Disodium cromoglycate suppresses the induction of cysteinyl leukotriene synthesis during granulocytic differentiation in HL-60 cells

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Summary Objectives: Disodium cromoglycate (DSCG) and nedocromil sodium are anti-asthma drugs that have a variety of physiological and biological effects. We examined whether DSCG affects the induction of cysteinyl leukotriene (cysLT) synthesis during dimethyl sulfoxide (DMSO)-induced granulocytic differentiation in HL-60 cells.

Methods: HL-60 cells were differentiated to mature granulocyte-like cells by DMSO in the presence or absence of DSCG for 5 days. Then, we measured A23187-stimulated production of LTC4, an initial product of cysLTs. We also examined the mRNA expression and enzyme activity of LTC4 synthase and other LT-synthetic enzymes.

Results: The amount of LTC4 production was 732.0 ± 19.0 pg/10^6 cells in DMSO-differentiated HL-60 cells. The value was significantly decreased to 420.7 ± 22.7 pg/10^6 cells in the presence of DSCG at 100 μg/ml. The DMSO-induced mRNA expression and enzyme activity of LTC4 synthase was also suppressed by DSCG.

Conclusions: Our results indicate that DSCG suppresses the DMSO-induced LTC4 synthase-activity by inhibiting mRNA expression of LTC4 synthase, which might be a novel anti-allergic action of DSCG.

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Introduction

Cromons, disodium cromoglycate (DSCG) and nedocromil sodium, are classified as anti-allergy drugs that are clinically effective for allergic diseases, including bronchial asthma.1–6 DSCG inhibits mediator releases from a wide range of cells2,5 and affects cell differentiation.7–9

The human leukemia (HL)-60 is a cell line that can be used in an experimental model providing the possibility to examine the regulation of arachidonic acid (AA) metabolism in myeloid cells. HL-60 cells can be differentiated into morphologically mature granulocyte like cells,10,11 which produce a various kind of arachidonate metabolites including leukotrienes. Leukotriene (LT) B4 and cysteinyl leukotrienes (cysLTs; LTC4, LTD4 and LTE4) are important chemical mediators of allergic diseases including bronchial asthma. Messenger RNA (mRNA) and enzyme activities for LT-synthetic enzymes and protein, such as cytosolic phospholipase A2 (cPLA2), five-lipoxygenase (5-LO), five-lipoxygenase activating protein (FLAP), LTA4 hydrolase and LTC4 synthase, is known to be induced during DMSO-initiated differentiation.
differentiation of HL-60 cells.\textsuperscript{12–16} We examined whether DSCG affected the induction of LT-synthetic activities in the process of DMSO-initiated cell differentiation.

**Materials and methods**

**Materials**

HL-60 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Oligo (dT) primer, RNA guard, ethidium bromide and a 100 bp DNA ladder were obtained from Pharmacia (Uppsala, Sweden), RPMI 1640, antibiotic-antimycotic liquid (penicillin, streptomycin, amphotericin B), fetal bovine serum, Moloney murine leukemia virus reverse transcriptase, dithiothreitol and 5\textsuperscript{m} reaction buffer were from Gibco BRL (Grand Island, NY), deoxyribonucleoside triphosphate (dNTP) mixture, recombinant Taq DNA polymerase and 10\textsuperscript{m} triphosphate (dNTP) mixture, recombinant Taq DNA polymerase and 10\textsuperscript{m} triphosphate (dNTP) mixture, recombinant Taq DNA polymerase and 10\textsuperscript{m} triphosphate (dNTP) mixture, recombinant Taq DNA polymerase and 10\textsuperscript{m} triphosphate (dNTP) mixture, recon.

**Methods**

Culture and manipulation of HL-60 cells

Cells were cultured in RPMI-1640 medium supplemented with an antibiotic-antimycotic solution (penicillin G 100 unit/ml, streptomycin 100 \textmu g/ml, amphotericin B 0.25 \textmu g/ml), 26 mM NaHCO\textsubscript{3} and 10% FBS at 37°C under a 5% CO\textsubscript{2} atmosphere. After incubation, the cells were collected and washed twice with phosphate buffered saline (PBS) before experiments. Cell viability was checked using Trypan blue stain. Differentiation of the cells was determined by morphological changes using May-Grunwald-Giemsa staining.

In a separate experiment, we conducted a time-course study in order to clarify the time-dependent action of DSCG on LT-synthetic activity. DSCG was added into the medium on days 0, 1, 2, 3, and 5 at 100 \textmu g/ml (incubation periods with DSCG; 5, 4, 3, 2, 1 and 0 days, respectively).

