4-Methylcoumarin Derivatives with Anti-inflammatory Effects in Activated Microglial Cells

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Inflammation contributes to the pathogenesis of neurodegenerative diseases and anti-inflammatory compounds may have a role in prevention or treatment of these pathologies. 4-Methylcoumarins are effective antioxidants with anti-inflammatory properties. In this study, the inhibitory effects of two 4-methylcoumarin derivatives, 7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin (DHEMC) and 7,8-diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (DAEMC) were examined on the inflammatory processes induced by lipopolysaccharide (LPS) in activated primary rat microglial cultures. LPS-induced production of nitric oxide (NO, measured by Griess method) and other pro-inflammatory mediators, thromboxane (TX) B₂ and prostaglandin (PG) E₂ (both determined by radioimmunoassay (RIA)), as well as tumor necrosis factor (TNF)- α (determined by enzyme-linked immunosorbent assay (ELISA)) were inhibited in the presence of 100 μ M DHEMC and DAEMC. DAEMC was able to significantly inhibit NO, TXB₂ and TNF- α production also at 50 μ M. Both compounds at 100 μ M significantly lowered cyclooxygenase-2 (COX-2) protein expression in LPS-stimulated microglial cells measured by Western blot, but only DAEMC showed an inhibitory effect on inducible nitric oxide synthase (iNOS) protein expression at 100 μ M. In conclusion, our findings show that 4-methylcoumarin derivatives can modulate inflammatory pathways in microglial cells, probably by acting at the protein expression level.

Key words 4-methylcoumarin; inflammation; microglia; inducible nitric oxide synthase (iNOS); cyclooxygenase-2 (COX-2)

Genetic studies have shown that genes involved in inflammation are associated with an increased risk of Alzheimer's disease.¹⁾ Further, epidemiological reports have demonstrated that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) can lower the risk of development of Alzheimer's disease.²⁾ Although, it is not very clear whether inflammation is the cause or the consequence of neurodegeneration, persistent inflammation can be harmful to the central nervous system (CNS),³⁾ especially in the early stages of development of Alzheimer's disease.⁴⁾ Therefore, anti-inflammatory compounds that can modulate neuroinflammation could prove useful for prevention of neurodegenerative diseases.⁴⁾

Coumarins are a large class of naturally occurring compounds with several biological activities such as antitumoral⁵⁾ and antioxidant activities.⁶⁾ Recently, much attention has been focused on the anti-inflammatory activity of coumarin derivatives.^{7–10)}

4-Methylcoumarins, a subclass of coumarin compounds, are effective antioxidant^{11–14)} and anti-inflammatory agents.^{9,11,15,16)} 4-Methylcoumarins bearing 7,8-*ortho*-dihydroxy or 7,8-*ortho*-diacetoxy moieties have been found to be better radical scavengers and antioxidants.^{12,17)}

On the other hand, the insertion of ethoxycarbonylmethyl moiety at C3 position does not lower the activity of these compounds and may increase the lipophilicity.¹⁴⁾ Increased lipophilicity is a very important physicochemical property that enables the compound to cross the cell membranes and reach

its intracellular targets.

In this study, the effects of two 4-methylcoumarin derivatives, 7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin (DHEMC) and 7,8-diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (DAEMC), on the lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators as well as protein expression levels of pro-inflammatory enzymes were studied in microglial cells.

MATERIALS AND METHODS

Chemicals DHEMC and DAEMC were synthesized and characterized as described earlier.¹⁸⁾ DHEMC was obtained from 7,8-dihydroxy-4-methylcoumarin (synthesized by using Pechmann condensation of pyrogallol with ethyl acetoacetate) by introduction of an ethoxycarbonylmethyl group at the C3 position; DAEMC was obtained by acetylation of DHEMC

Table 1. Structures of Tested 4-Methylcoumarin Derivatives

	R ₁	R ₂	R ₃	R ₄	R ₅
DHEMC ^{a)}	–CH ₂ COOCH ₂ CH ₃	–H	–H	–OH	–OH
DAEMC ^{b)}	–CH ₂ COOCH ₂ CH ₃	–H	–H	–OOCCH ₃	–OOCCH ₃

a) 7,8-Dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin.

b) 7,8-Diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin.

The authors declare no conflict of interest.

