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Advances in Brief

Lipid Hydroperoxides Greatly Increase the Rate of Oxidative Catabolism of All-*trans*-Retinoic Acid by Human Cell Culture Microsomes Genetically Enriched in Specified Cytochrome P-450 Isoforms[1]

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Abstract

Cytochrome P-450 enzymes have been implicated in the oxidative catabolism of all-*trans*-retinoic acid (RA), a process that is accelerated by exposure to RA in cultured cells and rodents, and also in patients receiving RA as treatment for cancer (J. F. R. Muindi *et al.*, Cancer Res., *52:* 2138, 1992; Blood, *79:* 299, 1992). Accelerated oxidation of RA could arise from an induction of RA-catabolizing P-450 isoforms or from an increase in oxidative cofactors. We have examined the efficiency of NADPH/O_2 and lipid hydroperoxides (LOOH) to support oxidation of RA using human cell microsomes genetically enriched in different P-450 isoforms. The observed rate of RA oxidation using the NADPH/O_2 system was slow for all isoforms (6–23 pmol/mg protein/min). LOOH-mediated oxidation was much faster (24–1078 pmol/mg protein/min), not isoform specific, but dependent upon the chemical nature of the LOOH. The order of efficiency of RA oxidation using LOOH was 13-hydroperoxy[*S*-(*E,Z*)]-9,11-octadecadienoic acid > 5-hydroperoxy[*S*-(*E,Z,Z,Z*)]-6,6,11,14-eicosatetraenoic acid > prostaglandin G_2 > cumene hydroperoxide > *tert*-butylhydroperoxide > H_2O_2. Whereas submicromolar concentrations of 13-hydroperoxy[*S*-(*E,Z*)]-9,11-octadecadienoic and 5-hydroperoxy[*S*-(*E,Z,Z,Z*)]-6,6,11,14-eicosatetraenoic acid oxidized RA at appreciable rates, micromolar concentrations were required for the other LOOH. These observations suggest that physiological LOOH, generated by the arachidonic acid-lipoxygenase system, may be involved in the self-induced oxidative catabolism of RA.

Introduction

RA[3] induces complete remission in a high proportion of patients with acute promyelocytic leukemia (1–3); unfortunately, in the absence of aggressive postinduction cytotoxic chemotherapy, relapse and clinical resistance to further RA therapy occurs rather rapidly in the majority of patients (1–4). We have previously reported that RA induces its own accelerated clearance in patients with APL and have postulated that this accelerated drug catabolism could contribute to the rapid appearance of clinical resistance (4, 5). A similar acceleration of the drug clearance has been reported in pediatric patients treated chronically with RA (6). The pharmacokinetic observations in patients are fully consistent with the established capacity of RA to induce and accelerate its own oxidative catabolism in rodents and cells in culture (7–10). Although cultured cell populations vary in their constitutive rates of RA oxidation, they are broadly capable of induced catabolism after 24 h of exposure to the compound. Some tumor cell varieties that respond to high concentrations of RA *in vitro* demonstrate an elevated *constitutive* capacity to oxidize the agent (9, 10). We are exploring the biochemical mechanisms involved in the autoinduction of RA catabolism because of its inherent scientific interest and because accelerated catabolism could influence response to therapy with RA, particularly if it occurred within the neoplastic cell population.

It is generally accepted that the first step in the oxidative catabolism of RA, the generation of 4-hydroxy-all-*trans*-RA, requires participation of cytochrome P-450 enzymes (7, 11, 12); the P-450 system is also involved in some of the subsequent steps which produce increasingly polar metabolites. The capacity of ketoconazole, a rather broad inhibitor of P-450-based oxidations, to inhibit RA catabolic oxidation *in vivo* and in cultured cells is consistent with this concept (10, 13). The P-450 enzymes are genetically related membrane proteins which utilize a noncovalently bound heme iron, dissolved oxygen, and electrons provided in two steps by NADPH, and sometimes NADH, to oxygenate substrates (14). The P-450 system can also carry out hydroxylation and other oxidative reactions utilizing lipid hydroperoxides through the "peroxide shunt"; in LOOH-mediated oxidations neither molecular oxygen nor additional electrons are required (15).