**Measurement of LTB\textsubscript{4}, LTC\textsubscript{4} and TXB\textsubscript{2} synthesis**

After incubation with DMSO and DSCG as described above, the cells (5 \times 10\textsuperscript{5} cells) were seeded into 24-well dishes in 1 ml of RPMI-1640 per well. The cells were stimulated with A23187 (10\textsuperscript{-5} M) for 15 min at 37°C, the medium was collected and then LTB\textsubscript{4} was measured using a radioimmunoassay (RIA), as previously described.\textsuperscript{17} In brief, 100 \textmu l of medium was incubated at 4°C for 10 h with 100 \textmu l each of anti-LTB\textsubscript{4} anti-serum, \textsuperscript{3}H-LTB\textsubscript{4}, and 50 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and gamma-globulin (1 mg/ml). Free \textsuperscript{3}H-LTB\textsubscript{4} was separated by centrifugation at 3000 rpm for 10 min after adding of 0.5 ml of charcoal-coated gamma-globulin to the assay mixture. The supernatant was transferred to scintillation vials, and radioactivity was measured using a liquid scintillation counter (model LS 7500; Beckman, Irvine, CA). The amount of LTB\textsubscript{4} was calculated from a standard curve. The detectable range of LTB\textsubscript{4} was from 16 to 100 pg/100 \textmu l. RIA for LTC\textsubscript{4} was performed under the same conditions, except that anti-LTC\textsubscript{4} anti-serum and \textsuperscript{3}H-LTC\textsubscript{4} were used instead of anti-LTB\textsubscript{4} anti-serum and \textsuperscript{3}H-LTB\textsubscript{4}. The detectable range of LTC\textsubscript{4} was from 8 to 1000 pg/100 \textmu l. RIA for TXB\textsubscript{2} was performed under the same conditions, except that anti-TXB\textsubscript{2} anti-serum and \textsuperscript{3}H-TXB\textsubscript{2} were used instead of anti-LTB\textsubscript{4} anti-serum and \textsuperscript{3}H-LTB\textsubscript{4}. The detectable range of TXB\textsubscript{2} was from 16 to 1000 pg/100 \textmu l.

**RNA extraction and RT-PCR**

Cells (5 \times 10\textsuperscript{5} cells) were lysed after adding 1 ml of lsofen, then chloroform was added and the mixture was centrifuged. The aqueous (upper) phase was removed to another tube. Isopropanol was added and centrifuged. The supernatant was discarded, the pellet was resuspended in 75% ethanol and centrifuged. Total RNA extracted from the pellet was dissolved in 10 \textmu l of DEPC-treated water and quantified by measuring the absorbance at 260 nm with a spectrometer (model DU-600; Beckman, Irvine, CA). Complementary DNA (cDNA) was
synthesized from the reverse transcription mixture (final volume, 20 μl) containing 1 μg of total RNA, 1 μg of oligo (dT) primer, 4 μl of 5 × reaction buffer, 2 μl of 0.25 M diithothreitol, 4 μl of dNTP mixture (2.5 mM each), 1 μl of RNA guard (5 units), 1 μl of Moloney murine leukemia virus reverse transcriptase (200 units), and DEPC-treated water. PCR proceeded at a total volume of 50 μl containing 1 μl of the reverse transcription reaction products, 5 μl of 10 × PCR buffer, 4 μl of dNTP mixture (2.5 mM each), 0.25 μl of Taq DNA polymerase (5 units/μl), 100 pmol of paired primers (cPLA2, 5-LO, FLAP, LTA4 hydrolase, LTC4 synthase or beta-actin), and 37.5 μl of DEPC-treated water. The DNA was amplified with each paired primer with 28 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. The primers were: cPLA2, 5′-ATTCTGGATTGTCCTACC TAC, 5′-AACCAAGGACTGGAATGGG; FLAP, 5′-CACGAAAGCAGGACC CAGAA, 5′-CAGACACAGCGAGAAAGT; LTA4 hydrolase, 5′-CCACCATCTTCTCATAT, 5′-AACATCGTC CGAAAT; LTC4 synthase, 5′-GCAGTCCTGCT AAACAATCGTC; beta-actin, 5′-GCAGTCCTGCTCAGAAG, 5′-TCCTGTGGCATCACGAAACT, 5′-TCCTGTGGCATCACGAAACT.

