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Mechanisms of Free-Radical Induction in Relation to Fenretinide-Induced Apoptosis of Neuroblastoma

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Abstract The mechanisms of fenretinide-induced cell death of neuroblastoma cells are complex, involving signaling pathways mediated by free radicals or reactive oxygen species (ROS). The aim of this study was to identify mechanisms generating ROS and apoptosis of neuroblastoma cells in response to fenretinide. Fenretinide-induced ROS or apoptosis of SH-SY5Y or HTLA 230 neuroblastoma cells were not blocked by Nitro L-argenine methyl ester (L-NAME), an inhibitor of nitric oxide synthase. Flavoprotein-dependent superoxide-producing enzymes such as NADPH oxidase were also not involved in fenretinide-induced apoptosis or ROS generation. Similarly, ketoconazole, a cytochrome P450 inhibitor, and inhibitors of cyclooxygenase (COX) were also ineffective. In contrast, inhibition of phospholipase A_2 or lipoxygenases (LOX) blocked the induction of ROS and apoptosis in response to fenretinide. Using specific inhibitors of LOX, blocking 12-LOX but not 5- or 15-LOX inhibited both fenretinide-induced ROS and apoptosis. The effects of eicosatriynoic acid, a specific 12-LOX inhibitor, were reversed by the addition of the 12-LOX products, 12 (*S*)-hydroperoxyeicosatetraenoic acid and 12 (*S*)-hydroxyeicosatetraenoic acid. The targeting of 12-LOX in neuroblastoma cells may thus be a novel pathway for the development of drugs inducing apoptosis of neuroblastoma with improved tumor specificity. J. Cell. Biochem. 89: 698–708, 2003.

Key words: lipoxygenase; fenretinide; neuroblastoma; free radicals; apoptosis

Retinoic acid induces neuroblastoma cells to differentiate [Sidell et al., 1983]. In contrast, fenretinide, a synthetic derivative of retinoic acid in which the carboxyl end has been modified by the addition of an *N*-4-(hydroxyphenyl) group, induces apoptosis of neuroblastoma cells rather than differentiation [Ponzoni et al., 1995]. The inhibition of apoptosis by retinoic acid receptor (RAR) antagonists and antioxidants suggests that signaling pathways involving RARs and reactive oxygen species (ROS) are both required for the fenretinide-induced apoptosis of neuroblastoma cells [Lovat et al., 2000a], with the latter also involving a ROS-dependent induction of the stress-response transcription factor GADD153 [Lovat et al., 2002]. Recent studies have also suggested that a p53-independent pathway of fenretinide-induced apoptosis of neuroblastoma may operate through increased intracellular levels of the lipid secondary-messenger ceramide [Maurer et al., 1999, 2000]. Since fenretinide synergizes with chemotherapeutic drugs to induce apoptosis in vitro [Lovat et al., 2000b], defining the mechanism of free radical

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induction by fenretinide will be important in the therapeutic application of fenretinide or the search for other compounds that synergize with conventional chemotherapeutic drugs.

Intracellular ROS in neuroblastoma cells resulting from fenretinide treatment accumulate rapidly within 2 h after treatment, peaking at 6 h [Lovat et al., 2000a]. Fenretinide itself may have antioxidant properties [Takahashi, 2000], so the increase in ROS must result from disturbances in cellular metabolic pathways. A number of candidate enzymes and biochemical pathways are known to be important in generating ROS leading to apoptosis in various cell types (Fig. 1). Nitric oxide (NO), produced by nitric oxide synthase (NOS), is a powerful inducer of apoptosis in some cell types, generating peroxynitrite radicals and shifting cells to a more oxidative state. Flavoprotein-dependent superoxide-producing enzymes such as NADPH oxidase are implicated in ROS generation in response to drugs such as tamoxifen [Lee et al., 2000] and growth factors such as the ROS induced by NGF in PC12 phaeochromocytoma cells [Suzukawa et al., 2000]. Since NADPH oxidase can also contribute to neuronal apoptosis [Tammariello et al., 2000], this enzyme could be important in fenretinide-induced ROS in neuroblastoma cells. Cytochrome P450 enzymes are involved in many intracellular metabolic processes, and the induction of cytochrome P450 activity can result in increased levels of ROS [Cross and Jones, 1991]. For example, cytochrome P450 mediates ROS generation in rat hepatocytes, the resulting oxidative stress leading to apoptosis [Shiba and Shimamoto, 1999; Ferrara et al., 2001]. The release and metabolism of unsaturated fatty acids such as arachidonic acid (AA) could also play an important role in ROS generation. AA released from cell membranes can affect cell proliferation and induce apoptosis directly [Surette et al., 1999] or via activation of NADPH oxidase [Brash, 2001]. Subsequent metabolism of AA or other fatty acids by the lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 enzymes of the prostaglandin, thromboxane, leukotriene, and eicosatetraenoid synthesis pathways may generate ROS resulting in membrane lipid peroxidation, ER stress [Van Leyen et al., 1998] and apoptosis [Maccarrone et al., 1997]. The aim of this study was to identify cellular pathways generating ROS in neuroblastoma cells in response to fenretinide. Using a range of enzyme inhibitors, we present evidence that the primary source of ROS in response to fenretinide in neuroblastoma cells stems from 12-LOX activity.