Conceptually, an accelerated oxidation of RA could arise from an autoinduced increase in the total number of efficiently RA-catabolizing P-450 units or by an increase in the cofactors which support or facilitate that oxidative process. CYPs can be induced in the liver by a variety of compounds; in preclinical studies, compounds which induce CYPs 1A1, 1A2, 2B4, 2E1, and members of the 3A series have been reported to increase the oxidative capacity of the liver with regard to RA. We report herein studies on the RA-oxidative capacity of microsomes from human cell lines which genetically constitutively hyperexpress specified CYP isoforms (16). We have compared oxidative rates in the presence of NADPH/O_2 or a series of physiological and nonphysiological hydroperoxides. We have placed emphasis on examining the effects of LOOHs that are generated physiologically by inducible reactions, notably those that are derived from phospholipase-released fatty acids through the actions of PGHS or cellular lipoxygenases.

Materials and Methods

Reagents. Cell microsomes genetically enriched in human P4501A1, P4501A2, P4502B6, P4502D6, P4502E1, and P4503A4 and unenriched cell microsomes, containing principally P4501A1, were obtained from Gentest Corp. (Woburn, MA). The protein content of all microsomal preparations was 10 mg/ml. [11,12-^{3}H]Retinoic acid (specific activity, 49.3 Ci/mmol) was purchased from NEN Research Products (Boston, MA). Reagent grade 1-butanol and hydrogen peroxide (30%) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Nonradioactive RA, 13-*cis*-RA, NADPH, *tert*-butylhydroperoxide, and cumene hydroperoxide were from Sigma Chemical Co. (St. Louis, MO). The internal standard (all-*trans*-9-nosityl-3,7-dimethyl-2,4,6,8-nonatetraenoic

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[3] The abbreviations used are: RA, all-*trans*-retinoic acid; LOOH, lipid hydroperoxides; 13(S)-HpODE, 13-hydroperoxy[*S*-(*E,Z*)]-9,11-octadecadienoic acid; 5(S)-HpETE, 5-hydroperoxy[*S*-(*E,Z,Z,Z*)]-6,6,11,14-eicosatetraenoic acid; CYP, a cytochrome P-450 gene, the isoform is specified, *e.g.*, CYP1A1; P4501A1, the protein product of CYP1A1; BSA, bovine serum albumin; PGHS, prostaglandin H synthase; 13-*cis*-RA, 13-*cis*-retinoic acid; HPLC, high performance liquid chromatography; CRABP, cellular retinoic acid-binding protein; 12(S)-HpETE, 12-hydroperoxy[*S*-(*E,Z,Z,Z*)]-5,8,10,14-eicosatetraenoic acid; 15-(S)-HpETE, 15-hydroperoxy-[*S*-(*E,Z,Z,Z*)]-5,8,11,13-eicosatetraenoic acid.

acid) and 4-oxo metabolites of both RA and 13-*cis*-Ra were a gift from Dr. F. M. Vane of Hoffmann LaRoche (Nutley, NJ). Prostaglandin H synthase, prostaglandins G_2 and H_2, arachidonic acid, 5(S)-HpETE, 12(S)-HpETE, 15(S)-HpETE, and 13(S)-HpODE were purchased from Cayman Chemical Co. (Ann Arbor, MI). HPLC grade acetonitrile was obtained from T. J. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were obtained from either Fisher or T. J. Baker.

