Biochemical Pharmacology, Vol. 52, pp. 991–998, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00386-3

Cytokine Production in Human and Rat Macrophages and Dicatechol Rooperol and Esters

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ABSTRACT. The ability of dicatechol rooperol and esters to inhibit the production of cytokines in endotoxin-stimulated human alveolar macrophages, human blood monocyte/macrophages, histiocytic cell line U937, and rat alveolar macrophages was examined *in vitro*. Rooperol derivatives inhibited the production of tumour necrosis factor-α, interleukin-1β and interleukin-6. Of the esters tested on human cells, rooperol diacetate and tetraacetate were more potent inhibitors of cytokine production (IC_{50} in the range of 10–20 μM) than rooperol disulphate (IC_{50} in the range of 25–75 μM). The acetate esters also inhibited cytokine production in rat alveolar macrophages, whereas the sulphate had little effect. Rooperol and acetate esters, in the same concentration range, decreased the production of nitric oxide by rat alveolar macrophages stimulated by endotoxin. These concentrations of rooperol had no effect on cell viability, as indicated by incorporation of ^{14}C-labelled leucine into macrophage proteins and their content of lactate dehydrogenase. The results obtained suggest that rooperol esters are potentially useful antiinflammatory agents. BIOCHEM PHARMACOL 52;7:991–998, 1996.

KEY WORDS. cytokine synthesis; macrophages; endotoxin; nitric oxide; rooperol

Evidence has accumulated that the cytokine TNFα[‖] and IL-1 play an important role in inflammatory reactions involved in the pathogenesis of many diseases, including septicaemia, trauma, burns, rheumatoid arthritis, and asthma [1–4]. Injury and shock involve a cascade of events leading to a highly complex homeostatic reaction known as the acute phase response, in which the key role is played by various cytokines [5, 6]. Macrophages stimulated by bacterial endotoxin, other bacterial products, and lymphokines are the most important source of TNFα, IL-1β and IL-6 [7–9].

A major focus of asthma research over the past 5 years has been the role of inflammatory cells, mediators, cytokine, and adhesion molecules in the maintenance of airway inflammation and bronchoconstriction [10]. Pulmonary alveolar macrophages release TNFα and IL-1, which induce the expression of adhesion molecules on endothelial cells, thereby increasing the infiltration of leukocytes, including eosinophils. Recently, these cytokines have been shown [4] to augment the expression of iNOS in pulmonary airway epithelial cells. Asthmatic patients demonstrate an increased expression of iNOS and an increased level of nitric oxide (NO) in exhaled air.

To suppress systemic and local inflammatory responses, it is desirable to inhibit the production of proinflammatory cytokines and other mediators by macrophages and other cells. Glucocorticoids are widely used for this purpose, inhibiting as they do, the synthesis of TNFα and IL-1β by macrophages [2] and suppressing induction by TNFα and IL-1 of iNOS expression in airway epithelial cells [4] and endothelial cells [11]. Glucocorticoids also inhibit the production of eicosanoids. Despite the usefulness of glucocorticoids in inflammatory diseases and asthma, their side effects are limiting. It is therefore desirable to identify other classes of drugs that can be administered topically or by inhalation. This paper describes rooperol, which has activities suggesting a promising profile as a drug.

Rooperol is a dicatechol (Fig. 1) derived from the South African plant, *Hypoxis rooperi*. Rooperol has been shown [12] to be a potent inhibitor of 5-lipooxygenase, but not of cyclooxygenase. The compound has no activity when tested against the two isoforms of PGH synthase in concentrations up to 10 μM, which are clinically attainable with topical use. Because peptidoleukotrienes are major mediators of bronchoconstriction, whereas PGE_2 has bronchodilator activity [13], inhibiting the production of the former, but not the latter, is desirable in asthma. In this

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‖ *Abbreviations:* DMEM, Dulbecco's Modified Eagle Medium; FCS, foetal calf serum; IL-1, interleukin-1; IL-6; interleukin-6; iNOS, inducible NO synthase; LDH, lactate dehydrogenase; LPS, endotoxin; NDGA, nordihydroguaiaretic acid; PMA, phorbol myristate acetate; TNF, tumour necrosis factor-α.

Received 18 December 1995; accepted 25 April 1996.