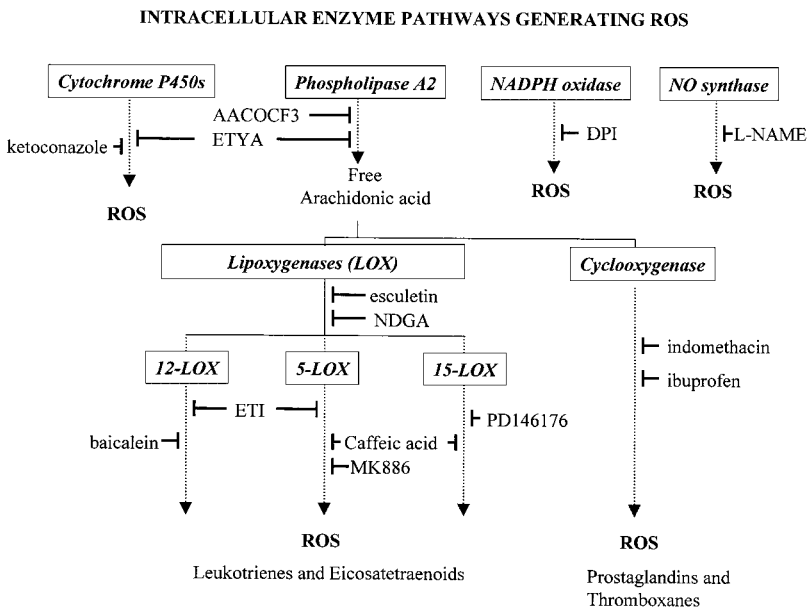


Fig. 1. Intracellular enzyme systems generating reactive oxygen species (ROS). The relevant enzymes are given in italics (boxed), and inhibitors of these enzymes are indicated alongside the arrows indicating the products of each pathway.

MATERIALS AND METHODS

Growth of Human Neuroblastoma Cell Lines and Treatment With Fenretinide and Free Radical Inhibitors

The human neuroblastoma cell lines, SH-SY5Y (without amplification of *N*-myc [Biedler et al., 1973]) and HTLA 230 (*N*-myc amplified [Matsushima and Bogenmann, 1992]) were grown at 37°C in Dulbecco's modified Eagle's medium (Life Technologies Ltd., Paisley, UK), supplemented with 10% foetal bovine serum (FCS, Life Technologies Ltd.) (culture medium) and in a humidified atmosphere of 5% CO_2 in air. For all experiments, 2×10^6 cells were seeded into 25 cm^2 tissue culture flasks (Costar, Cambridge, UK) in 5 ml of culture medium and allowed to attach overnight before treatment. For experiments with 12 (*S*)-hydroxyeicosatetraenoic acid (12-HETE) and 12 (*S*)-hydroperoxyeicosatetraenoic acid (12-HPETE) (both Sigma Chemical Co., Poole, UK), 2×10^6 cells were seeded into 25 cm^2 tissue culture flasks, allowed to attach overnight and treated with inhibitors for 2 h, before washout and subsequent incubation in serum free medium with inhibitors for a further 2 h prior to addition of fenretinide ±12-HETE or 12 HPETE for 22 h. Two hours after addition of fenretinide ±12-HETE or 12 HPETE, 10% FCS was added back into cultures until the time of harvest.

Fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) was added to cultures in ethanol and an equal volume of ethanol (<0.1% of culture volume) was used to treat control cells. Inhibitors of ROS-generating enzymes, obtained from Calbiochem (La Jolla, CA) unless stated otherwise, were added in DMSO (diaphenylene iodonium (DPI), esculetin, and ketoconazole) or ethanol to specifically inhibit the target pathway at the following concentrations for 2 h prior to 22 h treatment with fenretinide in the presence of inhibitor: Nitro L-argenine methyl ester (L-NAME, Sigma Chemical Co.), 400 μM; AACOCF3, 10 μM; diaphenylene iodonium (DPI), 10 μM; indomethacin, 40 μM; ibuprofen (Sigma Chemical Co.), 10 μM; nordihydroguairetic acid (NDGA), 50 μM; esculetin (Sigma Chemical Co.), 5 μM; 5,8,11,14-eicosatetraynoic acid (ETYA), 30 μM; ketoconazole (Sigma Chemical Co.), 5 μM with SH-SY5Y cells and at 2 μM with HTLA 230 cells; MK886, 1 μM; caffeic acid, 10 μM; PD146176 (Parke-Davis Pharmaceutical Research, MI), 0.3 μM with SH-SY5Y cells and 0.1 μM with HTLA 230 cells; baicalein, 1 μM; 5, 8, 11-eicosatriynoic acid (ETI), 40 μM with SH-SY5Y cells and 50 μM with HTLA 230 cells. An equivalent amount of diluent was added to control cells. The ROS-generating pathways inhibited by these reagents are shown in Figure 1.

