La Jolla, CA, USA). 3-Hydroxyflavone and other flavones were purchased from Roth Chem. (Karlsruhe, Germany). Hanks' balanced salt solution (HBSS) without phenol red, phosphate-buffered saline solution (PBS), RPMI 1640 medium without phenol red, and heat-inactivated fetal calf serum (FCS) low in endotoxin, penicillin – streptomycin (10 000 IU/ml – 10 000 μg/ml) were purchased from GIBCO BRL Life Technologies Ltd. (Paisley, UK). Neutral 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). Phorbol myristate acetate (PMA), kit for α-naphthyl acetate esterase determination and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was bought from LKB (Turku, Finland). The monoclonal antibody (mAb) anti-mouse Thy-1,2 fluorescein conjugate was purchased from Becton Dickinson Systems (Mountain View, CA, USA). The mAb F4/80 anti-mouse macrophages fluorescein conjugate was purchased from SEROTEC Ltd. (Oxford, UK). Fluid thioglycolate medium was purchased from DIFCO Lab. (Detroit, MI, USA).

Collection and cultivation of mouse peritoneal exudate macrophages

Macrophages were obtained from mice given an i.p. injection of sterile thioglycolate broth (1 ml) 4d prior to harvest and the solution of Con A (100 μg/ml) in PBS (1 ml) 18 h prior to harvest. Mice were sacrificed by cervical dislocation, and cells were collected by washing the peritoneum with PBS (5ml). The macrophages populations were enriched by adherence to plastic or glass 24-well plates (Falcon Becton Dickinson, Lincoln Park, NJ, USA) (nitrite assay), 16-well TC chamber slides (Nunc, Roskilde, Denmark) (morphological study of macrophages) or test tubes (chemiluminescence) with calculated 10^6 macrophages per well or tube. Non-adherent cells were removed after 2 h of incubation in RPMI 1640 without phenol red, supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) (culture medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. The adherent population cells were assessed by May-Grünwald/Giemsa staining and biochemical criteria whereas immunological specificity (mAbs to mouse leukocyte differentiation antigens) by fluorescence microscopy.

Nitrite assay

The accumulated nitrite as a stable end product of NO was determined colorimetrically by a diazotization reaction using the standard Griess reagent (Green, Wagner, Głogowski, Skipper, Wishnak & Tannenbaum, 1982), as described in detail previously (Król, Czuba, Thredgill, Cunningham & Pietsz, 1995a) after 48 h culture of macrophages

in the absence or presence of various flavonoids, SOD, N^GMMA in the presence of LPS. The absorbance of culture medium and Griess reagent at 550 nm was determined with automated microplate reader ELx 800 (Bio-Tek Instruments Inc., Winooski, VT, USA). 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.

Chemiluminescence

Chemiluminescence was measured as described previously (Król, Czuba, Scheller, Gabryś, Grabiec & Shani, 1990; Czuba, Król, Scheller & Shani, 1992) using a special low noise-count rate photomultiplier 9514s (EMI, Middlesex, UK). The sample volume was usually 1 ml. The reactions were initiated by dispensing aliquots of solutions of luminol (100 μM) to macrophages (10^6) and tested compounds in HBSS in glass test tubes. The resting intensity of chemiluminescence was recorded after 5 min. PMA solution was then added to give a final concentration of 0.8 μM. The light emitted was then recorded continuously for 20 min.

RESULTS

The adherent cells populations contained > 98% macrophages as assessed by May-Grünwald/Giemsa staining and biochemical criteria (nonspecific esterase staining), > 98% F4/80-positive, and < 2% T-lymphocytes by fluorescence microscopy after fluorescent antibody labelling with anti Thy-1,2 mAb. More than 98% of the cells were viable as determined by exclusion of neutral red, also after incubation with all reagents.

Con A-stimulated macrophages produced L-arginine-dependent NO in the presence of LPS (100 ng/ml). The production of nitrite as a stable final product of NO was 44.2 ± 1.0 nmol/10^6 cells after 48 h. N^GMMA (300 μM) inhibited nitrite production by 87 ± 2% (Table 1) SOD (200 U) also decreased the production of nitrite. In an other experiment, upon addition of PMA (0.8 μM), macrophages luminol-dependent chemiluminescence increased 25 times over baseline light emission, reaching the maximum about 9 min after addition of the stimuli. The NOS inhibitor, N^GMMA (300 μM) plus SOD, significantly diminished route and peak intensities of PMA-stimulated macrophages chemiluminescence (85 ± 1%) (Table 1).