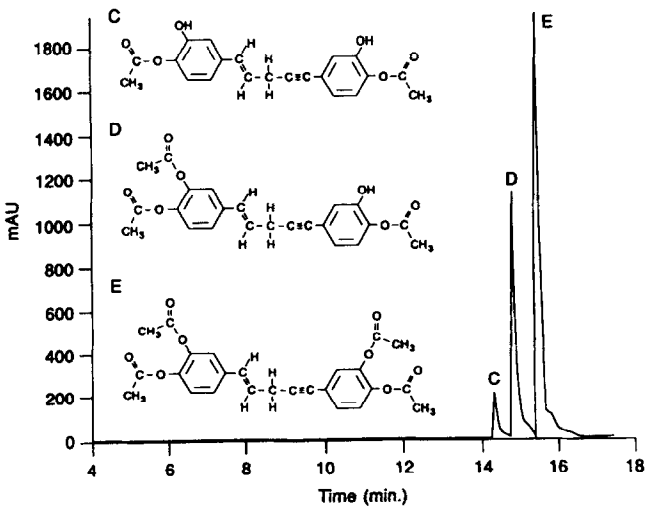

FIG. 1. The structures of rooperol acetates and their separation by HPLC (see Methods).

paper, experimental results show that rooperol inhibits the production of TNFα, IL-1β, and IL-6 by human and rat alveolar macrophages and NO production in the latter cells. In a complementary study, we found that rooperol inhibits cytokine-induced iNOS and VCAM-1 expression in murine endothelial cells.* Repeated administration of rooperol by inhalation may therefore decrease production of proinflammatory cytokines and NO in asthmatics, thereby reversing the imbalance between subsets of helper T-lymphocytes characteristic of that disorder [4].

A dicatechol structurally related to rooperol, nordihydroguaiaretic acid (NDGA), has been found clinically active in actinic keratosis [14]. However, NDGA is an irritant when applied to the skin [14], possibly because the catechol moieties, in the presence of iron in the skin, exert oxidant activity leading to the release of inflammatory mediators [15]. Free catechols are relatively unstable, and unsuitable for pharmaceutical formulation. Therefore, we have prepared acetyl esters of rooperol that are rapidly converted in the gastrointestinal tract and by cultured macrophages into free rooperol, as shown by HPLC determination [16]. Acetyl esters seem to be suitable for inhalation, application to the skin, and treatment of inflammatory diseases of the small bowel. To treat inflammation of the large bowel, oral administration of rooperol diglucoside is proposed. The diglucoside is pharmacologically inactive [12], but is converted in the large intestine of humans and experimental animals into rooperol by bacterial β-glucosidase activity. Rooperol is recoverable in concentrations of 10 μM or higher from the intestines, but is not detectable in circulating blood. It is quantitatively converted into phase 2 metabolites (glucuronide or sulphate) on the first pass through the liver. For these reasons, rooperol would be expected to exert desirable antiinflammatory activity locally (in the lungs, intestinal tract, and skin), but have few systemic effects.

In the experiments described here, we have studied the effects of acetate and sulphate esters of rooperol on human and rat pulmonary macrophages, and also on human blood mononuclear cells and the human histiocytic cell line U937.

MATERIALS AND METHODS

Materials

The sources of reagents were as follows: kits for determination of TNFα, IL-1β and IL-6 were from Genzyme (Cambridge, MA, U.S.A.); antiserum to rat C3 complement from Cappel Oregon Teknika Corp. (Durham, NC, U.S.A.); antisera to rat fibrinogen, α-2-macroglobulin and α-1-acid glycoprotein were prepared by us in the rabbit; antisera to human haptoglobin and fibrinogen were from ATAB (Stillwater, MI, U.S.A.); and to human transferrin from Behringwerke AG (Marburg, Germany); ^{14}C-leucine was from Polatom (Świerk, Poland); RPMI tissue culture medium, FCS, antibiotics from Gibco (New York, NY, USA); DMEM tissue culture medium, L-glutamine, TRIS, dexamethasone, PMA, agarose, and NADH were from Sigma (St. Louis, MO, U.S.A.); all other reagents of analytical grade were from POCH (Gliwice, Poland).

Endotoxin (LPS, lipopolysaccharide from E. coli 026-86, Sigma) was dissolved in sterile PBS to the concentration of 1 mg/mL. The working concentration of LPS was 500 ng/mL.

Rooperol was isolated from *Hypoxis rooperi* as described by Van der Merwe *et al.* [12]. The diacetate and tetraacetate were prepared by esterification with acetic anhydride in the presence of pyridine. Products were separated and identified by HPLC (Fig. 1) as described by Kruger *et al.* [16]. Rooperol 4,4′-disulphate was recovered from the urine of human volunteers to whom a rooperol prodrug had been administered, and purified by HPLC. Rooperol acetates and sulphate were dissolved in DMSO to prepare stock 100 mM solutions. The stock solution was further diluted in 10% bovine serum albumin in PBS. The working concentration of rooperol acetate in cell culture media was 5–25 μM and of rooperol sulphate 25–75 μM.

Culture of Macrophages

Mononuclear cells were prepared from fresh human blood by centrifugation with Mono-Poly resolving medium (ICN Biomedicals, Aurora, OH, U.S.A.) [17].