NADPH/O_2-dependent Oxygenation. NADPH/O_2 dependent oxidation of RA by microsomes was performed in 0.1 M potassium phosphate buffer, pH 7.4. The standard assay mixture consisted of 100 μg of microsomal protein, 0.44 mM NADPH, and 0.162 to 5.0 μM [^{3}H]RA diluted with nonradioactive RA. The amount of [^{3}H]RA was the same in all assays. The assay volume was 250 μl. The reaction was initiated by the addition of NADPH. Following a 10-min incubation at 37°C, the reaction was stopped by adding 175 μl of acetonitrile:1-butanol (1:1) containing 125 ng of the internal standard. Following addition of 150 μl of saturated K_2HPO_4 solution and liquid-liquid partition, RA and its oxidative products were extracted into the organic phase and analyzed by HPLC. An assay mixture with 100 μg of BSA instead of microsomal protein was included in all experiments in order to determine nonenzymatic break down of RA under the assay conditions. NADPH was used routinely; however, comparable results were obtained when NADPH was replaced with a NADPH-generating system consisting of NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase.

LOOH-dependent Oxygenation. The assay conditions for lipid hydroperoxide-mediated RA catabolism were similar to those used for the NADPH/O_2-mediated catabolism except that NADPH was replaced by the appropriate lipid hydroperoxide; the reaction was terminated after 5 min incubation. To determine the role of adventitious iron and other metal in these reactions, experiments were repeated in the presence of SKF-525A, a known cytochrome P-450 inhibitor, and with heat-inactivated microsomal preparations. Studies on the oxidation of RA by PGHS used 25 units of purified ursine PGHS and 50 μM arachidonic acid. The reaction was initiated by addition of arachidonic acid and stopped after 5 min incubation at 37°C. Products of the reaction were extracted as described for cytochrome P-450 assays.

HPLC/Scintillation Counting. RA and its oxidative products were separated by reverse phase HPLC using published methods (4, 5). RA and its oxidative products were monitored by UV at 365 nm and by collection of column eluate for measurement of radioactivity. Fractions were collected at 1-min intervals, mixed with 10 ml of hydrofluor scintillation liquid, and counted for 3 min in a Packard Tri-Carb 4000 Series liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Radioactivity cpm were converted to dpm using an appropriate quench curve.

Data Analysis. The oxidative catabolism of RA produces compounds of increasing polarity which elute from a reverse phase HPLC column before the parent compound. Based on the retention times of known RA oxidative products under the HPLC conditions used in this study, radioactivity eluting after the solvent front and up to the appearance of the internal standard peak (fractions 3–14) represented metabolized RA; counts in the subsequent fractions represented unmetabolized RA. The quantity of RA catabolized (R) (pmol/assay) at each RA concentration was calculated as

$$R = S \times \left(\frac{M_{P450}}{M_{P450} + P_{P450}} - \frac{M_{BSA}}{M_{BSA} + P_{BSA}} \right)$$

where S is pmol of RA in the assay mixture and M_{P450} and M_{BSA} are oxidative metabolite radioactivity counts for the microsomal preparation and BSA assays, respectively. P_{P450} and P_{BSA} represent radioactive counts present as the parent compound, *i.e.*, unmetabolized RA, in the microsomal and BSA assays, respectively. Kinetic parameters for the NADPH/O_2-mediated oxidation of RA were calculated by the Michaelis-Menten equation using the PHARM/PCS computer program of Tallarida and Murray (17).