In the next experiment, the flavonoids were investigated for their involvement in release of NO from Con A and LPS-activated macrophages. The structures of the flavonoids used in this work are shown in Table 2. Concentrations of the flavonoids in the range 10–50 μM reduced the production of nitrite (Table 3). 3-Hydroxyflavone and 3'-amino-4'-hydroxyflavone were the most potent inhibitors. 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.

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.

The purpose of the present study was also to evaluate quantitatively the activity of flavonoids on the chemiluminescent activity of PMA-stimulated macrophages.

Table 1

Inhibitory effects of the superoxide dismutase and of N^G-monomethyl-L-arginine on the nitrite and luminol-dependent chemiluminescence production by murine macrophages

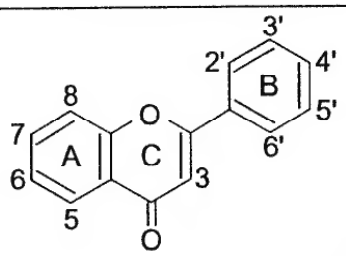
Inhibitor	Inhibition (%)	
	NO_2^- production[a]	CL production[b]
SOD (200 U)	10 ± 1	81 ± 1
N^GMMA (300 μM)	87 ± 2	45 ± 1
SOD (200 U) + N^GMMA (300 μM)	88 ± 2	85 ± 1

[a] Nitrite concentrations in the culture medium were measured after 48 h. Macrophages were incubated with LPS (100 ng/ml) in the presence of the inhibitors. Concentration of nitrite (nmol/10^6 cells, mean ± SD of three wells from three independent experiments) produced by control macrophages were 44.2 ± 1.0.

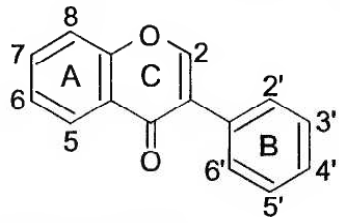
[b] The intensity of CL was determined by measuring counts/min and by calculating the area under CL intensity curve (integral counts). The rates of CL produced by 1×10^6 macrophages for 20 min is the absence of the inhibitors were 85000 ± 2000 (integral counts). Results are expressed as mean ± SD, n = 2–4.

Table 2

Structures of flavonoids evaluated.



flavo-noid	name	substituent						
		3	5	6	7	8	3'	4'
1	Flavone	H	H	H	H	H	H	H
2	3-Hydroxyflavone	OH	H	H	H	H	H	H
3	Flavone-8-acetic acid	H	H	H	H	CH_3CO_2H	H	H
4	4'-Aminoflavone	H	H	H	H	H	H	NH_2
5	3'-Amino-4'-hydroxyflavone	H	H	H	H	H	NH_2	OH
6	3'-Amino-4'-metoxyflavone	H	H	H	H	H	NH_2	OCH_3
7	4'-Amino-6-hydroxyflavone	H	H	OH	H	H	H	NH_2
8	Chrysin	H	OH	H	OH	H	H	H
9	Galangin	OH	OH	H	OH	H	H	H
10	Genkwanin	H	OH	H	OCH_3	H	H	OH
11	Kaempferol	OH	OH	H	OH	H	H	OH
12	Kaempferide	OH	OH	H	OH	H	H	OCH_3
13	Quercetin	OH	OH	H	OH	H	OH	OH



		substituent		
		5	7	4'
14	Daidzein	H	OH	OH
15	Genistein	OH	OH	OH

The level of inhibition of all fourteen compounds tested is presented in Table 4. 3-Hydroxyflavones (3-hydroxyflavone, galangin, kaempferol, kaempferide and quercetin) and 3'-amino-4'-hydroxyflavone were the most potent inhibitors. PMA-stimulated production of chemiluminescence was enhanced to a small but statistically insignificant extent in the presence of low (10 μM) concentrations of 4'-aminoflavone and 3'-amino-4'-methoxyflavone. However, PMA-stimulated production of chemiluminescence was progressively inhibited in the presence of increasing concentrations of these agents.