Evaluation of Free-Radical Generation and Apoptosis

Free radical generation in SH-SY5Y cells was detected by staining with dihydrodichlorofluorescein diacetate [Possel et al., 1997] and evaluated by flow cytometry as previously described [Lovat et al., 2000a,b]. We have previously characterized fenretinide-induced apoptosis in neuroblastoma cells using a number of techniques [Lovat et al., 2000a]; in the present study, apoptosis in SH-SY5Y and HTLA 230 cells was evaluated by flow cytometry of propidium iodide-stained cells as previously described [Lovat et al., 2000a,b].

Western Blotting

Western blotting was performed as previously described [Lovat et al., 2002]. All primary antibodies were diluted 1:2,000 in blocking solution (10% w/v non-fat milk powder, 5% w/v bovine serum albumin, 0.1% v/v Tween 20 in PBS) and incubated with the blots for 2 h at room temperature. The rabbit polyclonal 5-LOX and leukocyte12-LOX antibodies were from Alexis Corporation (Nottingham, UK, Cat. No. 160402 and ALX-210-717, respectively); although the 12-LOX anibody was raised to murine leukocyte 12-LOX, this is reported to cross react with human 12-LOX (manufacturers data sheet). The sheep 15-LOX antibody and 15-LOX protein standard were gifts from Dr. Joe Cornicelli (Parke-Davis Pharmaceutical Research). Appropriate secondary antibodies were diluted 1:3,000 in blocking solution and incubated for 1 h at room temperature before detection by chemiluminescence [Lovat et al., 2002].

RESULTS

Fenretinide-Induced ROS and Apoptosis of SH-SY5Y Cells are Not Blocked by Inhibitors of Nitric Oxide Synthase

To determine whether fenretinide-induced ROS and apoptosis result from NOS activity, cells were treated with 3 μM fenretinide in the presence or absence of L-NAME, a NOS inhibitor

[Maccarrone et al., 1998]. This inhibitor did not block ROS or apoptosis induced by fenretinide in SH-SY5Y cells (Fig. 2), suggesting that NOS does not participate in ROS generation or apoptosis induced by fenretinide in SH-SY5Y neuroblastoma cells.

The Role of NADPH Oxidase in Fenretinide-Induced Apoptosis

NADPH oxidase and other flavoprotein-dependent superoxide-producing enzymes are inhibited by DPI [Cross, 1990]. To test for a role of NADPH oxidase and other DPI-inhibitable enzymes in fenretinide-induced apoptosis and ROS generation, SH-SY5Y cells were treated with DPI prior to incubation with fenretinide. As with the inhibitors of NOS, DPI was unable to block fenretinide-induced ROS or apoptosis in SH-SY5Y cells (Figs. 3 and 4). Similar results with respect to apoptosis were obtained with HTLA 230 cells (Fig. 5). These data imply that flavoprotein-dependent superoxide-producing enzymes such as NADPH oxidase are not involved in fenretinide-induced apoptosis or ROS generation in neuroblastoma cells.

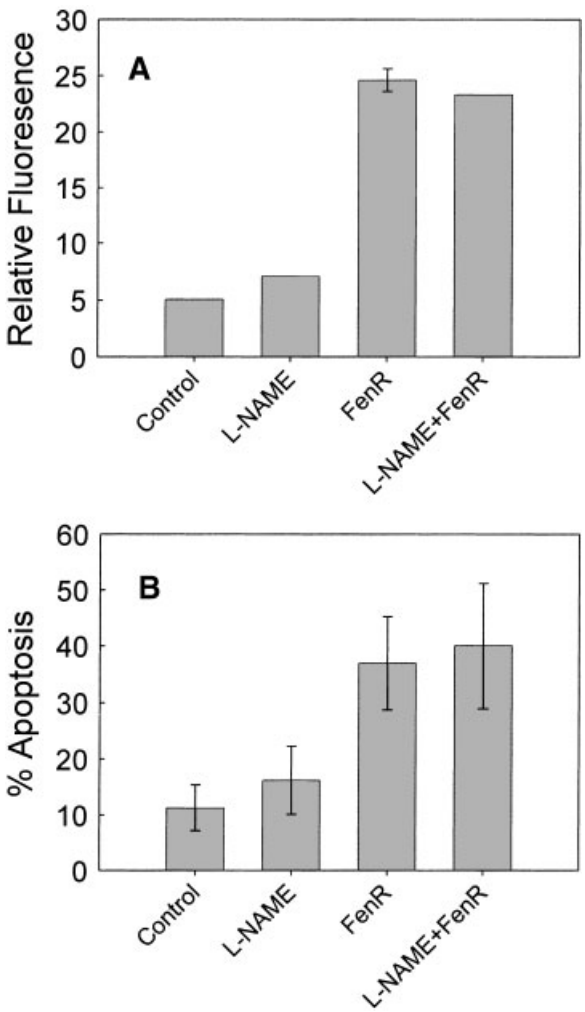


Fig. 2. Lack of inhibition of fenretinide-induced apoptosis or ROS by inhibitors of nitric oxide synthase. DCFDA fluorescence (**A**, arbitrary units) or % apoptosis (**B**) of SH-SY5Y cells treated for 24 h in the presence of vehicle control (ethanol), 400 μM nitro L-argenine methyl ester (L-NAME), 3 μM fenretinide (FenR) or pretreated for 2 h with 400 μM L-NAME followed by the addition of fenretinide to 3 μM and culture for a further 22 h (L-NAME + FenR). Each bar is the mean ± SD.