**Measurement of LTA4 hydrolase- and LTC4 synthase activities**

The cells were washed twice with 10 ml of HEPES buffer (137 mM NaCl, 2.6 mM KCl, 0.36 mM NaH2PO4, 10 mM HEPES and 1 mM EDTA at pH 7.5) containing 1 mM diithothreitol, and resuspended in the same buffer at a concentration of 10⁶ cells/ml. The cells were sonicated three times on ice (model W-225R, Heat Systems-Ultrasonics, Inc., Plainview, NY). The activities of LTA4 hydrolase and LTC4 synthase in the lysates were measured. LTA4 methylester was saponified in 95% ethanol in 0.2 N NaOH for 30 min to produce LTA4 free acid. LTA4 free acid was diluted with 20 μl of HEPES buffer containing 10 mg/ml bovine albumin (LTA4 solution, at pH 11.5–12.1) and used within 30 min as the substrate. A mixture of cell lysates and GSH (180 μl) was incubated for 1 min at 37°C, then 20 μl of the LTA4 solution was added, and the mixture was incubated for a further 5 min at 37°C. The reaction was stopped by adding 2 ml of methanol. The production of LTB4 and LTC4 was measured using high performance liquid chromatography (HPLC) as described. In brief, the samples were partially purified, concentrated by passing through a Sep Pak C18 cartridge (Waters, Milford, MA) and applied to HPLC system (TOSO, model 8010, Tokyo, Japan) equipped with a Novapak C18 column (Waters, Milford, MA). The samples were eluted with acetonitrile:methanol:water:acetic acid [31:17.68:51.24:0.68 (v/v)] at 0.8 ml/min and the absorbance was monitored using a spectrophotometer at 280 nm. The concentration of LTs was normalized by the protein concentration determined by the Lowry method.

**Statistical analysis**

The data are shown as means ± SEM. The data were statistically analyzed by one-way analysis of variance. A P-value of < 0.05 was defined as statistically significant.

**Results**

**Morphological effect of DSCG on DMSO-induced granulocytic differentiation of HL-60 cells**

Fig. 1 shows that the ratio (%) of differentiated cells was significantly increased by DMSO treatment, when compared with that of the untreated cells. DSCG had no effect on morphological change in granulocytic differentiation in HL-60 cells.
Neither ketotifen, an oral anti-allergic drug, nor pranlukast, a cysLT1 receptor antagonist, changed the ratio of differentiated cells (data not shown).

**Effect of DSCG on LTC₄⁻, LTB₄⁻ and TXB₂⁻ synthesis during DMSO-induced granulocytic differentiation of HL-60 cells**

Table 1 shows that the production of LTC₄, LTB₄ and TXB₂ in DMSO-differentiated cells was significantly increased, when compared to that in untreated cells. The production of LTC₄ was significantly suppressed by DSCG. The production of LTB₄ and TXB₂ was also decreased in accordance with an increasing DSCG dose. After HL-60 cells were differentiated by exposure to DMSO without DSCG for 5 days, production of LTC₄, LTB₄ and TXB₂ was not influenced by DSCG at 1–100 μg/ml; incubation periods for 1–12 h (data not shown).

The production of LTC₄, LTB₄ and TXB₂ in DMSO-treated cells tended to be decreased by ketotifen and pranlukast, but it was not significant (Table 1).

**Effect of DSCG on mRNA expression for cPLA₂, 5-LO, FLAP, LTC₄ synthase and LTA₄ hydrolase during DMSO-induced granulocytic differentiation of HL-60 cells**

The expression of the mRNA for cPLA₂, 5-LO, FLAP, LTC₄ synthase, LTA₄ hydrolase, and beta-actin in DMSO-induced HL-60 cells is shown in Fig. 2A. The densitometric analysis of the PCR products is demonstrated in Fig. 2B. The abundance of mRNA for 5LO, LTC₄ synthase and LTA₄ hydrolase in DMSO-differentiated cells was significantly increased, when compared to that of untreated cells. The abundance of cPLA₂ mRNA was decreased, whereas the abundance of mRNA for FLAP and beta-actin was not changed by the treatment of DMSO. The DMSO-induced enhancement of mRNA for LTC₄ synthase was dose-dependently inhibited by DSCG. The mRNA expression for cPLA₂, 5-LO, FLAP, LTA₄ hydrolase was not changed by DSCG.

**Effect of DSCG on the induction of LTC₄ synthase- and LTA₄ hydrolase activities during DMSO-induced granulocytic differentiation of HL-60 cells**

Fig. 3A and B shows LTC₄ synthase and LTA₄ hydrolase activities. Five-day incubation with DMSO (1.5%) significantly increased both of these activities. DSCG significantly inhibited LTC₄ synthase activity, but not LTA₄ hydrolase activity.

**Time course study for the effect of DSCG on LTC₄ synthesis during DMSO-induced granulocytic differentiation of HL-60 cells**

As shown in Fig. 4, induction of LTC₄ synthetic activity was suppressed when DSCG was added into the medium within 48 h after DMSO treatment.

