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Biochemical and Biophysical Research Communications 314 (2004) 104–109
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Induction of cytochrome CYP4F3A in all-*trans*-retinoic acid-treated HL60 cells☆

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Received 3 December 2003

Abstract

Cytochrome P-450 CYP4F3A catalyzes the inactivation of leukotriene B_4 by ω-hydroxylation, an activity of which is specifically expressed in human neutrophils. Here, we examined expression of the LTB_4 ω-hydroxylating activity during the differentiation of HL60 cells, an acute promyelocytic leukemia cell line, in the presence of various inducers. Among the inducers used, all-*trans*-retinoic acid (ATRA) most strongly induces the LTB_4 ω-hydroxylating activity in a dose-dependent manner. The time course of the induction of the ω-hydroxylating activity correlates well with that of the superoxide-generating activity, indicative of cell differentiation. ATRA-treated cell microsomes convert LTB_4 to its 20-hydroxyl derivative under aerobic conditions in the present of NADPH. The reaction is inhibited by carbon monoxide, an inhibitor of cytochrome P-450, and by antibodies raised against NADPH-P-450 reductase. CYP4F3A appears to be responsible for the LTB_4 ω-hydroxylase activity, based on the following observations: expression of the mRNA for CYP4F3A is observed together with the induction of LTB_4 ω-hydroxylating activity in ATRA-treated HL60 cells; and the apparent K_m values for the ω-hydroxylation of LTB_4 and lipoxin B_4 by ATRA-treated cell microsomes are essentially the same as those of CYP4F3A in human neutrophil microsomes.

Leukotriene B_4 (LTB_4), which is produced from arachidonic acid via the 5-lipoxygenase pathway, is a potent chemotactic and chemokinetic agent for human neutrophils. This leukotriene also induces neutrophil aggregation, degranulation, and superoxide production, suggesting it to be a mediator of inflammation and hypersensitivity reactions [1,2]. Human neutrophils rapidly and specifically metabolize LTB_4 via ω-oxidation to 20-hydroxy-LTB_4 (20-OH-LTB_4) and then to 20-carboxy-LTB_4 (20-COOH-LTB_4) [3–6]. Since the ω-oxidized products show less potent activities toward neutrophils, oxidation is considered to limit the activities of this mediator at inflammatory sites [7,8]. Hydroxylation at the ω-site, the first step in the oxidation pathway, is catalyzed by an LTB_4 ω-hydroxylase, a cytochrome P-450 with a high affinity for LTB_4 that is unique to neutrophils (designated P-$450_{LTB\ \omega}$) [9–12]. This P-450 is also involved in the subsequent oxidation to 20-COOH-LTB_4, which also appears to be important in the inactivation of LTB_4. Furthermore, P-$450_{LTB\omega}$ converts lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4), arachidonic acid metabolites, to their ω-oxidized products [13,14]. Although LTB_4 and LXB_4 are also metabolized through ω-oxidation by distinct enzymes from P-$450_{LTB\omega}$ in neutrophil and rat liver, the affinity for these substrates is much lower than that of P-$450_{LTB\omega}$ [12,15–18].

A cDNA clone corresponding to P-$450_{LTB\omega}$ was isolated from human neutrophil library and designated CYP4F3 based on its deduced amino acid sequence [19,20]. The gene encodes two functionally distinct

☆ *Abbreviations:* LTB_4, leukotriene B_4 or (5*S*,12*R*)-5,12-dihydroxy-6,8,10,14-*Z, E, E, Z*-eicosatetraenoic acid; 20-OH-LTB_4, 20-hydroxy-LTB_4; 20-COOH-LTB_4, 20-carboxy-LTB_4; LXB_4, lipoxin B_4 or (5*S*,14*R*,15*S*)-5,14,15-trihydroxy-6,8,10,12-*E,Z,E,E*-eicosatetraenoic acid; PGA_1, prostaglandins A_1; P-450, cytochrome P-450; P-$450_{LTB\omega}$, cytochrome P-$450_{LTB\omega}$ or human neutrophil LTB_4 ω-hydroxylase; RP-HPLC, reverse-phase high performance liquid chromatography; PMA, phorbol-12, 13-myristate acetate; O_2^-, superoxide.