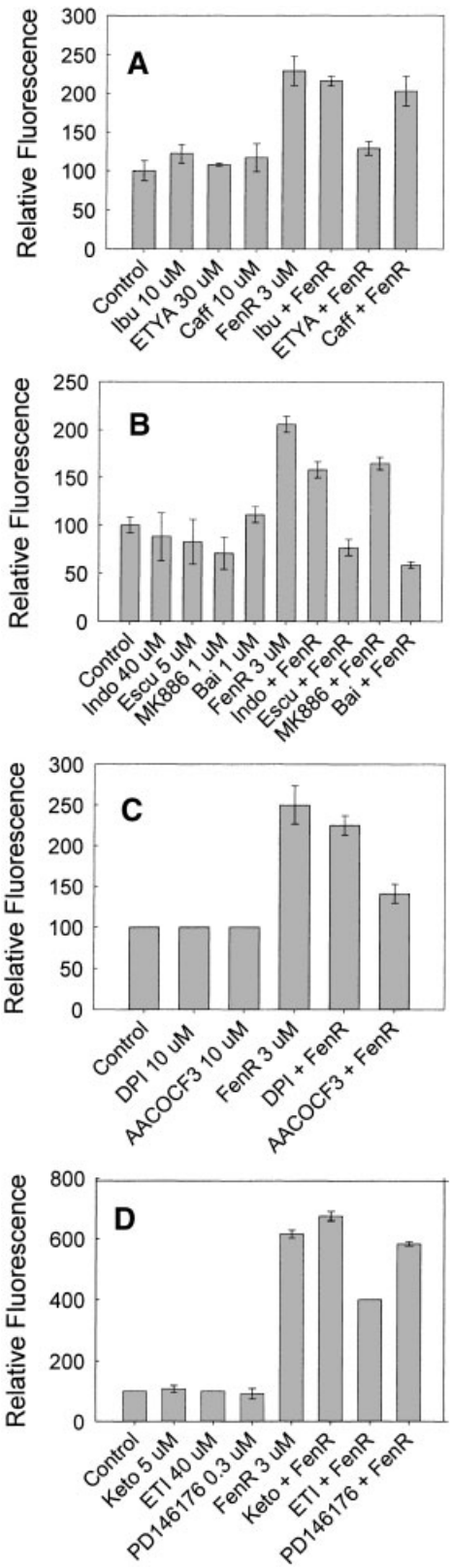
No Evidence for Cytochrome P450 Enzymes as Meditators of Fenretinide-Induced ROS

Ketoconazole, a general inhibitor of cytochrome P450 enzymes [Rizzo et al., 1999], was used to investigate the role of cytochrome P450s in fenretinde-induced ROS and apoptosis of neuroblastoma cells. Ketoconazole was unable to block fenretinide-induced ROS (Fig. 2) in SH-SY5Y cells or fenretinide-induced apoptosis of either SH-SY5Y (Fig. 3) or HTLA 230 cells (Fig. 5). These results suggest that cytochrome P450 is not involved in either fenretinide-induced ROS or apoptosis of neuroblastoma cells.

