

GADD153 and 12-Lipoxygenase Mediate Fenretinide-induced Apoptosis of Neuroblastoma¹

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ABSTRACT

The synthetic retinoid fenretinide induces apoptosis of neuroblastoma cells and *in vitro* acts synergistically with chemotherapeutic drugs used to treat neuroblastoma. The mechanisms of fenretinide-induced cell death of neuroblastoma cells are complex, involving cellular signaling pathways as yet incompletely defined but, in part, involving the generation of reactive oxygen species (ROS). In an attempt to characterize the mechanism of action of fenretinide, cDNA array filters were screened to identify apoptotic genes regulated in response to treatment of SH-SY5Y cells with fenretinide. Expression of the stress-induced transcription factor, GADD153, was up-regulated at both the protein and mRNA levels in response to fenretinide. Overexpression of GADD153 increased apoptosis in the presence and absence of fenretinide, whereas reduced expression of GADD153 by expression of antisense DNA abrogated the response to fenretinide. Although fenretinide is a partial retinoic acid receptor (RAR)- β/γ agonist, RAR β/γ antagonists did not block the induction of GADD153 by fenretinide; conversely, the induction of GADD153 was blocked by antioxidants. Enzyme inhibitors were used to identify pathways mediating the ROS-dependent effects of fenretinide: inhibitors of phospholipase A₂ and lipoxygenases (LOX), and specific inhibitors of 12-LOX, but not 5-LOX or 15-LOX, inhibited the induction of ROS, apoptosis, and GADD153 in response to fenretinide. The inhibition of ROS and apoptosis was reversed by the addition of the 12-LOX products, 12 (S)-hydroperoxyeicosatetraenoic acid (12-HpETE) and 12 (S)-hydroxyeicosatetraenoic acid (12-HETE). Fenretinide did not increase free arachidonic acid levels, but increased LOX activity without a detectable increase in 12-LOX protein. These results suggest that fenretinide induces apoptosis via RAR-dependent and -independent pathways in which the RAR-independent pathway is characterized by a fenretinide-dependent increase in 12-LOX activity, leading to the induction of GADD153. The targeting of 12-LOX and/or GADD153 in neuroblastoma cells may thus present a novel pathway for the development of drugs inducing apoptosis of neuroblastoma with improved tumor specificity.

INTRODUCTION

Neuroblastoma is a common extra-cranial tumor of childhood, responsible for 15% of all pediatric deaths from malignancy. Although an aggressive tumor, biologically most tumors show some form of differentiation that in a small group of patients can result in spontaneous regression (1). Retinoic acid has long been known to induce differentiation of neuroblastoma *in vitro*, and the observation

that 13-*cis* retinoic acid increases event-free survival when used to treat children with residual disease after chemotherapy and bone marrow transplantation (2) has led to the inclusion of retinoids in most treatment regimes. However, it has been reported that retinoic acid-induced differentiation of neuroblastoma cells may render them resistant to chemotherapy (3). Synthetic derivatives of retinoic acid such as fenretinide are more effective than 13-*cis* retinoic acid in that fenretinide is able to directly induce apoptosis of neuroblastoma *in vitro* (4–6), and this may overcome the problem of retinoic acid-induced differentiation increasing the resistance to chemotherapeutic drugs. In addition, the fact that fenretinide is synergistic with cisplatin, etoposide, or carboplatin (7) in the induction of apoptosis of neuroblastoma cells suggests that this retinoid may be a valuable adjunct to retinoid therapy for neuroblastoma.

The mechanisms of fenretinide-induced cell death of neuroblastoma cells are complex and probably involve several overlapping pathways. Fenretinide-induced apoptosis of neuroblastoma cells has been suggested to involve RARs³ (6). However, oxidative stress via the induction of ROS is also involved in mediating the fenretinide-induced apoptosis of neuroblastoma (7, 8) and prostate cancer cells (9, 10). Induction of apoptosis of other cancer cells by chemotherapeutic drugs results from DNA damage leading to cell death, probably as a result of increased p53 activity (11, 12), and, therefore, the synergistic induction of apoptosis between cisplatin, etoposide, and carboplatin and fenretinide in the induction of apoptosis of neuroblastoma cells may result from the activation of different pathways of cell death (7).

In neuroblastoma cells, the inhibition of apoptosis by RAR antagonists and antioxidants suggests that signaling pathways involving RARs and ROS are both required for fenretinide-induced apoptosis (6). 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 (8, 13). Because fenretinide synergizes with chemotherapeutic drugs to induce apoptosis *in vitro* (7), defining the mechanism of apoptosis induction by fenretinide will be important in the therapeutic application of fenretinide or in the search for other compounds that synergize with conventional chemotherapeutic drugs. Therefore, the aim of this study was to identify gene(s) induced by fenretinide that might be implicated in mediating the synergistic induction of apoptosis with chemotherapeutic reagents in neuroblastoma cells, and the mechanisms and role of ROS in these processes. We show that fenretinide, unlike 13-*cis* retinoic acid, or other retinoic acid isomers, induces the expression of GADD153, also known as CHOP (CEBP homology protein), a growth arrest and DNA damage-inducible tran-