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doi:10.1016/j.bbrc.2003.12.062

enzymes, CYP4F3A and CYP4F3B, generated by alternative splicing by the selection of exons 4 and 3, respectively [21,22]. CYP4F3A is expressed mainly in neutrophils and shows high affinity for LTB_4 with a K_m value of $<1\,\mu M$, which is consistent with the enzymatic property of $P\text{-}450_{LTB\omega}$. On the other hand, CYP4F3B is expressed in liver and shows a much lower efficiency of LTB_4 inactivation. The expression of CYP4F3 isoforms is regulated not only by alternative splicing but also by a distinct promoter in each tissue. The promoter of CYP4F3A is present in the region 468–872 bp upstream of the initiation ATG codon, whereas that of CYP4F3B is in the region 123–155 bp upstream of the ATG codon [23]. The CYP4F3A mRNA can be detected in cells expressing markers of myeloid differentiation, such as CD11b, suggesting that differentiation to neutrophils may be related to CYP4F3A expression. However, PU.1 and MZF, known myeloid transcription factors, do not display a transcriptional activity for CYP4F3A, and the promoter is activated by Zeb-2, a factor primarily characterized as a transcriptional repressor in the various cells including lymphocytes [23]. Thus, it has remained unknown whether the CYP4F3A-related LTB_4 ω-hydroxylase activity is actually induced according to differentiation into neutrophils.

We investigated the induction of the LTB_4 ω-hydroxylating activity during the differentiation of HL60 cells, an acute promyelocytic leukemia cell line that can be induced to differentiate into neutrophils, monocytes, or macrophages in response to a variety of inducers. In this study, we demonstrate that all-*trans*-retinoic acid (ATRA) specifically induces LTB_4 ω-hydroxylase in HL60 cells, in coordination with the induction of superoxide-generating activity. The LTB_4 ω-hydroxylase is likely identified as CYP4F3A, on the basis of enzymatic analyses and the results of a reverse transcription-PCR method.

Materials and methods

Chemicals. Antibodies raised against NADPH-cytochrome P-450 reductase in rat liver microsomes were prepared as previously described [13,14,16,17]. LXB_4 was purchased from Cascade Biochem., UK; LTB_4 and prostaglandin A_1 (PGA_1) were from Cayman Chemical, Ann Arbor, USA. The standard for 20-OH-LXB_4 was prepared by incubating LXB_4 with human neutrophil microsomes and purified by reverse-phase high performance liquid chromatography (RP-HPLC) [14]. Water passing through a MilliQ filter was used in all experiments (synthesis A10, Millipore, Bedford, MA). All other chemicals were of the highest purity commercially available.

Cell culture and differentiation. HL60 cells were cultured and induced to differentiate by ATRA as described previously [24,25]. Superoxide production was determined as previously described with minor modification. Briefly, HL60 cells (approximately 5×10^5 cells/ml) were suspended in saline buffered with 20 mM Hepes (pH 7.4) containing 50 μM cytochrome *c*. Before the addition of a stimulant, the suspension was preincubated for 10 min at 37 °C in a thermostated cuvette, which was placed in a Hitachi 557 dual-wavelength spectrophotometer. After the addition of 20 μg/ml phorbol-12, 13-myristate acetate (PMA), the change in absorbance at 550 nm in reference to 540 nm was recorded and converted to superoxide release using a molar absorption coefficient for reduced-minus oxidized cytochrome *c* of $19.1 \times 10^3\ 10^{-1}\ cm^{-1}$.