LOXs Mediate Fenretinide-Induced ROS and Apoptosis of Neuroblastoma Cells

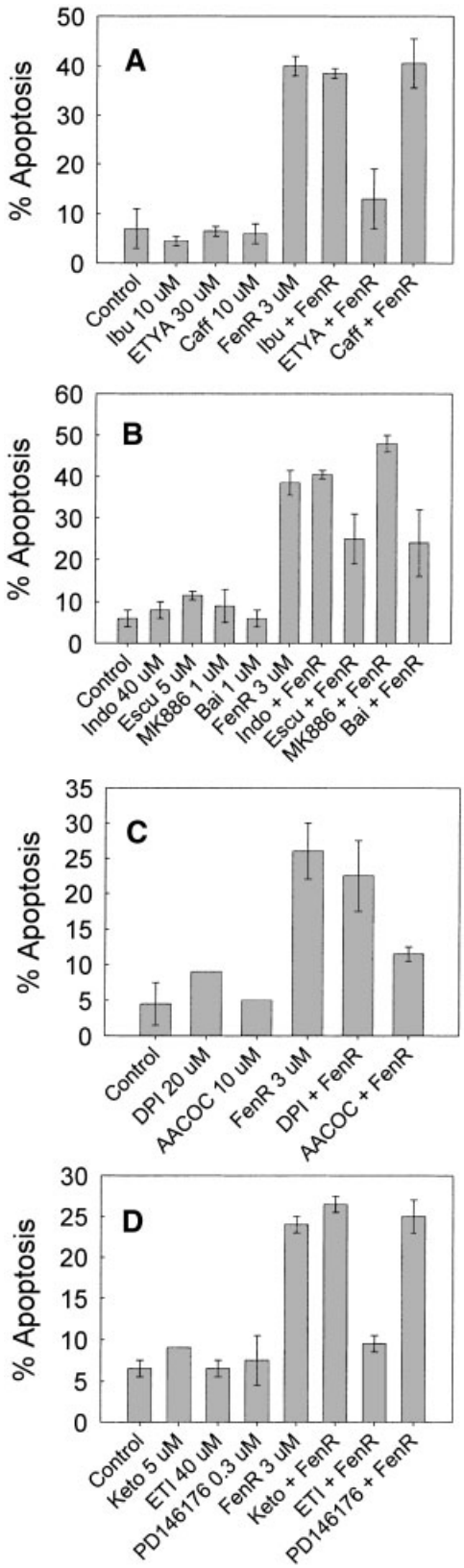
The potential involvement of AA in fenretinide-induced ROS and apoptosis was investigated using AACOCF3, a selective inhibitor of PLA_2 [Kuwata et al., 1998]. The treatment of SH-SY5Y and HTLA 230 cells with AACOCF3 blocked fenretinide-induced ROS in SH-SY5Y cells (Fig. 3) and also fenretinide-induced apoptosis of both SH-SY5Y (Fig. 4) and HTLA 230 cells (Fig. 5). These results imply that the release of free AA is necessary for the apoptotic effects of fenretinide on neuroblastoma cells. Indomethacin [Chen et al., 1998] and ibuprofen [Fadwa et al., 2000] were used as inhibitors to investigate the role of COX in fenretinide activity. Although indomethacin demonstrates cross reactivity with PLA_2 [Chen et al., 1998], at the concentrations used here, inhibition is relatively specific for COX. Both COX inhibitors had no effect on fenretinide-induced ROS in SH-SY5Y cells, or apoptosis in SH-SY5Y or HTLA 230 cells (Figs. 3–5).

Fenretinide-induced ROS are inhibited by the antioxidant, vitamin E [Lovat et al., 2000a],



implying that fenretinide-induced ROS may be due to lipid hydroperoxides. To investigate the role of LOX enzymes in mediating the effects of fenretinide, we used a range of LOX inhibitors, at appropriate concentrations to prevent cross specificity, to treat SH-SY5Y and HTLA 230 cells 2 h prior to addition of fenretinide. Two pan-LOX inhibitors, esculetin, which blocks activity by binding to LOX [Moore et al., 1991], and ETYA, which blocks LOX activity by competing with AA [Takami et al., 2000], did not induce ROS or apoptosis on their own but were effective in blocking fenretinide-induced ROS and apoptosis of SY-SY5Y cells (Figs. 3–4). Similar results with respect to apoptosis were obtained with HTLA 230 cells (Fig. 5). The pan-LOX inhibitor NDGA also effectively blocked fenretinide-induced free radicals in SH-SY5Y cells (DCFDA fluorescence, arbitrary units $\pm$ SD, $n = 3$: control, 5.17 ± 0.06; 50 μM NDGA, 13.02 ± 1.57; 10 μM fenretinide, 34.5 ± 2.84; 50 μM NDGA + 10 μM fenretinide, 9.4 ± 0.63); however, it was not possible to measure an effect of NDGA on fenretinide-induced apoptosis as this agent produced high levels of cell death alone (data not shown). Apoptosis of SH-SY5Y and HTLA 230 cells, and the induction of ROS in SH-SY5Y cells was also blocked by baicalein, a specific inhibitor of 12-LOX [Huang et al., 1994], and by ETI which inhibits 5- and 12-LOX at an IC_{50} of 20 μM [Takami et al., 2000] (Figs. 3–5). Conversely, MK886 an inhibitor of 5-LOX [Ford-Hutchinson et al., 1994], and PD146176, a highly specific inhibitor of 15-LOX [Sendobry et al., 1997], did not block the induction of ROS and apoptosis in response to fenretinide in SH-SY5Y cells, or fenretinide-induced apoptosis in HTLA 230 cells (Figs. 3–5). The same result was obtained with caffeic acid, a 5-LOX

Fig. 3. Inhibition of fenretinide induced ROS in SH-SY5Y cells by inhibitors of PLA_2, NADPH oxidase, cytochrome P450, cyclooxygenase (COX), or lipoxygenase (LOX). In each of **A**, **B**, **C**, **D**, DCFDA fluorescence (arbitrary units) is indicated for SH-SY5Y cells treated with ROS inhibitors or 3 μM fenretinide (FenR) alone for 24 h or after pre-treatment with inhibitors for 2 h with subsequent addition of fenretinide (to 3 μM) for 22 h. A: results for treatment with ethanol vehicle control (control), ibuprofen (Ibu, 10 μM), ETYA (30 μM), caffeic acid (caff, 10 μM) (B), DMSO vehicle control (control), indomethacin (Indo, 40 μM), esculetin (Escu, 5 μM), MK886 (1 μM), baicalein (Bai, 1 μM), (C) DMSO vehicle control (control), diaphenylene iodonium (DPI, 10 μM), AACOCF3 (10 μM). (D) ethanol vehicle control (control), ketoconazole (keto, 5 μM), ETI (40 μM), PD146176 (0.3 μM). Each bar is the mean ± range of duplicate treatments.