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(Table 1). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Invitrogen. LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-(1-naphthyl)-ethylenediamine dihydrochloride, penicillin/streptomycin, *o*-phosphoric acid (H_3PO_4), sodium nitrite and sulfamylamide were purchased from Sigma-Aldrich. Tumor necrosis factor (TNF)- α kit was from IBL (Hamburg, Germany). Polyclonal anti-inducible nitric oxide synthase (iNOS) and anti-cyclooxygenase-2 (COX-2) antibodies were from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Western blot enhanced chemiluminescence detection system was from Bio-Rad Laboratory (Hercules, CA, U.S.A.).

Primary Rat Microglial Cell Cultures All the animal-related procedures were conducted in accordance with European Communities Council Directive n° 86/609/EEC. Primary rat microglial cells were obtained, as previously described by Giulian and Baker¹⁹⁾ with some modifications. The glial cells, derived from 1- or 2-d-old Wistar rats, were cultured for 11–14 d in Dulbecco's MEM containing 10% inactivated fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin). Afterwards, microglial cells were detached from the astrocyte monolayer by gentle shaking and re-suspended in D-MEM/F12 supplemented with 10% fetal calf serum and antibiotics as above. Then, the cells were plated into 24-well plates at a density of 5×10^5 cells/well. Purity of microglial cell populations ($>98\%$) was verified by staining with IBA-1 (1:1000) antibody. After 2 h the medium was replaced with fresh medium. Compounds DHEMC and DAEMC dissolved in dimethylsulfoxide (DMSO), were added at different concentrations (10, 50 and 100 μM) to microglial cultures 30 min before stimulation by LPS 10 ng/mL (from *Escherichia coli* serotype 026:B6). The control group was treated with DMSO diluted in the culture medium at the same final concentration used for 4-methylcoumarin compounds (0.5%). The supernatants were collected after 24 h incubation and kept at -80°C for measurements of nitric oxide (NO), prostanoids [thromboxane B_2 (TXB $_2$) and prostaglandin E_2 (PGE $_2$)], and TNF- α production. Cells were used for subsequent protein measurement, MTT assay and western blot analysis. These experimental conditions were kept constant in all performed tests in this study.

Cell Viability Cell viability was assessed by MTT assay. In this assay, mitochondrial dehydrogenase enzyme of living cells converts yellow MTT to purple formazan, which is spectrophotometrically measured. In brief, microglial cells at a density of 5×10^4 cells/well were seeded in into 96-well plates and treated with DHEMC and DAEMC at 50 and 100 μM for 24 h. Then, the medium was removed and the cells were incubated with MTT (0.5 mg/mL) for 4 h at 37°C . Formazan crystals in the cells were solubilized with DMSO. The level of formazan in each well was determined by measuring its absorbance at 570 nm.

NO Production in Microglial Cells NO production in microglial culture supernatants was evaluated by measuring nitrite, a stable end product of NO. Nitrite was determined by a colorimetric assay with Griess reagent. One-hundred microliter of culture medium reacted with an equal volume of Griess reagent (one part of 1% sulfanilamide dissolved in 5% H_3PO_4 and one part of 0.1% naphthylethylenediamine dissolved in distilled water) in 96-well culture plates for 10 min at room temperature. The absorbance was measured with

a microplate reader at 545 nm using a calibration curve of sodium nitrite standards (0.7–50 μM).

Prostanoid and TNF- α Production in Microglial Cells PGE $_2$ and TXB $_2$ (the stable breakdown product of TXA $_2$) concentrations were measured in supernatants by radioimmunoassay (RIA).²⁰⁾ The least detectable concentration was 2 pg/mL for both assays. TNF- α was measured in supernatants by a commercially available kit.

Western Blot Analysis Microglial cells were lysed in 100 μL of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol) containing a protease inhibitor cocktail at 4°C . After 1 h, lysates were collected, cellular debris were pelleted by centrifugation ($1000 \times g$, 10 min, 4°C) and supernatants were collected and stored at -80°C until use for immunoblotting. Protein concentration of each lysate was determined by Bradford assay. Equal amounts of total protein (30 μg) were electrophoretically separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBST) for 1 h and then washed three times with TBST. Primary antibodies for iNOS and COX-2 were added at proper dilutions (1:1000 for polyclonal anti-iNOS, and 1:100 for polyclonal anti-COX-2) in TBST containing 5% non-fat milk and incubated overnight at 4°C . After several washes, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5000) for 1 h at room temperature and immunoreactivity was visualized using chemiluminescence (ECL) reagent (Bio-Rad) according to manufacturer's instructions. Scanning densitometry was performed using the ImageJ 1.47 program, and signal density was normalized to β -actin density.