Human lung macrophages were obtained by Dr. K. Sładek (Department of Internal Medicine, Collegium Medicum, Jagiellonian University) by fiberoptic bronchoscopy from patients examined for diagnostic purposes following the guidelines of the American Thoracic Society [18]. They were collected in sterile PBS and plated within 2 hr. To increase macrophage yield and obtain uniform pathologic background, lung lavage fluid from patients with

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mild sarcoidosis was used as the source of cells. Earlier, we confirmed observations of Homolka and Muller-Quernheim [19] suggesting that unstimulated macrophages from sarcoidosis patients produce slightly more IL-6 than do those from control patients. However, preliminary experiments indicated that LPS-induced production of cytokines was similarly inhibited by rooperol in macrophages from control and sarcoidosis patients.

Rat alveolar macrophages were collected by rinsing lungs freshly isolated from adult Wistar rats with cold, sterile PBS.

Human and rat monocytes/macrophages were seeded in 6- or 24-well plates (Nunclon, Kamstrup, Denmark) in RPMI with 8% fetal calf serum (FCS) and cultured for 2 hr/37°C in 5% CO_2. Nonadherent cells were removed and attached cells were cultured for an additional 40 hr under the same conditions. Then, the media were replaced with RPMI containing 2% FCS and the tested factors added at indicated concentrations.

Human histiocytic lymphoma line U937 cells were seeded at a density of 1×10^6 cells/mL in 6-well plates in RPMI medium with antibiotics and 5% FCS. Cells were induced to macrophage phenotype by culturing with PMA (34 ng/mL) for 48 hr. The nonadherent cells were discarded and attached cells cultured for 24 hr. Then, the medium was replaced, supplemented with 2% FCS and analyzed factors added for an additional 24 hr.

Cytokine Assays

The content of acute phase cytokines in the macrophage media was carried out either directly with specific kits or by bioassay with rat or human hepatoma cells. These cells, with stimulation, produce acute phase proteins that can be estimated using immunoelectrophoresis [20].

In 3 experiments, the amounts of TNF, IL-1, and IL-6 released to the media from human alveolar macrophages cultured for 24 hr were compared with the amounts of these cytokines retained in the cells. At the end of culture, macrophages were disrupted by repeated freezing and thawing. For direct human cytokine assay, culture supernatants from human blood monocyte/macrophages, alveolar macrophages, and U937 cells were centrifuged, and TNFα, IL-1β, and IL-6 were evaluated by commercially available immunoenzymatic kits based on ELISA (Genzyme). Rooperol esters at 20 μM concentrations had no effect on cytokine assay.

For bioassay of human or rat cytokines, H35 rat hepatoma cells and human hepatoma HepG2 were grown to subconfluency in 6-well plates in DMEM with antibiotics and FCS. The cells were washed with serum-free DMEM and then supplemented (1:3) with macrophage-conditioned medium (bioassay) and 1 μM dexamethasone. The cells were cultured for 24 hr and culture media collected, dialyzed, and freeze-dried. Selected proteins were then determined by rocket immunoelectrophoresis [21].

Nitric Oxide Assay

Human or rat macrophage culture media were centrifuged and 80 μL samples were transferred to a microtiter plate. Then, 80 μL of Griess reagent (1% sulphanilamide/0.1% naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4) was added to each well, the plate was incubated at room temperature for 10 min, and absorbance was read using a 540-nm filter. Nitrite concentration was determined by using dilutions of sodium nitrite in water as a standard [22]. Rooperol esters at 20 μM concentration had no effect on the nitrite assay.

Radioisotopic Experiments

^{14}C-DL-leucine (Polatom, Świerk, Poland, specific activity of 765 Mbq/mM) served as a marker for macrophage protein synthesis. Labeled leucine dissolved in PBS was added to the medium (10 μCi/mL) simultaneously with the tested factors and cells were cultured for 16 hr. In some experiments, ^{14}C-leucine was added for the last 2 hr of culture of cells already exposed to rooperol or/and LPS for 22 hr. Total proteins were precipitated from the medium with an equal volume of 10% TCA, the precipitate collected by centrifugation, washed twice with 5% TCA, and dissolved in 0.3 M NaOH. The cells were rinsed twice with cold PBS, and proteins were precipitated with 5% TCA, washed twice with 5% TCA, and dissolved in 0.3 M NaOH. ^{14}C-radioactivity was measured in an LKB liquid-scintillation spectrometer using dioxan-based Bray scintillation fluid.

Lactate Dehydrogenase Activity Assay

The activity of the enzyme was estimated in rat alveolar macrophages and U937 cells and their culture media. Cells were washed twice with cold PBS, scraped in 0.1 M Tris:HCl buffer pH 7.5, and submitted to 3 cycles of freezing and thawing. After centrifugation of the medium or cell lysate, LDH activity was measured spectrophotometrically (340 nm) [23]. The mixture of buffer, NADH, and medium or cell lysate was allowed to stand for 20 min at room temperature and, after the addition of sodium pyruvate, the decrease of absorbance at 340 nm was measured within 5 min.

Statistical analysis

Data were analyzed by Student's *t* test.