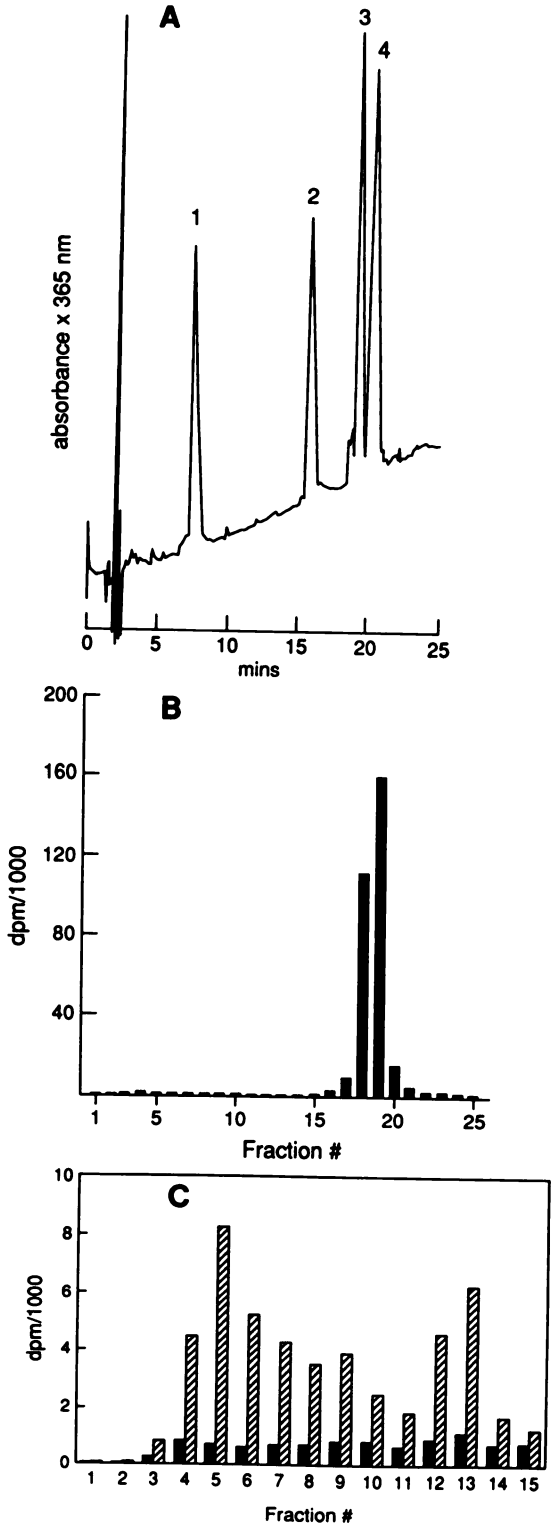

Fig. 1. HPLC elution profiles of RA and relevant metabolites or standards. *A*, *Peak 1*, 4-oxo-RA; *Peak 2*, internal standard; *Peak 3*, 13-*cis*-retinoic acid; *Peak 4*, RA. *B*, radioactivity of sequential fractions of the lipid-phase extract from an incubation mixture containing 5(S)-HpETE and BSA. *C*, radioactivity of sequential fractions 1 through 15 of the lipid-phase extract from incubation mixtures comparing 5(S)-HpETE + P4502D6 microsomes (▨) to 5(S)HpETE + BSA (■).

Results

Fig. 1 demonstrates the elution pattern of UV absorbance (Fig. 1*A*), radioactivity in the sequential chromatographic fractions of [^{3}H]RA prior to oxidation (Fig. 1*B*), and following incubation with 5(S)-HpETE, in the presence of P4502D6-enriched microsomes *versus* BSA (Fig. 1*C*). The present assay conditions are designed to assess global rates of oxidation rather than resolving specific metabolites; however, fractions 8–10 probably contain 4-hydroxy-RA and 4-oxo-RA, and fractions 11–14 may contain epoxide derivatives of RA. The early exiting fractions 3–7 represent oxidative products beyond 4-oxo-RA. In time course studies, the putative 4-hydroxy- and 4-oxo-RA

metabolites appeared rapidly but rose to a plateau, while the content of the more polar products continued to increase as the incubation proceeded (data not shown).

$NADPH/O_2$-mediated oxidation of RA was observed with all cytochrome P-450 isoforms examined; the rate of RA oxidation under these assay conditions was relatively slow (6–23 pmol/mg protein/min); however, it was consistent with published rates obtained in hepatic microsomes from rodents (12, 18). Apparent K_m and V_{max} values for RA oxidation by the various preparations are provided in Table 1. We observed no major differences in the K_m values between the P-450 isoforms; neither was there a correlation between the degree of enrichment of a particular isoform and the V_{max}.