DISCUSSION

The toxicity of NO is primarily determined in the presence of H_2O_2 rather than O_2^-. This synergism may have important implications for the understanding of the pathophysiology of sepsis, reperfusion injury and shock. Locally disregulated productions of either species (e.g. after the exposure of cytokines) may be fatal when are produced together, but regulate normal cellular functions when acting separatly. A second possible explanation may arise from a regulated coproduction when cytotoxicity is required. Macrophages are capable

Table 3

Effects of flavonoids on the production of nitrite by murine macrophages

Flavonoid	Inhibition (%)[a]		
	Concentration (μM)		
	10	20	50
1	2 ± 1	3 ± 1	75 ± 10
2	ND	96 ± 1	ND
3	−1 ± 1	3 ± 2	9 ± 4
4	49 ± 1	66 ± 1	86 ± 3
5	30 ± 4	89 ± 3	ND
6	3 ± 1	23 ± 1	83 ± 7
7	3 ± 1	ND	86 ± 2
8	3 ± 1	86 ± 1	80 ± 3
9	15 ± 2	27 ± 3	ND
10	42 ± 3	67 ± 8	ND
11	22 ± 5	70 ± 2	ND
12	22 ± 6	80 ± 6	ND
13	4 ± 1	2 ± 1	33 ± 20
14	ND	ND	97 ± 2
15	ND	ND	57 ± 9

[a]Macrophages were incubated with LPS (100 ng/ml), without flavonoids or with flavonoids, for 48 h. Concentration of nitrite (nmol 10^6 cells, mean ± SD of three wells from three independent experiments) produced by control macrophages were 42.8 ± 5.9.
ND: not determined.

Table 4

Effects of flavonoids on luminol – dependent chemiluminescence of murine macrophages

Flavonoid	Inhibition (%)[a]		
	Concentration (μM)		
	10	50	100
1	11 ± 5	63 ± 1	79 ± 2
2	91 ± 2	99 ± 1	100
3	−12 ± 4	−14 ± 6	−19 ± 6
4	−4 ± 1	74 ± 4	78 ± 3
5	86 ± 1	98 ± 1	100
6	−4 ± 2	55 ± 2	82 ± 8
7	41 + 2	ND	43 ± 3
8	49 ± 9	96 ± 1	98 ± 1
9	92 ± 1	99 ± 1	100
10	30 ± 7	32 ± 8	37 ± 9
11	93 ± 1	100	100
12	95 ± 1	100	100
13	98 ± 1	100	100
14	29 ± 4	86 ± 1	88 ± 1
15	56 ± 10	88 ± 1	90 ± 1

[a]The rates of CL produced by 1×10^6 macrophages for 20 min is the absence of flavonoids were 85000 ± 2000 (integral counts). A negative value indicates stimulation. Results are expressed as mean ± SD, $n = 3–5$.
ND: not determined.

of secreting an array of cytotoxic products that include ROIs and RONIs.

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 vitro*) and LPS (*in vitro*). This model for activation of macrophages has been reported in our previous study (Król *et al.*, 1995a).

Thioglycollate elicited and Con A + LPS – primed macrophages express cytostatic activity against P815 tumour cells, and release interleukin-6 and tumour necrosis factor-α. Similarly, activated macrophages secrete of ROIs (Król *et al.*, 1995b)

We have shown here that flavones and isoflavones inhibit production of nitrite, a chemical product of NO. These compounds also inhibit luminol-dependent chemiluminescence. The cellular mechanism of the effects are not clear, but may by related to known biological effects of the flavones, such as antioxidant and antiradical properties (Robak & Gryglewski, 1988; Cotelle, Bernier, Catteau, Pommery, Wallet & Gaydon, 1996), inhibition of oxido-reductases (Tauber, Fay & Marletta, 1984) and inhibition of cellular enzymes involved in a signal transduction (Cunningham *et al.*, 1992; Cushman, Zhu, Geahlen & Kraker, 1994). 3'-Amino-4'-hydroxyflavone and 3-hydroxyflavone was the most potent inhibitors of NO production in murine macrophages and were evaluated for inhibition of the generation of ROIs by macrophages. In a previous study from this laboratory we demonstrated that the flavonols (3-hydroxyflavone derivatives) also was the most decreased neutrophil chemiluminescence (Król, Shani, Czuba & Scheller, 1992; Król, Czuba, Scheller, Paradowski & Shani, 1994). Interestingly, the concentration of release of NO by the macrophages closely match these values. These data strongly suggest that production of NO is a major controlling factor in generation of chemiluminescence upon activation of the macrophages. Also these results show that the flavones can modulate the immune responses and the inflammatory reactions by controlling productions of NO.

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