RESULTS

Rooperol Acetate and Rooperol Sulphate Inhibition of Cytokine Production by Human Alveolar Macrophages and Human Blood Monocyte/Macrophages Stimulated by LPS

As shown in Table 1, the amount of TNF released into the medium during 2-hr incubation of human alveolar macrophages ranged considerably (from 0.02 to 0.14 ng/mL), but

TABLE 1. Effects of rooperol diacetate (RodiA), rooperol tetraacetate (RoteA), and rooperol sulphate (RoS) on LPS-stimulated TNF production by human alveolar macrophages

	TNFα content (ng/mL)		
	Exp. No. 1	**Exp. No. 2**	**Exp. No. 3**
Control	0.10	0.02	0.14
LPS (2 hr)	2.09	1.26	0.80
	RodiA	**RoteA**	**RoS**
LPS 2 hr + Ro 3 hr	1.32	0.42	0.07
LPS 2 hr + Ro 2 hr	1.83	0.42	0.03
LPS 2 hr + Ro 1 hr	2.29	1.05	0.20

Macrophages were stimulated with 500 ng per mL of LPS for 2 hr, and rooperol was added either 1 hr before (3 hr), together (2 hr) or 1 hr after LPS. Concentrations of the tested compounds: RodiA 20 μM, RoteA 10 μM, RoS 70 μM. TNF estimation by ELISA test (Genzyme).

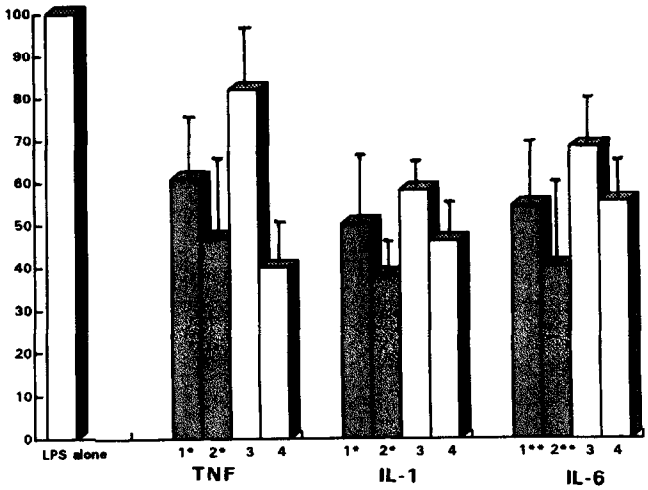

FIG. 2. Inhibition by rooperol esters of LPS-stimulated cytokine production in human macrophages seeded at a density of 2 × 10^5 cells per 1 mL. Rooperol ester was added 1 hr before LPS and the cells cultured for 24 hr. The amount of cytokines produced by macrophages stimulated with LPS was assumed as 100%, 1, LPS + 10 μM rooperol diacetate; 2, LPS + 25 μM rooperol diacetate; 3, LPS + 25 μM rooperol sulphate; 4, LPS + 50 μM sulphate. The results are means of 6 experiments. Vertical bars show SD and asterisks statistical significance at *$P < 0.05$, and **$P < 0.02$. Cytokine synthesis (ng/mL) in control culture was in the range of: TNF 0.01–0.7; IL-1β 0.02–0.37; IL-6 0.01–0.42. Cytokine synthesis in cultures of LPS-stimulated macrophages (mean ± SD): TNF 13.69 ± 4.91; IL-1β 1.72 ± 0.90; IL-6 14.56 ± 4.93.

was greatly enhanced by LPS. Rooperol esters inhibited TNF synthesis (or release), especially when added before LPS. However, with prolonged culture of cells (up to 24 hr), the inhibitory effect of rooperol on TNF synthesis was clearly manifested, even when rooperol esters were added 1 hr after endotoxin (data not shown).

In contrast to TNF, only traces of IL-1β and IL-6 were found in the culture medium after 2 hr of exposure to endotoxin, so that the early effects of rooperol could not be tested. However, when human alveolar macrophages were cultured for 24 hr with LPS and rooperol, inhibitory effects of this dicatechol on the synthesis of all three cytokines assayed were clearly visible (Fig. 2). Again, great individual variations were observed in the amounts of released cytokines but, in all cases, relative inhibitory activities of rooperol esters within each experiment were comparable.

In these experiments, the inhibitory capacity of rooperol diacetate was somewhat greater than that of rooperol sulphate: IC_{50} values were in the range of 10–20 μM and 25–75 μM, respectively (see also Fig. 2). IC_{50} values were calculated from 7 experiments, in which concentrations of 5 to 25 μM (rooperol diacetate) and 25 to 75 μM (rooperol sulphate) were tested. The dose-dependent inhibition of cytokine synthesis by rooperol diacetate was confirmed by the results obtained from bioassay (Fig. 3).