### Table 1  Action of DSCG, ketotifen and pranlukast on A23187stimulated LTC₄, LTB₄, TXB₂ synthesis in DMSO-differentiated HL-60 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>LTC₄ (pg/10⁶ cells)</th>
<th>LTB₄ (pg/10⁶ cells)</th>
<th>TXB₂ (pg/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (−)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DMSO (+)</td>
<td>732.0±19.0</td>
<td>2463.4±73.7</td>
<td>2313.0±73.3</td>
</tr>
<tr>
<td>+ DSCG (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>548.0±29.9*</td>
<td>2363.3±72.6</td>
<td>2310.0±73.3</td>
</tr>
<tr>
<td>10</td>
<td>473.3±34.1*</td>
<td>2299.3±47.8</td>
<td>2260.0±38.8</td>
</tr>
<tr>
<td>100</td>
<td>420.7±22.7*</td>
<td>2007.6±45.1*</td>
<td>1926.7±34.1*</td>
</tr>
<tr>
<td>+ Ketotifen (M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>693.3±44.1</td>
<td>2443.3±88.2</td>
<td>2333.3±128.2</td>
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<tr>
<td>10⁻⁶</td>
<td>645.3±54.1</td>
<td>2313.3±76.7</td>
<td>2113.3±122.7</td>
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<tr>
<td>10⁻⁵</td>
<td>611.0±38.9**</td>
<td>2110.0±68.9***</td>
<td>1957.6±78.2***</td>
</tr>
<tr>
<td>+ Pranlukast (M)</td>
<td></td>
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<tr>
<td>10⁻⁵</td>
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<td>2160.0±122.2</td>
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<td>2250.0±186.2</td>
<td>2275.0±153.2</td>
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<tr>
<td>10⁻⁵</td>
<td>623.5±45.4****</td>
<td>2055.0±104.1****</td>
<td>2,046.0±248.2****</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. DMSO, dimethyl sulfoxide; DSCG, disodium cromoglycate. ND, not detectable.

*P<0.05 vs. cells with DMSO and without drug. **P=0.07; ***P=0.09; ****P=0.08.
Discussion

This study demonstrated that DSCG inhibited DMSO-induced LTC₄ synthesis and mRNA expression for LTC₄ synthase during granulocytic differentiation of HL-60 cells. Other investigators previously reported that DSCG inhibited the synthesis of cysLTs.²⁻²² However, these studies did not examine the mRNA expression of LT-synthetic enzymes such as cPLA₂, 5-LO, FLAP, LTA₄ hydrolase and LTC₄ synthase. Among these LT-synthetic enzymes, LTC₄ synthase is a key enzyme for cysLT synthesis, and it may be an important target for asthma treatment. In this study we demonstrated that DSCG suppressed LTC₄ synthesis.
synthesis, an initial product of cysLTs, by inhibiting the induction of mRNA for LTC4 synthase during granulocytic differentiation of HL-60 cells. These results indicate a novel anti-allergic mechanism of DSCG. Several weeks may be necessary before the effects of DSCG appear in patients with asthma. Our results may explain this time lag of the appearance of clinical efficacy and the initiation of the drug. Marquardt et al. reported that long-term exposure to cromolyn inhibited LTC4 generation in mouse mast cells, although they did not mention the effect of DSCG on LTC4 synthase activity in these cells. DSCG may affect induction of the LT-synthetic enzymes during differentiation or maturation in other inflammatory cell types including mast cell, although there is no definite evidence.

In this study, a higher dose of DSCG inhibited DMSO-induced LTB4- and TXB2-synthetic activities. This mechanism could not be explained from our study. DSCG did not reduce mRNA expression for cPLA2, 5-LO, FLAP, LTA4 hydrolase or cyclooxygenases (data not shown). A previous study demonstrated that DSCG stabilized the membrane and eliminated voltage-dependent Ca2+ currents. A higher dose of DSCG may affect the regulation of Ca2+ influx and lead to the inhibition of AA metabolism in HL-60 cells. The synthesis of LTs and TX is initiated by transmembranous stimulation via various factors that increase the Ca2+ influx, leading to cPLA2 activation. It is possible that the activity of cPLA2, a common pivotal enzyme for production of cyclooxygenase- and 5-lipoxygenase-metabolism, is inhibited due to the suppression of Ca2+ influx by high doses of DSCG.

In conclusion, we have demonstrated that DSCG suppressed cysLT synthesis during DMSO-induced granulocytic differentiation in HL-60 cells. These actions may be clinically relevant to the effect of DSCG, although the inhibition by DSCG is modest our data might not correctly reflect the inhibitory effect of DSCG on cysLT synthesis in vivo.

Acknowledgements

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References