inhibitor [Sud'ina et al., 1993] which may also have activity against 15-LOX [Shureiqi et al., 2000a,b]. These results suggest that 12-LOX is the mediator of ROS and apoptosis signaling in response to fenretinide in SH-SY5Y and HTLA 230 neuroblastoma cells. To ask whether levels of LOX increased in response to fenretinide, extracts from SH-SY5Y cells treated with 3 μM fenretinide were analyzed by Western blotting. 15-LOX was neither expressed nor induced in these cells (Fig. 6A). The 12- and 5-LOX antibodies both cross-reacted with the 15-LOX protein standard, and with proteins of similar size in SH-SY5Y cells; although there was an apparent increase in 5-LOX in response to fenretinide, there was no evidence for an increase in protein cross-reacting with the 12-LOX antibody in response to fenretinide (Fig. 6A).

Inhibition of Fenretinide-Induced Apoptosis by ETI is Reversed by the Addition of 12-LOX Products

SH-SY5Y cells were treated for 2 h with ETI before washout and incubation in serum-free medium in the presence of this inhibitor for a further 2 h prior to treatment with either 12-HETE or 12-HPETE in the presence or absence of fenretinide. The effects of ETI on fenretinide-induced apoptosis or ROS were irreversible and the same results were obtained whether cells were treated briefly (2 h) with the inhibitors or cultured in their continued presence (data not shown). Although 12-HETE or 12-HPETE did not induce apoptosis on their own when added to SH-SY5Y cells, the addition of these eicosatetraenoids to cells pre-treated with ETI in the presence of fenretinide restored the level of apoptosis to that obtained with cells treated with

Fig. 4. Inhibition of fenretinide-induced apoptosis of SH-SY5Y cells by inhibitors of PLA_2, NADPH oxidase, cytochrome P450, COX, and LOX. In each of **A**, **B**, **C**, **D**, % apoptosis is indicated for SH-SY5Y cells treated with ROS inhibitors or 3 μM fenretinide (FenR) alone for 24 h or after pretreatment with inhibitors for 2 h with subsequent addition of fenretinide (to 3 μM) for 22 h. A: results for treatment with ethanol vehicle control (control), ibuprofen (Ibu, 10 μM), ETYA (30 μM), caffeic acid (caff, 10 μM); (B), DMSO vehicle control (control), indomethacin (Indo, 40 μM), esculetin (Escu, 5 μM), MK886 (1 μM), baicalein (Bai, 1 μM); (C) DMSO vehicle control (control), diaphenylene iodonium (DPI, 10 μM), AACOCF3 (10 μM); (D) ethanol vehicle control (control), ketoconazole (keto, 5 μM), ETI (40 μM), PD146176 (0.3 μM). Each bar is the mean ± range of duplicate treatments.