In Vitro Cyclooxygenase Inhibition COX-1 activity was measured by determination of TXB $_2$ production by platelets, after spontaneous clotting of human whole blood.^{21,22)} COX-2 activity was determined by assessment of LPS-induced PGE $_2$ production by monocytes in human whole blood.^{21,22)}

Briefly, fresh human venous blood from healthy donors was collected in glass tubes without anticoagulant (COX-1 assay) or containing heparin 15 IU/mL (COX-2 assay) (1 volunteer for each experiment). The volunteers had not taken any NSAIDs during the last 2 weeks before sampling. Aliquots of blood (0.5 mL) were immediately transferred to tubes containing 2 μL of test compound dissolved in DMSO or DMSO alone (control samples).

For COX-1 assay, samples were vortex-mixed and incubated at 37°C for 1 h. The tubes were then centrifuged at $5000 \times g$ for 10 min and 120 μL of serum was taken and mixed with 480 μL of methanol to precipitate the proteins. Samples were centrifuged again and the supernatants were used for measurement of TXB $_2$ by RIA.²⁰⁾ Each compound was tested 3–4 times in independent experiments performed on the blood obtained from different volunteers.

For COX-2 assay, samples were vortex-mixed and incubated at 37°C for 15 min. Then, LPS was added to each tube except for negative control vials. Samples were incubated overnight and plasma was separated by centrifugation and proteins were precipitated as described above. PGE $_2$ was measured in samples by RIA.²⁰⁾

Statistical Analysis Data are presented as means $\pm$ standard deviation (S.D.). The statistical significance of differences among groups was performed with one-way ANOVA, followed by *post hoc* Dunnett's test. The level of significance was set at $p < 0.05$.

RESULTS

Effect on Cell Viability Treatment of microglia with DHEM or DAEMC (50 and 100 μ M) for 24h did not cause any change of absorbance in MTT assay, indicating that these compounds did not affect the viability of microglial cells (Fig. 1).

Inhibition of NO Production in Microglial Cells NO production by microglia was increased after treatment with LPS (Fig. 2). DHEMC significantly inhibited LPS induced NO production by microglial cells at the concentration of 100 μ M, while DAEMC elicited a significant inhibitory effect at the concentration of 50 and 100 μ M. No inhibitory effect was re-

corded at 10 μ M for any of the two compounds.

In the experiments performed on microglia in the absence of LPS stimulation, none of the test compounds altered NO production by microglial cells (data not shown).

Inhibition of Prostanoid Production in Microglial Cells

Effect of 4-methylcoumarin derivatives was also investigated on LPS-induced production of inflammatory prostanoids, TXB₂ and PGE₂ (Fig. 3). Compound DHEMC was capable of inhibiting the production of both prostanoids at 100 μ M, however no effect was observed at lower concentrations of 10 and 50 μ M. Pretreatment of microglial cells with DAEMC significantly inhibited LPS-induced production of TXB₂ at 50 and 100 μ M (Fig. 3A), but its inhibitory effect on PGE₂ reached significance only at 100 μ M (Fig. 3B).

In the experiments performed on microglia in the absence of LPS stimulation, none of the test compounds altered prostanoids production by microglial cells (data not shown).

Fig. 1. Effect of 4-Methylcoumarin Derivatives on Microglial Cell Viability

Primary rat microglial cells were treated with DHEMC and DAEMC for 24h. The cell viability was assessed by MTT reduction assay. Values are expressed as mean $\pm$ S.D. of 4 different experiment.

Fig. 2. Inhibitory Effect of 4-Methylcoumarin Derivatives on Rat Microglial Lipopolysaccharide (LPS)-Induced Production of Nitric Oxide (NO)

NO production was evaluated in microglial culture supernatants by measuring nitrite, a stable end product of NO. Primary rat microglial cultures were pre-incubated with DHEMC and DAEMC for 30min and then stimulated with 10ng/mL LPS. NO was measured in the culture medium by the Griess method after 24h. The bars and error bars represent the mean and S.D., respectively. Significantly different from the value in LPS alone treated control cells: * $p < 0.05$ ($n = 4$).