Assay of LTB_4 ω-hydroxylation. LTB_4 ω-hydroxylating activity was determined as described [26] with minor modification. For the assay of LTB_4 ω-hydroxylating activity in intact HL60 cells, 4×10^6 cells suspended in 200 μl Hepes-buffered saline (20 mM Hepes, 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.6 mM $CaCl_2$, and 2 mM glucose, pH 7.4) were incubated for the indicated times at 37 °C with LTB_4. For the assay of LTB_4 ω-hydroxylating activity in the microsome fraction, microsomes suspended in 100 μl of 340 mM sucrose, 20 mM Hepes (pH 7.4), and 1 mM NADPH were incubated for the indicated times at 37 °C with LTB_4. The reactions were terminated by the addition of 400 μl ethanol containing 1.0 nmol PGB_2, acidified to pH 4.2 with 100 mM H_3PO_4, and extracted with 1 ml ethyl acetate. The extract was evaporated to dryness under a stream of N_2 and the residue was dissolved in 100 μl methanol/acetonitrile/water/acetic acid (40/25/65/0.02, by vol.) and subjected to RP-HPLC using a Shimadzu LC-6A gradient system and a Chemcosorb 5-ODS-H column (5 μm, 4.6×150 mm, Chemco Scientific, Osaka) with continuous monitoring of ultraviolet (UV) absorbance at 270 nm. Solvents A and B consisted of methanol/acetonitrile/water/acetic acid (40/25/65/0.02, by vol.) and methanol/acetonitrile (70/30, by vol.), respectively. The flow rate was maintained at 1.0 ml/min. After equilibrating the column with solvent A, elution was performed isocratically with solvent A for the first 6 min, followed by a linear increase in solvent B from 0 to 40% over 6 min. From 12 to 24 min the column was eluted isocratically with a mixture of 60% solvent A and 40% solvent B. After 24 min, the ratio of solvent B was increased to 100% at a rate of 15%/min. Typical elution times for 20-COOH-LTB_4, 20-OH-LTB_4, LTB_4, and PGB_2 were 8.2, 9.4, 19.7, and 24.5 min, respectively.

Assay of LXB_4 ω-hydroxylation. LXB_4 ω-hydroxylating activity was determined as described [14,17] with minor modification. Microsomes, suspended in 100 μl of 100 mM potassium phosphate buffer (pH7.4), were incubated for the indicated times at 37 °C with LXB_4 in the presence of 1 mM NADPH. The reaction was terminated by the addition of 100 μl methanol. After centrifugation, 40 μl of the supernatant was diluted with 160 μl methanol/50 mM ammonium acetate, pH 5.6 (50/50, by vol.), and the sample (200 μl) was subjected to RP-HPLC. This was carried out using a Shimadzu LC-6A gradient system and a Chemcosorb 5-ODS-H column with continuous monitoring of UV absorbance at 301 nm. The flow rate was maintained at 1.0 ml/min. Elution was performed isocratically with methanol/50 mM ammonium acetate (50/50, by vol.) for the first 17 min, followed by a linear increase to 75% methanol over the next 13 min. From 30 to 40 min, the column was eluted isocratically with methanol/50 mM ammonium acetate (75/25, by vol.). Typical elution times for LXB_4, 20-hydroxy LXB_4, and 20-carboxy LXB_4 were 34.2, 12.9, and 4.4 min, respectively.

PCR analysis. Total RNAs were isolated from HL60 cells as described previously [27]. To synthesize single-stranded cDNA, 2 μg of total RNA was incubated at 37 °C for 60 min according to the manufacturer's instructions (Amersham–Pharmacia Biotech, Buckinghamshire, UK). The 5′ antisense primer (Primer 1) covered the nucleotide from position 281–302 and the 3′ antisense primer (Primer 2) covered the nucleotide from position 760–781 of the LTB_4 ω-hydroxylase cDNA [19]. The single strand cDNA was amplified by PCR in 45 μl of 1.4 mM $MgCl_2$, 1.0 μg (5 μl) cDNA template, 0.5 pmol of primer, 220 μM dNTP, 2.5 U *Taq* DNA polymerase, and 50 μl of mineral oil. Amplification was carried out in 28 cycles in a DNA thermal cycler, using a cycle of denaturation for 3 min at 95 °C, annealing for 1 min at 55 °C, and extension at 55 °C for 7 min. The PCR-amplified DNA fragments were subjected to electrophoresis in 2% agarose gels. DNAs were visualized by staining with ethidium bromide.