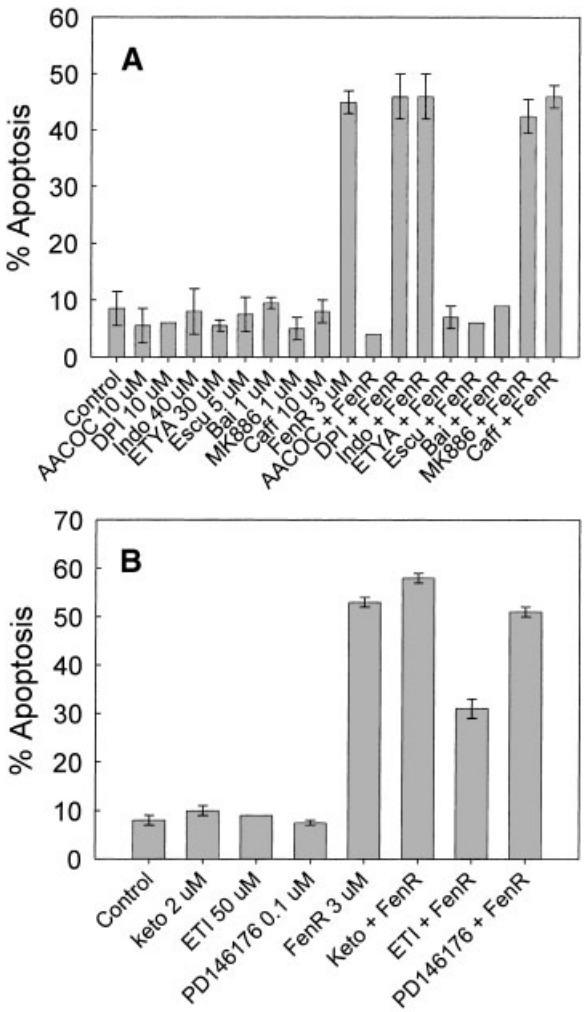


Fig. 5. Inhibition of fenretinide-induced apoptosis of HTLA 230 cells by inhibitors of PLA_2, NADPH oxidase, cytochrome P450, COX, and LOX. In each of **A** and **B**, % apoptosis is indicated for HTLA 230 cells treated with ROS inhibitors or 3 μM fenretinide (FenR) alone for 24 h or after pretreatment with inhibitors for 2 h with subsequent addition of fenretinide (to 3 μM) for 22 h. A: results for treatment with ethanol vehicle control (control), AACOCF3 (AACOC, 10 μM), diaphenylene iodonium (DPI, 10 μM), indomethacin (Indo, 40 μM), ETYA (30 μM), esculetin (Escu, 5 μM), baicalein (Bai, 1 μM), MK886 (1 μM), caffeic acid (caff, 10 μM); (B), DMSO vehicle control (control), ketoconazole (keto, 2 μM), ETI (50 μM), PD146176 (0.1 μM). Each bar is the mean ± range of duplicate treatments.

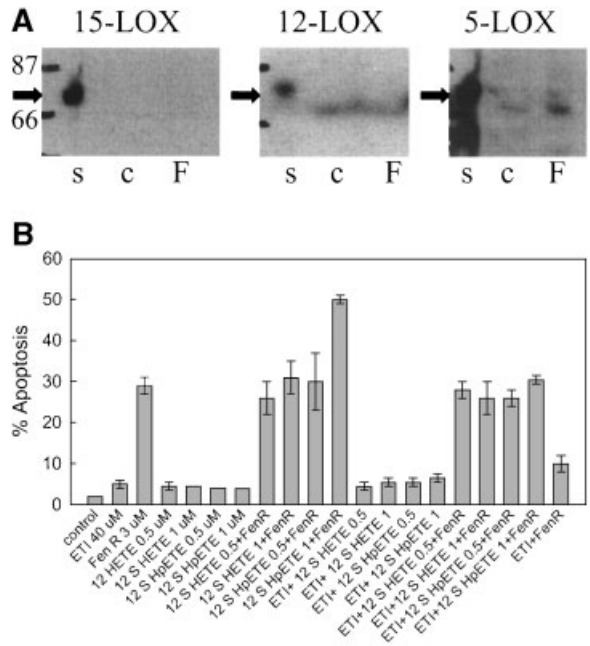


Fig. 6. **A**: Western blot probed with antibodies to 15-, 12-, and 5-LOX. **Lanes**: s, standard 15-LOX protein; c, 30 μg of lysate from SH-SY5Y cells treated with vehicle control (ethanol); F, 30 μg of lysate from SH-SY5Y cells treated with 3 μM fenretinide for 22 h. The arrows mark the position of the 15-LOX protein standard, which was detected by all antibodies. Molecular weights in kilodaltons are indicated on the left of the figure. **B**: reversibility of ETI inhibition of fenretinide-induced apoptosis of SH-SY5Y cells by 12-HETE and 12 (*S*) HPETE. % apoptosis after treatment with ETI (40 μM) alone for 24 h or treatment with fenretinide (Fen R, 3 μM) alone for 22 h, 12 (*S*) HETE or 12-HPETE (0.5 or 1 μM) alone for 22 h, or with 12 (*S*) HETE or 12-HPETE (0.5 or 1 μM) + Fen R (3 μM) together for 22 h. The % apoptosis is also shown for pre-treatment with ETI (B) for 4 h prior to addition of 12 (*S*) HETE or 12-HPETE (0.5 or 1 μM) ± Fen R (3 μM) for 22 h or with just the addition of Fen R (3 μM) for 22 h. Each bar is the mean ± range of duplicate treatments.

fenretinide in the absense of ETI (Fig. 6B). These results provide further evidence for the involvement of 12-LOX in fenretinide-induced ROS and apoptosis of neuroblastoma.

DISCUSSION

This study suggests that of the enzyme systems frequently implicated in ROS generation, only PLA_2 and 12-LOX were apparently involved in ROS generation and apoptosis in response to fenretinide in both SH-SY5Y and HTLA 230 neuroblastoma cells. PLA_2 activity releases AA from membrane phospholipids; AA released in this way acts as a substrate for 12-LOX which catalyses the stereospecific oxygenation of AA to form 12-HPETE and 12-HETE [Nie et al., 2000]. The production of 12-HPETE by 12-LOX may lead to the production of ROS via glutathione peroxidase. AA has been shown to be part of the signal transduction pathway of IFN γ-induced differentiation and apoptosis of neuroblastoma [Ponzoni and Cornaglia-Ferraris, 1993], and is also clearly necessary for fenretinide-induced apoptosis of these cells. Fenretinide increases LOX activity in SH-SY5Y cells

without increasing free AA levels [Lovat et al., 2002]. Since levels of protein cross-reacting with the 12-LOX antibody did not increase in response to fenretinide, it is possible that fenretinide mediates apoptosis in SH-SY5Y and HTLA 230 neuroblastoma cells by increasing 12-LOX activity.