LOOH-mediated oxidation of RA was much faster than that observed with the $NADPH/O_2$ system; observed rates for the different P-450 isoforms and cumene hydroperoxide are presented in Table 1. Detailed studies of RA oxidation by different LOOHs and H_2O_2 were performed using the P4502D6 isoform; the results of these observations are presented in Fig. 2. When compared at 64 μM LOOH, the rates ranged from 24 to 1078 pmol/mg protein/min; the order of efficiency of RA oxidation using hydroperoxides was 13(S)-HpODE > 5(S)-HpETE > prostaglandin G_2 > cumene hydroperoxide > *tert*-butylhydroperoxide > H_2O_2. Furthermore, whereas submicromolar concentrations of 13(S)-HpODE and 5(S)-HpETE oxidized RA at appreciable rates, micromolar concentrations were required for the other LOOH. The three lipoxygenase generated substances 5(S)HpETE, 12(S)-HpETE, and 15(S)-HpETE were equally effective in supporting RA oxidation by P4502D6; moreover, 5(S)-HpETE also effectively supported RA oxidation by unenriched and P4501A2- and P4503A4-enriched microsomes (data not shown).

Heat inactivation of the P4502D6 preparation decreased the yield of 5(S)-HpETE-dependent RA oxidative products by 50%, but it did not eliminate that oxidation. Inclusion of 50 μg/ml of SKF-525A (a standard inhibitor of P-450 systems) in the assay inhibited 5(S)-HpETE-mediated RA oxidation by 40%. These observations suggest that contribution of both enzymatic and nonenzymatic components to RA oxidation in these assays.

Discussion

The focus of these studies is to try to generate information in human cell-derived microsomal systems that might be germane to the rapid induction of enhanced oxidative catabolism by exposure to RA itself, a process that can be demonstrated both in intact animals and in cells in culture. We are seeking supportive data bearing on the following hypothesis. RA exposure could induce an increase in the cellular content of either (*a*) a P-450 isoform that is particularly efficient in oxidizing RA or (*b*) a cofactor which increases the oxidative efficiency for RA of the existing P-450 machinery.

Table 1 *Kinetic characteristics of retionic acid oxidation by cytochrome P-450 isoforms supported by NADPH/O_2 or by cumene hydroperoxide*

V_{max} and oxidation rates are expressed as pmol/mg protein/min. The listed cumene hydroperoxide rates were determined at 100 μM; the rate was linear for at least 10 min for all isoforms. Reference substrates for the P-450 isoforms: Unenriched, P4501A1, and P450–1A2, 7-ethoxyresorufin; P4502B6, 7-ethoxycoumarin; P4502D6, bufuralol; P4502E1, chlorzoxazone; P4503A4, testosterone.

P-450 isoform	Reference substrate activity	RA/NADPH/O_2		RA/cumene hydroperoxide (initial rate)
		K_m (μM)	V_{max}	
Unenriched	2	0.9	5.2	87
P4501A1	68	2.2	11.7	62
P4501A2	67	0.9	7.6	nd
P4502B6	9	3.5	22.5	nd
P4502D6	2230	1.4	12.1	334
P4502E1	75	0.7	7.6	68
P4503A4	170	2.7	15.7	94

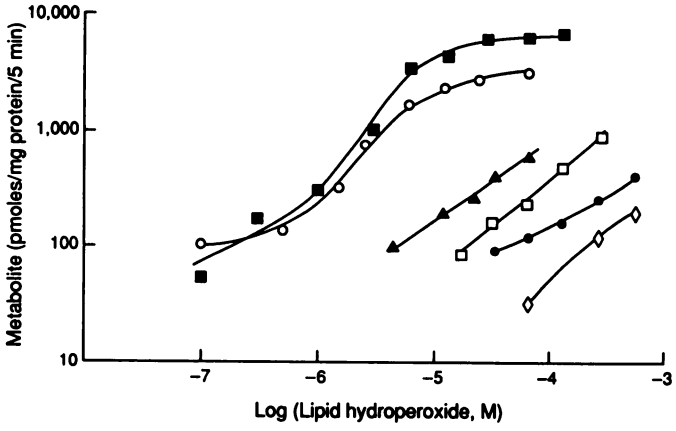

Fig. 2. Comparison of the capacity of physiological and nonphysiological hydroperoxides to support the oxidation of RA by P4502D6-enriched human cell microsomes: 13(S)-HpODE (■); 5(S)-HpETE (○); prostaglandin G_2 (▲); cumene hydroperoxide (□); *tert*-butylhydroperoxide (●); H_2O_2 (◇).