Rooperol diacetate also inhibited cytokine synthesis by LPS-stimulated human blood monocytes/macrophages and human histiocytic lymphoma U937 cells induced by PMA to macrophage phenotype. As shown in Fig. 4, human hepatoma cells produced less haptoglobin in the presence of conditioned medium of monocytes cultured with the mixture of LPS and rooperol diacetate than in the presence of LPS alone. The bioassay results were correlated with those of the immunoassay of TNF and IL-6 (Fig. 4). The synthesis of transferrin, which does not belong to typical acute phase proteins, was unaffected by the addition of macrophage medium.

The concentrations of cytokines measured in macrophage medium may not correspond to total cytokine synthesis in the period of culture. To elucidate the effect of rooperol esters on eventual cytokine retention in macrophages, the concentrations of TNFα, IL-1β, and IL-6 were determined in the media and cell lysates. As shown in Table 2, almost 99% of IL-6, but only approximately 20% of IL-1β, was secreted to the media from macrophages stimulated with LPS and cultured for 24 hr. In both cases, this proportion was almost unaffected by rooperol, although considerable individual variations were observed, especially for IL-1β. On the other hand, approximately 2.5% of total TNF-α produced by LPS-stimulated human alveolar macrophages was found intracellularly, but rooperol increased this retention to over 6%. However, the total synthesis of all three cytokines was always considerably inhibited by rooperol, although scatter of absolute values in individual experiments adversely affected statistical significance.

Inhibition of Cytokine Synthesis by Rooperol Esters and Unspecific Inhibition of Protein Synthesis or Cytotoxic Effects

We observed that rooperol esters slightly enhanced incorporation of ^{14}C-leucine into total macrophage proteins, both in the presence and absence of LPS. However, when the ratios of ^{14}C-labelled proteins in the cells and media were compared, it was found that rooperol diacetate increased protein secretion (or release) in human alveolar macrophages (Table 3). This phenomenon was consistently

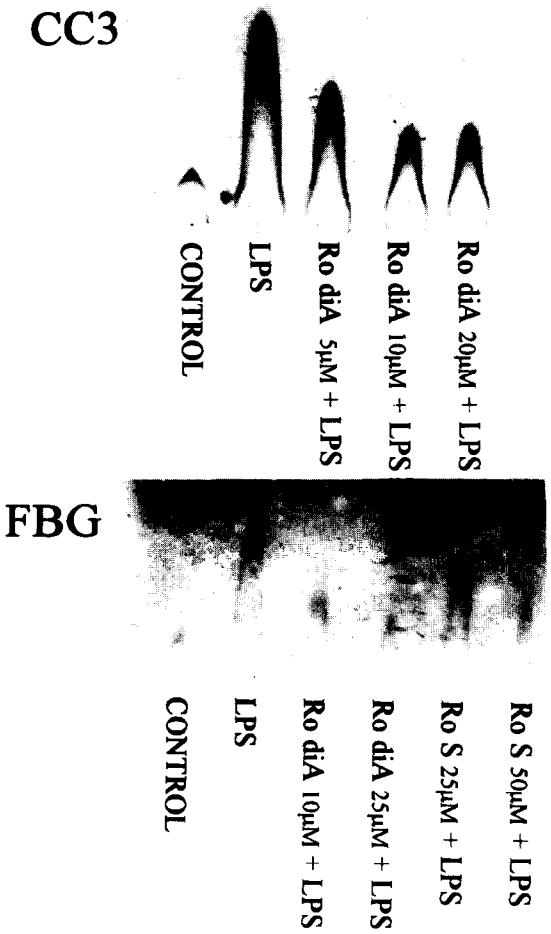

FIG. 3. Bioassay of acute phase cytokines with rat hepatoma H35 cells using C3 component of the complement (CC3) and fibrinogen (FBG). The hepatoma cells were cultured for 24 hr in the presence of supernatants of control, LPS, and LPS plus rooperol diacetate-treated human alveolar macrophages.