Individually, different LOX enzymes have been implicated in modulating apoptosis in several cellular systems, and the inhibition of LOX activity is often associated with the promotion of apoptosis. For example, inhibition of 5- and 12-LOX triggers apoptosis in human prostate cancer [Ghosh and Myers, 1998; Pidgeon et al., 2002], and inhibition of 12-LOX induces apoptosis of gastric cancer cells [Wong et al., 2001]. In contrast, increased15-LOX expression mediates drug-induced apoptosis in colorectal cancer [Shureiqi et al., 2000a,b]. Clearly, the role of LOX enzymes in carcinogenesis and tumor biology may vary according to cell type and it has been suggested that LOX pathways may exist in a dynamic balance of procarcinogenic (5- and 12-LOX) and anticarcinogenic (15-LOX) forms [Shureiqi and Lippman, 2001]. The apoptosis induced by specific LOX inhibitors in some cell types could also result from shifts in AA metabolism to other LOX enzymes, generating pro-apoptotic AA metabolites such as products of the 15-LOX pathway [Avis et al., 2001].

Nevertheless, in CHP100 neuroepithelioma cells, hydroperoxides generated from 5-, 12-, and 15-LOX all induce apoptosis and cellular LOX activity clearly has a pro-apoptotic effect [Maccarrone et al., 2000, 2001]. There is increasing evidence to suggest that 12-LOX is a key enzyme of AA metabolism in brain and neuronal cells [Palluy et al., 1994], and is involved in aspects of synaptic transmission as well as nerve cell death [Li et al., 1997; Christie et al., 2000]. Since activation of 12-LOX also leads to apoptosis in fibroblasts [Gu et al., 2001], the response to changes in 12-LOX activity varies according to cell type. Although the effects of 12-LOX inhibition in neuroblastoma cells could be reversed by addition of 12-LOX products to the cells, restoring the apoptotic effects of fenretinide, 12-HETE, and 12-HPETE did not induce apoptosis on their own. Evidence from other studies suggests that RAR activation via a conventional retinoid activity of fenretinide is also required in order for fenretinide to induce apoptosis [Lovat et al., 2000a]. The nature of this RAR-dependent activity is unknown. However, LOX products can be potent ligands for peroxisome proliferator-activated receptors (PPARs) [Murakami et al., 1999; Shappell et al., 2001], and, since these work as heterodimers with RXRs, as do RARs, it is possible to speculate that interactions between RXR-dependent signaling pathways is a potential mechanism underlying the requirement [Lovat et al., 2000a] for fenretinide to have RAR-dependent and ROS-dependent activities in order to induce apoptosis of neuroblastoma cells.

With respect to the ROS-dependent pathway of fenretinide action in neuroblastoma cells, other studies have shown that ceramide levels increase in response to treatment with high (10 μM) doses of fenretinide [Maurer et al., 1999, 2000]. Since both AA and ROS can induce ceramide production [Andrieu-Abadie et al., 2001; Chen et al., 2001], the PLA_2/12-LOX pathway may be an upstream event in fenretinide-induced apoptosis of neuroblastoma cells with ceramide activation as a consequence of increased ROS or AA levels. Alternatively, ceramide accumulated in response to fenretinide may contribute to apoptosis by conversion to ganglioside GD3 [De Maria et al., 1997; Rippo et al., 2000], which may stimulate 12-LOX activity [Bezuglov et al., 1991], recruit ROS by other mechanisms [Bhunia et al., 2002], and/or directly participate in cytochrome c release from mitochondria. Further studies are clearly required to elucidate relationships between ceramide, AA signaling pathways, and fenretinide-induced apoptosis.

Downstream signaling events of fenretinide-induced apoptosis involve mitochondria, and in neuroblastoma cell lines, the effector pathway of fenretinide-induced apoptosis is caspase-dependent, involving the mitochondrial release of cytochrome c independently of changes in the mitochondrial permeability transition [Lovat et al., 2000a]. Since cytochrome c may catalyze the ability of 12-LOX to metabolize linoleic acid, increasing hydroperoxide production [Iwase et al., 2000], cytochrome c release may be an important factor increasing cellular stress in addition to its function in activating caspases. In this context, a LOX-mediated alteration of membrane fluidity and permeability has been shown to be a basis for the dissipation of the membrane potential and increased cytochrome c release from isolated mitochondria [Maccarrone et al., 2001].

LOX pathways play an important role in growth-related signal transduction and may be important targets for the development of new drugs to arrest cancer progression [Cuendet and Pezzuto, 2000; Shureiqi and Lippman, 2001]. The results of this study suggest that 12-LOX in neuroblastoma cells may act as a new target for neuroblastoma therapy, in which drugs that activate 12-LOX or increase AA levels may be used in combination with retinoids to induce apoptosis. Such an approach may have greater tumor specificity than the chemotherapeutic agents currently in clinical use and hence the design of new compounds targeting components of the AA cascade may be of substantial benefit for the treatment of neuroblastoma in the future.

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