Results and discussion

Induction of the LTB_4 ω-hydroxylating activity in HL60 cells in the presence of various inducers

We determined the LTB_4 ω-hydroxylating and superoxide-releasing activities, when HL60 cells, an undifferentiated cell line, were treated with various inducers. PMA-induced production of superoxide, detected as the reduction of cytochrome *c*, was defined as a marker of cellular differentiation. Treatment of HL60 cells with ATRA, which is known to induce HL60 cells to differentiate into neutrophil-like cells, led to a significant, dose-dependent increase in the superoxide-producing activity (Table 1). Consistent with the production of superoxide, the LTB_4 ω-hydroxylase activity increased significantly in ATRA-treated HL60 cells. The addition of granulocyte-colony stimulating factor to ATRA-treated cells led to a further increase in the production of superoxide as compared with cells treated with ATRA alone; however, the cytokine had no effect on the LTB_4 ω-hydroxylase activity induced by ATRA. Dimethyl sulfoxide (DMSO) and dibutyryl cyclic-AMP (Bt_2-c-AMP), both of which also induce differentiation to neutrophil-like cells, were less effective in inducing the LTB_4 ω-hydroxylating activity than ATRA, consistent with the results reported previously [28]. The differentiation of HL60 cells into monocyte-like cells by treatment with PMA and vitamin D_3 did not result in induction of the LTB_4 ω-hydroxylating activity (data not shown). When undifferentiated HL60 cells were incubated with 2 μM LTB_4 for 30 min at 37 °C, we did not observe any LTB_4 ω-oxidized products, such as 20-OH-LTB_4 and 20-COOH-LTB_4, detectable by UV absorption following separation by RP-HPLC (data not shown). Thus, the LTB_4 ω-hydroxylating activity was strongly induced only in ATRA-treated HL60 cells, whereas all compounds tested in the present study were capable of inducing the PMA-stimulated superoxide-producing activity to various extents.

Table 1
Effect of various compounds on the superoxide-generating activity and LTB_4 ω-hydroxylating activity in HL60 cells

Compound	O_2^- production (nmol/min/10^5 cells)	LTB_4 ω-hydroxylation (pmol/min/10^7 cells)
Control	0	0
Bt_2-c-AMP (1 mM)	0.4	0.1
DMSO (1.25%)	2.6	0.1
G-CSF (10 ng/ml)	0	0
ATRA (0.1 μM)	5.3	1.84
ATRA (0.3 μM)	7.3	2.54
ATRA (0.1 μM) + G-CSF	12.6	1.75
ATRA (0.3 μM) + G-CSF	12.3	2.17

HL60 cells were cultured with 1 mM dibutyryl c-AMP (Bt_2-c-AMP), 1.25% dimethyl sulfoxide (DMSO), 10 ng/ml granulocyte-colony stimulating factor (G-CSF), and 0.1 or 0.3 μM ATRA for 7 days. Superoxide (O_2^-)-generating activity and LTB_4 ω-hydroxylating activity were determined as described under "Materials and methods." The values represent the average of two independent experiments.

Time course of the expression of the LTB_4 ω-hydroxylating activity in ATRA-treated HL60 cells

We examined the time course of superoxide production and LTB_4 ω-hydroxylating activity after treatment with ATRA. The production of superoxide was detected 4 days after the addition of ATRA and the activity increased linearly until 8 days after the ATRA treatment (Fig. 1A). The superoxide production was dependent on the concentration of ATRA. The time course of induction of the LTB_4 ω-hydroxylating activity was similar to that of the superoxide-producing activity (Fig. 1B). These observations suggest that the induction of the LTB_4 ω-hydroxylating activity is closely related to the ATRA-induced differentiation into neutrophil-like cells.