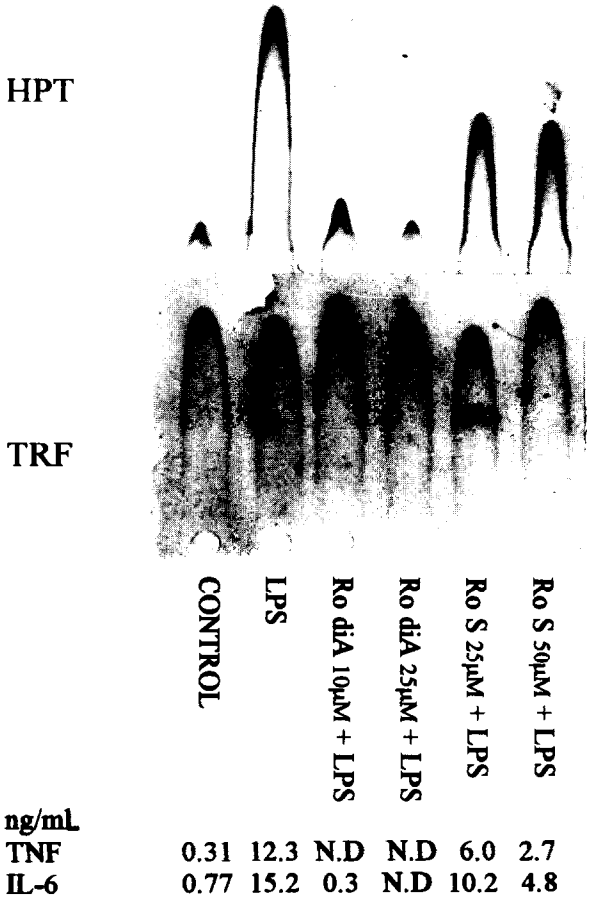

FIG. 4. Bioassay of acute phase cytokines with human hepatoma HepG2 cells using haptoglobin (HPT) and transferrin (TRF). The cells were treated for 24 h with the supernatants of human monocytes cultured with LPS or the LPS and rooperol diacetate mixture. The control corresponds to the supernatant of macrophages cultured without LPS and rooperol ester. The results of direct assay of TNF and IL-6 in monocyte/macrophage supernatant are shown below the corresponding rockets. N.D., not detectable.

observed in 6 consecutive experiments and was not caused by a cytotoxic effect of rooperol and resulting leakage of proteins from the cells, because the relative activities of lactate dehydrogenase (a marker of cell integrity) determined in the cells and media were almost unaffected by culturing of macrophages with rooperol esters (although total LDH activity was found to be higher in cultures containing rooperol esters). In rat alvolar macrophages, rooperol diacetate also enhanced release of labelled proteins to the media (Fig. 5) without parallel leakage of LDH when tested in concentrations up to 25 μM. A similar trend was observed in human U937 cells (data not shown).

Rooperol Acetate and Rooperol Sulphate Inhibition of Cytokine Production and Nitric Oxide Synthesis in Rat Alveolar Macrophages

We found that rat macrophages responded to rooperol similarly to human macrophages. Figure 6 shows the results of a representative experiment in which we evaluated the influence of conditioned medium of rat macrophages cultured with LPS or/and rooperol acetate and rooperol sulphate on the synthesis of fibrinogen in rat hepatoma cells. The results of the bioassay showed that rooperol did not influence basal synthesis of cytokines or fibrinogen, although it did inhibit LPS-stimulated cytokine production (Fig. 6 and Table 4). Rooperol sulphate was even less effective in rat macrophages than in human monocytes: this may be due to species differences in the activity of sulphatases, but further studies are required.

Because iNOS in rodent (but not human) macrophages is regulated by cytokines and endotoxin [24] and NO can enhance the release of TNF and IL-1 [25], it was reasonable to postulate that LPS-stimulated NO synthesis in rat macrophages might be inhibited by agents that diminish cytokine synthesis. Indeed, rooperol decreased not only LPS-stimulated cytokine synthesis in rat macrophages but also diminished LPS-stimulated nitric oxide production (Table

TABLE 2. The effect of rooperol diacetate on intra-/extracellular distribution of TNF, IL-1, and IL-6 (ng/2 × 10^5 cells) in human alveolar macrophages cultured for 24 hr with LPS

	TNF α		IL-1β		IL-6	
	T	C	T	C	T	C
LPS	17.66 ± 0.28	2.5 ± 1.4	8.47 ± 4.28	78 ± 1	13.39 ± 1.48	1.0 ± 0.07
LPS + RoA (10 μM)	15.82 ± 2.28	6.1 ± 1.9	6.05 ± 3.40	87 ± 7	8.35 ± 3.42	1.1 ± 0.20
LPS + RoA (20 μM)	13.06 ± 2.50	6.4 ± 1.5	4.31 ± 2.66	85 ± 7	6.39 ± 3.37	1.0 ± 0.26

Mean of 3 independent experiments, ±SD; T-total cytokine content (medium + cells). The amount of cytokine found in cell lysate (C) is expressed as % of total cytokine content.

5). The inhibitory capacities of rooperol diacetate and tetraacetate were similar and much higher than that of rooperol sulphate: at 25 μM concentrations, rooperol acetates almost totally blocked induced NO synthesis, but rooperol sulphate reduced it by only 10%.

DISCUSSION

Macrophages activated by bacterial products, such as LPS, or by other mechanisms, produce inflammatory cytokines. Excessive release of these cytokines can lead to inflammation or to shock. Because both TNF and IL-1 are involved in the pathogenesis of inflammation and shock and act independently, as well as sequentially, the pharmacological control of these disorders requires inhibition of the production and/or effects of at least these cytokines [2]. Several strategies have been proposed to achieve this aim. Lee *et al.* [26] found that a group of bicyclic imidazole derivatives, termed cytokine suppressive antiinflammatory drugs, bind to and inhibit a specific protein kinase required for translation of messages for TNF and IL-1. A piperazine derivative, CGP 47969A, was reported to decrease translational efficiency of IL-1β and TNF mRNAs by an unspecified mechanism [27]. Some anti-inflammatory and/or antirheumatic drugs, such as tenidap [28] or piroxicam [29], were reported as inhibitors of the synthesis of proinflammatory cytokines, but these findings are still disputed [2]. Eugui *et al.* [30] showed that some antioxidants can prevent the oxidant activation of transcription factors in cells of the monocyte-macrophage lineage, and thereby suppress the transcription of genes for proinflammatory cytokines.