A

B

Fig. 3. Inhibitory Effect of 4-Methylcoumarin Derivatives on Rat Microglial Lipopolysaccharide (LPS)-Induced Production of Thromboxane A₂ (TXA₂) and Prostaglandin E₂ (PGE₂)

Primary rat microglial cultures were pre-incubated with DHEMC and DAEMC for 30min and then stimulated with 10ng/mL LPS. TXB₂, the stable breakdown product of TXA₂, (A) and PGE₂ (B) were measured in the culture medium after 24h. The bars and error bars represent the mean and S.D., respectively. Significantly different from the value in LPS alone treated control cells: * $p < 0.05$ ($n = 4$).

Fig. 4. Inhibitory Effect of 4-Methylcoumarin Derivatives on Rat Microglial Lipopolysaccharide (LPS)-Induced TNF- α Production

Primary rat microglial cultures were pre-incubated with DHEMC and DAEMC for 30 min and then stimulated with 10 ng/mL LPS. TNF- α concentrations were assayed in the medium by ELISA after 24 h. The bars and error bars represent the mean and S.D., respectively. Significantly different from the value in LPS alone treated control cells: * $p < 0.05$ ($n = 3$).

Inhibition of TNF- α Production in Microglial Cells As showed in Fig. 4, TNF- α production induced by LPS in primary microglial cells was significantly reduced by both DHEMC and DAEMC at 100 μ M. Only DAEMC showed an inhibitory effect at 50 μ M.

Inhibition of Microglial Protein Expression of iNOS and COX-2 The effect of 4-methylcoumarin derivatives DHEMC and DAEMC (100 μ M) on LPS-induced iNOS and COX-2 expression in rat microglial cultures was examined by western blotting (Fig. 5). While both DHEMC and DAEMC were able to significantly inhibit COX-2 expression (Figs. 5A, B), only DAEMC significantly decreased LPS-induced iNOS protein expression (Figs. 5A, C).

Cyclooxygenase Inhibition COX-1 and COX-2 inhibitory activities of 4-methylcoumarin derivatives were measured in human whole blood assay. Although DAEMC induced a slight reduction in COX-1 activity, this effect was not significant and overall none of the compounds showed any significant effect on the activity of COX-1 and COX-2 enzymes (Fig. 6).

Fig. 5. Effect of 4-Methylcoumarin Compounds on Lipopolysaccharide (LPS)-Induced COX-2 and iNOS Expression in Rat Microglial Cells

Primary rat microglial cultures were pre-incubated with DHEMC and DAEMC for 30 min and then stimulated with 10 ng/mL LPS for 24 h. COX-2 and iNOS expressions were determined by Western blot in harvested cells (panel A). The figure is representative of 4 different experiments. β -Actin was used as protein loading control. The densitometric data of COX-2 (B) and iNOS (C) protein expression were expressed as fold increase compared to C (basal level of COX-2 and iNOS expression without LPS-treatment). Values are the mean $\pm$ S.D. of 4 different experiments. Significantly different from cells treated with LPS alone: * $p < 0.05$.

Fig. 6. COX-1 and COX-2 Inhibitory Activities of 4-Methylcoumarin Derivatives Determined with Whole Blood Assay

COX-1 and COX-2 inhibitory activities of DHEMC and DAEMC were measured with human whole blood assay by determining the levels of TXB₂ and PGE₂, respectively. Values are expressed as percent enzyme activity compared to control. None of the differences were statistically significant compared to control. Values are means of 4 different experiments.

DISCUSSION

In this study, the anti-inflammatory effects of two 4-methylcoumarin derivatives, DHEMC and DAEMC were tested in activated primary rat microglial cultures. These compounds were able to inhibit LPS-induced production of pro-inflammatory and neurotoxic mediators. These effects were accompanied with a decreased protein expression of inflammatory enzymes, iNOS and COX-2.

Inflammation in the CNS may contribute to the development of neurodegeneration.^{3,4,23} Microglia, which are the main cellular components of the brain immune system, play an important role in the process of inflammation in the CNS³ and their chronic activation may contribute to the development and progression of neurodegenerative diseases.²⁴ In this context, compounds that can modulate the neuroinflammatory processes may prove useful in the control of neurodegeneration.^{2,23}

Both 4-methylcoumarin compounds investigated in this study were able to exert anti-inflammatory effects in primary rat microglial cultures, evidenced by inhibition of pro-inflammatory and neurotoxic mediators NO, PGE₂, TXB₂ and TNF- α production and also by lowering iNOS and COX-2 protein expression, but without any significant effect on COX-1 and COX-2 enzyme activities in whole blood assay.