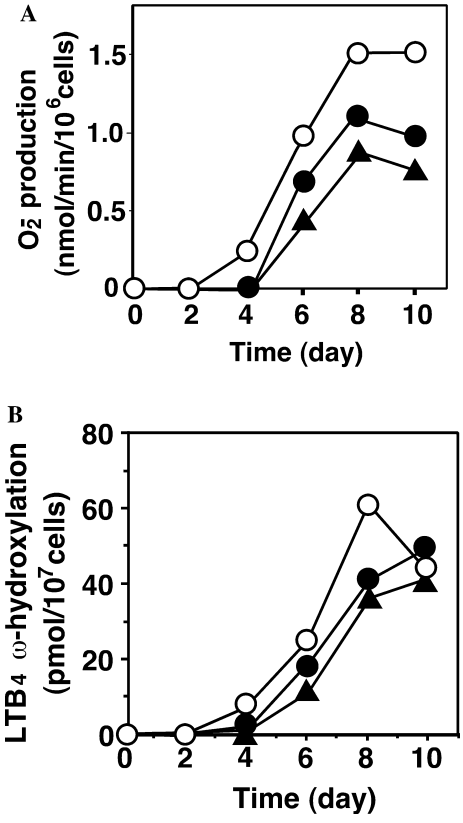

Fig. 1. Time course of superoxide-generating activity and LTB_4 ω-hydroxylating activity in ATRA-treated HL60 cells. HL60 cells were cultured with 1 μM (○), 0.1 μM (●), or 0.01 μM (▲) ATRA for the indicated times, and the superoxide (O_2^-)-generating activity (A) and LTB_4 ω-hydroxylating activity (B) were determined as described under "Materials and methods." The values represent the average of two independent experiments.

Identification of the ATRA-induced LTB_4 ω-hydroxylase as CYP4F3

We next investigated the enzymatic characteristics of the LTB_4 ω-hydroxylating activity in ATRA-treated HL60 cells. The LTB_4 ω-hydroxylation activity was localized in microsomes with negligible activities detected in the mitochondrial and cytosolic fractions (data not shown). No hydroxylation was observed in the absence of NADPH or when using microsomes heated at 80 °C for 10 min (Table 2). No reaction occurred under anaerobic conditions and the reaction was strongly inhibited by carbon monoxide (Table 2). These findings indicate that a P-450 is involved in the hydroxylation. The involvement of a P-450 is further supported by the fact that the reaction was inhibited by antibodies raised against NADPH-P450 reductase.

We added various concentrations of LTB_4 or LXB_4 to the ω-hydroxylation system to determine whether CYP4F3A participates in the hydroxylation in ATRA-treated HL60 cells. The apparent K_m value for LTB_4 ω-hydroxylation in differentiated cell microsomes is 0.38 μM, essentially the same as the K_m for the reaction in human neutrophil microsomes (0.30 μM) (Fig. 2). LXB_4, as well as LTB_4, is also converted to its ω-oxidized products by CYP4F3A as previously described [14]. The K_m value for the ω-hydroxylation of LXB_4 by the HL60 cell microsomes (10.2 μM) is almost the same as that (7.7 μM) by the neutrophil microsomes.

We finally examined the expression of the mRNA for CYP4F3A in differentiating HL60 cells by reverse transcription-PCR analysis. The synthetic PCR primers were designed to amplify the putative region of the full-length CYP4F3A cDNA [19]. Expression of the CYP4F3A mRNA was clearly observed 4 days after the addition of ATRA to undifferentiated HL60 cells, although there was no detectable *mRNA* for CYP4F3A in undifferentiated HL60 cells (Fig. 3). These findings indicate that the LTB_4 ω-hydroxylase induced in ATRA-treated HL60 cells is CYP4F3A, which is known to be expressed specifically in human neutrophils.

Table 2
Effects of various conditions on LTB_4 ω-hydroxylation by ATRA-treated HL60 cell microsomes

Condition	ω-hydroxylation (%)
Control	100
Heat-treated microsomes[a]	0
Without NADPH	0
Anaerobic[b]	6
CO/O_2 (80/20,%)	18
Anti-P-450 reductase IgG	22
Nonimmune IgG	104

HL60 cell microsomes (100 μg) treated with ATRA for 7 days were suspended in 100 μl of sucrose/Hepes buffered solution (pH 7.4) and incubated for 30 min at 37 °C with LTB_4 (2.0 μM) in the presence or absence of NADPH (1 mM) under the indicated conditions. The LTB_4 ω-hydroxylation activity was determined as described under "Materials and methods." The activity under aerobic conditions in the absence of inhibitors is set as 100%.