Elaborating the latter approach, we now report that the dicatechol rooperol can, in concentrations that show no cytotoxic effects (10–20 μM), suppress LPS-induced production of TNF, IL-1, and IL-6 in human alveolar macrophages, blood monocytes, and U937 cells, as well as in rat alveolar macrophages. Of the esters tested, rooperol diacetate and tetraacetate were more potent inhibitors of cytokine synthesis than rooperol sulphate. The sulphate had little activity in rat alveolar macrophages. These observations presumably reflect species differences in acetate esterase and sulphatase activities required to release rooperol. Although, from the clinical point of view, results obtained with human cells are most important, the use of rat macrophages permits broader biological conclusions to be drawn.

The compounds tested did not inhibit macrophage total protein synthesis at concentrations suppressing cytokine production by 50%. However, rooperol acetate augmented the release of labelled proteins from the cells. Because these concentrations of the test compound had no demonstrable cytotoxic effect and did not induce leakage of lactate dehydrogenase from the cells, enhanced secretion is a likely explanation. Testing this proposal and the mechanism by which it occurs will require further experimentation. However, we found that cell/medium ratios of three tested cytokines vary considerably, with IL-6 being the most efficiently secreted from human alveolar macrophages (Table 2). On the other hand, the majority of IL-1β was found intracellularly after 24 hr of culture. Because this may reflect not only impaired secretion, but also a prolonged period of IL-1 synthesis, further kinetic studies are required.

TABLE 3. Effect of rooperol diacetate on total protein synthesis and LDH content in LPS-stimulated human alveolar macrophages

	cpm TP	cpm MP/ cpm CP	LDH (T) (nmol/min)	C (%T)
LPS	5060 ± 2070	3.18 ± 1.24	24.83 ± 5.2	74 ± 7.6
LPS + RoA (10 μM)	6650 ± 2841	3.74 ± 1.30	30.68 ± 8.4	73 ± 10
LPS + RoA (20 μM)	7623 ± 3377	5.18 ± 0.60	33.85 ± 14.5	70 ± 20

Cells were cultured with LPS or the mixture of LPS and rooperol for 22 hr and isotope then added for an additional 2 hr. CP, cell proteins; MP, medium proteins; TP, total proteins (CP + MP). Mean of 6 experiments ± SD. LDH activity (nmole/min) was estimated in the medium (M) and cells (C) in the 24-hr culture of human macrophages. The activity of enzyme found in the cell was expressed as % of total (T).

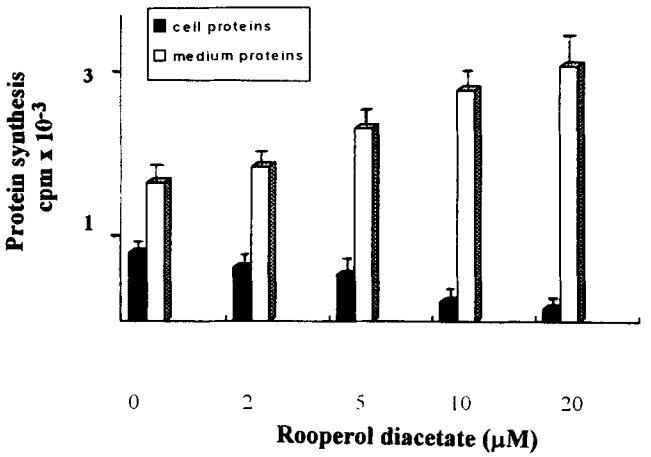

FIG. 5. Effect of rooperol diacetate on total protein synthesis in LPS-stimulated rat alveolar macrophages as measured by ^{14}C-leucine incorporation. Macrophages were cultured for 16 hr with tested factors and labelled amino acid and radioactivity of proteins measured in the cells and the media.

Highly variable macrophage responses concerning IL-1β synthesis and secretion involve complex mechanisms and are discussed by Dinarello [31].