None of the test compounds altered the base line prostanoids produced by microglial cells in the absence of LPS stimulation. Since base line prostanoids are mainly produced by the constitutive COX-1 enzyme, these compounds probably do not have a large effect on COX-1.

DAEMC appeared to be slightly more potent than DHEMC: DAEMC inhibited NO and prostanoid production starting from the dose of 50 μ M, while DHEMC inhibited their produc-

tion only at the dose of 100 μ M. Furthermore, DAEMC was able to lower both iNOS and COX-2 expression at 100 μ M, while DHEMC lowered the expression of COX-2, but not iNOS at the same concentration.

The reduced production of NO, TXB₂ and PGE₂ can result either from the inhibition of the enzymatic activities and/or protein expression of iNOS and COX isoforms. In order to answer this important question, the COX enzyme inhibitory activity of these compounds were tested in the whole blood assay, which is considered a reliable method to measure the enzyme inhibitory activities of various compounds.^{21,22} These experiments demonstrated that DAEMC and DHEMC had no significant inhibitory effect on the COX-1 and COX-2 enzyme activities. Therefore, this provides more evidence for the assumption that the mechanism of action of these compounds is through the modulation of expression of inflammatory enzymes.

However, regarding iNOS, inhibitory effect of the methylcoumarin derivatives on the enzyme activity can not be excluded with the present data.

Previous studies have shown that various coumarin derivatives are able to inhibit LPS-induced NO^{10,25,26} and PGE₂ production¹⁰ in macrophages and other investigators have reported the ability of coumarin related compounds in lowering the protein expression of inflammatory enzymes. It has been observed that 7,8-diacetoxy-4-methylcoumarin reduces COX-2 expression in A549 human lung adenocarcinoma cells²⁷ and other authors, studying the effect of 63 natural oxycoumarin derivatives on LPS-induced inflammation in RAW264.7 cells, showed that many of these compounds inhibited the protein expressions of iNOS and COX-2.⁸ Similarly, several naturally occurring coumarins including nodakenin,¹⁰ glycyrol²⁵ and 3-(1'-1'-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin²⁸ reduce iNOS and COX-2 expression in LPS-stimulated macrophages. Another natural coumarin, osthole, which has immunomodulatory activities, also inhibits COX-2 expression in LPS-stimulated macrophages.²⁶ Our observation that 4-methylcoumarin derivatives reduce COX-2 and iNOS expression is in line with the above mentioned investigation that show the target of these compounds is at the protein expression level.

To our knowledge, iNOS seems to be the main subtype of NOS present in microglia. Yao and colleagues²⁹ have stated that there are 3 known isoforms of NOS; The constitutively active isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), which are present in both neuronal and endothelial cells, in addition to the iNOS, which is expressed in response to cytokines and LPS in macrophages, monocytes and microglial cells. Therefore, this study was focused on the iNOS isoform.

According to some authors, the reduction in iNOS and COX-2 protein expression induced by coumarins is probably due to decreased mRNA transcription^{8,25} caused by blocking of NF- κ B activation, which has been previously reported by other authors about coumarin compounds.^{23,26}

4-Methylcoumarin derivatives bearing 7,8-dihydroxy and 7,8-diacetoxy moieties have been reported to be potent antioxidant compounds.¹²⁻¹⁴ Most of these reports have suggested that 7,8-dihydroxy confers higher activity compared to 7,8-diacetoxy,^{13,14} probably due the important contribution of catechol moiety to the antioxidant activity. However, in some papers this difference seems to be minimal.¹²

Our findings showed that DAEMC bearing 7,8-diacetoxy moiety had a slightly better activity compared to 7,8-dihydroxy bearing compound, DHEMC. The difference in lipophilicity of DHEMC and DAEMC may also explain these differences. More lipophilic DAEMC is probably more efficient in crossing the lipid membranes in microglia and reaching its targets.

In conclusion, the two 4-methylcoumarin compounds investigated in this study showed considerable activities in modulation of inflammatory processes in rat microglial cells. Since neuroinflammation is an important part of the etiopathogenesis of neurodegenerative diseases, compounds that can effectively inhibit inflammatory processes are valuable sources for further development of agents designed for prevention or treatment of these diseases.

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