[a] Microsomes pretreated for 10 min at 80 °C.

[b] Microsomes deoxygenated under a stream of nitrogen followed by incubation for 10 min at 25 °C with 10 mM glucose, glucose oxidase (50 μg/ml), and catalase (100 μg/ml) prior to the start of the reaction.

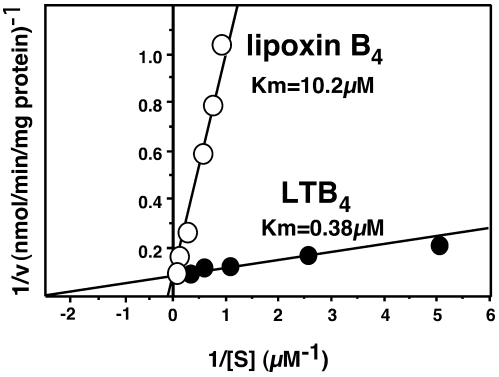

Fig. 2. Lineweaver–Burk plot of the ω-hydroxylation of LTB_4 and LXB_4 ATRA-treated HL60 cell microsomes (100 μg of protein) were incubated for 30 min at 37 °C with various concentrations of LTB_4 or LXB_4. LTB_4 and LXB_4 ω-hydroxylation were measured as described under "Materials and methods." The values represent the average of two independent experiments.

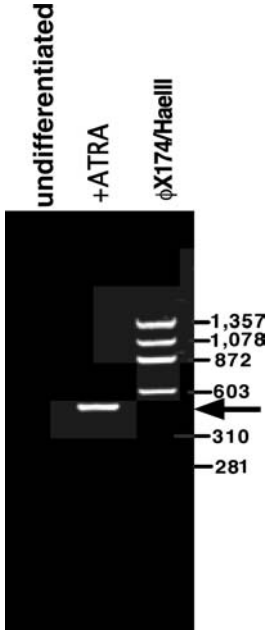

Fig. 3. Detection of the mRNA for CYP4F3A in ATRA-treated HL60 cells by an RT-PCR method. HL60 cells were cultured with or without 0.1 μM ATRA for 4 days, and the expression of CYP4F3A mRNA was determined as described under "Materials and methods." The PCR product specific for the CYP4F3A mRNA was generated and resolved by agarose gel electrophoresis. The position of the PCR product is indicated by the *arrow*. DNA molecular weight markers, *ϕX174/Hae*III digest. The figure shows the representative data obtained in two independent experiments.

Physiological significance of CYP4F3A expression

In the present study, we show that ATRA specifically induces the expression of CYP4F3A in parallel with the induction of the superoxide-generating activity. It is known that ATRA can induce complete remission in acute promyelocytic leukemia by inducing a terminal differentiation of leukemic cells into neutrophils [29,30]. The treatment acute promyelocytic leukemia patients with a genome translocation of the ATRA receptor with ATRA may produce an induction of CYP4F3A in vivo, which may play a role in recovery from the disease. The expression of CYP4F3A, which can inactivate LTB_4, might be necessary for the disappearance of the inflammatory reaction. Mice lacking 5-lipoxygenase, an enzyme involved in the synthesis of LTB_4, are unable to produce LTB_4, and so are resistant to the lethal shock effect of platelet-activating factor [31]. Endotoxin shock due to various infectious diseases is often observed during recovery from leukemia [32–34] and might be preventable by the induction of CYP4F3A.

The mechanism for the induction of CYP4F3A by ATRA remains unknown. ATRA may induce the CYP4F3A mRNA indirectly through expression of transcription factor(s) distinct from ATRA receptors, since it takes a long time, i.e., 4 days, for the induction of the LTB_4 ω-hydroxylating activity in HL60 cells after treatment with ATRA. Further studies on the promoter region of CYP4F3A will clarify the mechanism by which expression of CYP4F3 is regulated during myeloid development and also maybe in the inflammatory processes.

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