Another remarkable effect of rooperol esters, now reported on, is the inhibition of LPS-induced nitric oxide synthesis by rat alveolar macrophages. The roles of nitric oxide in immune mechanisms and inflammation are reviewed by Moncada *et al.* [32] and Barnes and Liew [4]. It is not clear to what extent findings in rodent macrophages can be extrapolated to human macrophages, but the latter can produce nitric oxide under some conditions [33], as can human endothelial and smooth muscle cells [32]. Macrophage lineage cells also produce superoxide when stimulated by LPS [34]. Nitric oxide reacts rapidly with superoxide to form the powerful oxidant peroxynitrite [35]. One permanent product of peroxynitrite attack on proteins is nitration of tyrosine residues on the 3-carbon atom adjacent to the oxygen. Immunohistochemical studies using antibodies to nitrotyrosine indicate extensive nitration in human lungs of patients dying of the respiratory distress

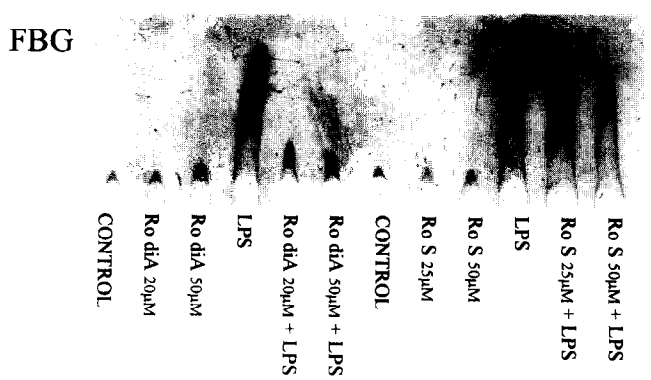

FIG. 6. Bioassay of acute phase cytokines with rat hepatoma H35 cells using fibrinogen (FBG). The cells were cultured for 24 hr with the supernatant of rat alveolar macrophages treated with rooperol diacetate, rooperol sulphate, LPS, or the mixture of LPS and rooperol diacetate or sulphate.

TABLE 4. Relative synthesis of acute phase proteins in rat hepatoma H35 cells, cultured with supernatants of rat macrophages treated with LPS or LPS and rooperol esters

	Relative protein synthesis			
	CC3	FBG	A2M	AGP
LPS alone	100	100	100	100
LPS + Rooperol diacetate (20 μM)	24 ± 2.8	24 ± 2.8	41 ± 13.4	36 ± 9.8
LPS + Rooperol sulphate (50 μM)	83 ± 6.6	64 ± 2.5	81 ± 31.8	83 ± 24.0

The net increase in protein synthesis induced by LPS was assumed as 100%. CC3, C3 component of the complement; FBG, figrinogen; A2M, α-2-macroglobulin; AGP, α-1-acid glycoprotein. The results are means ± SD of 3 experiments.

syndrome, sepsis, or pneumonia [35]. Asthmatic patients show increased expression of inducible nitric oxide synthase in airway epithelial cells and an increased level of nitric oxide in exhaled air [4]. Inhibition by rooperol acetate of the production of nitric oxide could be useful for treatment of these and other disorders.

Oxidants can also activate phospholipase A_2 and release arachidonic acid, which generates prostaglandins, leukotrienes, and other inflammatory mediators [36]. A dicatechol structurally related to rooperol, nordihydroguaiaretic acid, was found to inhibit such activation. Rooperol is also a potent inhibitor of leukotriene synthesis in polymorphonuclear leukocytes (IC_{50} approx. 1 μM) and a much weaker inhibitor of cyclooxygenase pathway in platelet microsomes [12]. By these mechanisms, as well as by suppression of the synthesis of TNF and IL-1, rooperol could exert clinically useful anti-inflammatory activity, especially with topical use. The irritant effect of rooperol when applied to the human skin and other sites, such as respiratory airways, can be avoided by using an ester prodrug. The experiments now reported show that rooperol diacetate and tetraacetate may be suitable prodrugs able to deliver rooperol, which can suppress the production of inflammatory cytokines and nitric oxide.

TABLE 5. NO production in rat alveolar macrophages (approx. 10^6 cells) cultured for 24 hr with LPS (500 ng/mL) or with the mixture of LPS and rooperol esters

	Relative NO synthesis		
μM	Rooperol diacetate	Rooperol tetraacetate	Rooperol sulphate
0	100	100	100
5	40 ± 8.6*	42 ± 10.4‡	N.D.
10	24 ± 14	18 ± 7.2‡	N.D.
25	9 ± 3.2*	10 ± 3.1†	93 ± 4.2
50	N.D.	N.D.	77 ± 7.6
75	N.D.	N.D.	69 ± 11.2

Differences statistically significant in comparison to LPS-stimulated macrophages cultured without rooperol: *$P < 0.05$; †$P < 0.02$; ‡$P < 0.001$.

The net increase of NO synthesis in LPS-stimulated macrophages was assumed as 100%. The results are means ± SD of 5 experiments. N.D., not determined; NO production in control culture, 17.9 ± 5.7 μM; NO production in LPS-stimulated macrophages, 218.2 ± 39.5 μM; NO production in human alveolar macrophages was in the range of 4–9 nM and was not stimulated by LPS.

This work was supported by grant No 1227/P05/95/08 to A.K. from the Committee of Scientific Research (KBN, Warsaw, Poland). The helpful suggestions of Dr. J. Bereta (Institute of Molecular Biology, Jagiellonian University) in respect to the NO assay are gratefully acknowledged.

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