Our studies demonstrate that multiple P-450 isoforms have the capacity to oxidize RA in the presence of NADPH and molecular oxygen; however, we did not observe a correlation between P-450 enrichment and capacity to oxidize RA. This is best demonstrated by the minimal difference in the V_{max} for RA oxidation between the control microsomes, which contain P4501A1, and the 34-fold-enriched P4501A1 microsomes (Table 1). Clearly, simply increasing the number of P-450 units did not comparably increase the rate of oxidation of RA under the present study conditions. Our studies also failed to disclose a major difference between the isoforms with respect to K_m values, including P4503A4, genetically closely related to P4503A2, the isoform reported to be induced in rat liver by prolonged feeding of vitamin A (19).

The remarkable increase in microsomal oxidation of RA in the presence of HpETEs and 13(S)-HpODE studies suggests that an increase in the cellular content of physiologically generated lipid hydroperoxides could accelerate the oxidative catabolism of RA. Our observations with microsomes parallel those made by Samokyszyn and Marnett (20) who demonstrated that 13-*cis*-retinoic acid is rapidly oxidized by PGHS in the presence of arachidonic acid or LOOH (20). We have demonstrated that PGHS also oxidizes the all-*trans*-retinoic acid isomer when incubated with arachidonic acid and [^{3}H]RA (data not shown); however, it is unlikely that PGHS was an operant factor in our microsomal studies because, in the latter system, exogenous arachidonic acid did not support oxidation of RA.

In the microsomal systems we have used, LOOH-mediated RA oxidation appears to have both enzymatic and nonenzymatic components. The enzymatic component is characterized by SKF-525A inhibition and heat inactivation; however, residual oxidative activity remains in heated preparations. Non-enzymatic oxidation may result from catalytic interactions involving iron molecules present in the microsomal preparation (21). In spite of the mixed enzymatic and nonenzymatic nature of the LOOH-mediated microsomal oxidation of RA, the fact that the reaction proceeds at reasonable rates when both HpETE and RA are present in submicromolar concentrations suggests that this could occur *in vivo*.

The following observations and reports suggest that LOOH-generating pathways are activated during drug-induced cytodifferentiation. Both retinoic acid and dimethyl sulfoxide induce granulocytic differentiation in HL-60 cells; temporally concomitant with that induction, we have observed that the RA-exposed cells HL-60 cells acquire the capacity to oxidize RA; dimethyl sulfoxide-treated cells have not been examined in this regard. Induction of the cytodifferentiation sequence

also activates both the prostaglandin and lipoxygenase-dependent leukotriene-synthetic pathways (22, 23).

CRABP, a RA-binding protein found in the cytosol that is unrelated the RA-nuclear receptors, may also contribute to the efficiency of RA catabolism. Fiorella and Napoli (24) have demonstrated that RA bound to CRABP had a 20-fold lower K_m for NADPH/O_2-dependent oxidation by rat testis microsomes than that observed with free RA; the V_{max} was unchanged. Although it is clear that cellular levels of CRABP increase with chronic exposure *in vivo*, it appears unlikely that an increase in CRABP can account for the rapid induction of RA catabolism in cultured cells. Cornic *et al.* (25) have demonstrated an increased content of CRABP in bone marrow mononuclear cells of patients with acute promyelocytic leukemia on long-term therapy with RA; however, they observed no change in CRABP content of cells in culture exposed to RA for a 6-day period, a time period in which induction of accelerated oxidation of RA is regularly observed.

It is reasonable to propose that increases in both CRABP and cellular lipid hydroxides could contribute to cellular modulation of RA metabolism. CRABP could serve as a cellular sponge for RA and then a catabolic delivery system that would be very useful at the extremely low concentrations of RA generated endogenously. While at the pharmacological tissue concentrations produced by exogenous administration of RA, the LOOH-mediated oxidation mechanism could play an increasingly important role.

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