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³ The abbreviations used are: RAR, retinoic acid receptor; AA, arachidonic acid; COX, cyclooxygenase; DCFDA, dihydrodichlorofluorescein diacetate; ETI, 5,8,11-eicosatriynoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; GSH, reduced glutathione; 12-HETE, 12 (S)-hydroxyeicosatetraenoic acid; 12-HpETE, 12 (S)-hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; NOS, nitric oxide synthase; PI, propidium iodide; PLA₂, phospholipase A₂; ROS, reactive oxygen species; Tet, tetracycline; GSSG, total glutathione; NAC, N-acetylcysteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase EC 1.2.1.12.

scription factor belonging to the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (14). The data indicate that GADD153 is essential for fenretinide-dependent apoptosis in SH-SY5Y neuroblastoma cells and is induced by the induction of ROS resulting from a fenretinide-dependent increase in 12-LOX activity. The results of this study suggest that GADD153 and 12-LOX are elements of a p53-independent pathway of apoptosis in neuroblastoma cells that may provide novel therapeutic targets to achieve synergistic responses with the activation of p53-dependent pathways by chemotherapeutic drugs.

MATERIALS AND METHODS

Growth of Human Neuroblastoma Cell Lines and Treatment with Retinoids, Chemotherapeutic Reagents, Antioxidants, RAR Antagonists, and Enzyme Inhibitors. The human neuroblastoma cell lines, SH-SY5Y (these cells do not have amplified N-myc; Ref. 15), HTLA230 (N-myc amplified; Ref. 16), and SH-SY5Y cells resistant to 1, 2, or 3 μM fenretinide (SH-SY5Y-R1, -R2 and -R3, respectively; Ref. 7) were grown in a 1:1 mixture of DMEM and Ham's F12 (Life Technologies, Inc. Ltd, Paisley, United Kingdom), supplemented with 10% fetal bovine serum (Life Technologies, Inc.; culture medium) and grown in a humidified atmosphere of 5% CO_2 in air. SH-SY5Y-R cells selectively resistant to 1, 2, or 3 μM fenretinide were treated with 1, 2, or 3 μM fenretinide every 3 days to maintain resistance. For all of the experiments, cells were seeded into tissue culture flasks and allowed to attach overnight before treatment. The seeding density varied according to the type of experiment; for apoptosis experiments with chemotherapeutic drugs and fenretinide: 0.4×10^6 cells were seeded into 100-mm tissue culture-grade Petri dishes (Costar) in 10 ml of culture medium; for RNA preparation; 8×10^6 cells were seeded into 75-cm² flasks (Costar, Cambridge, United Kingdom) in 10 ml of culture medium; and for immunofluorescence experiments, 2×10^6 cells were seeded into 25-cm² flasks (Costar) in 5 ml of culture medium.

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. Stock solutions of cisplatin (100 mM, freshly prepared in DMSO), carboplatin (10 mM, freshly prepared in culture medium), and etoposide (20 mM in DMSO, stored at -20°C ; all from Sigma Chemical Co., Poole, United Kingdom) were diluted in culture medium. The antioxidant vitamin C (ascorbic acid sodium salt; Sigma) was freshly diluted in PBS and was added at a final concentration of 100 μM , 2 h prior to a 22-h treatment with fenretinide; cell cultures were washed once with PBS after the removal of vitamin C prior to the addition of fenretinide. NAC (Sigma) was also added to cultures at a final concentration of 10 mM for 2 h prior to the addition of fenretinide but without washing cells with PBS. The RAR β/γ antagonists, CD2665 and CD2848, were dissolved in DMSO and were added to cultures at final concentrations of 1 μM in the presence or absence of fenretinide for 24 h.

Inhibitors of ROS-generating enzymes were added to the cultures for 2 h prior to the 22-h treatment with fenretinide as follows: nitro-L-arginine methyl ester (L-NAME; Sigma), a NOS inhibitor (17) was used at 400 μM ; AACOCF3 (Calbiochem, La Jolla, CA) was used to inhibit PLA₂ metabolism at a final concentration of 10 μM . Diaphenylene iodonium (Calbiochem; an inhibitor of NADPH oxidase and other flavoprotein-dependent superoxide-producing enzymes; Ref. 18) was used at 10 μM . Indomethacin (Calbiochem) and ibuprofen (Sigma) were used to specifically inhibit COXs, at 40 μM and 10 μM , respectively. Although indomethacin demonstrates cross-reactivity with PLA₂, at the concentration used here, inhibition is specific for COX (18). Nordihydroguaiaretic acid (NDGA; Calbiochem), esculetin (Sigma), and ETYA (Calbiochem) were used as pan-LOX inhibitors diluted to 50, 5, and 30 μM , respectively. Ketoconazole (Sigma) was used as a general inhibitor of cytochrome P450 enzymes at a concentration of 5 μM in experiments with SH-SY5Y cells and at 2 μM with HTLA230 cells.

MK886 and caffeic acid (Ref. 19; Calbiochem) were used to inhibit 5-LOX at concentrations of 1 μM and 10 μM , respectively. MK886 inhibits 5-LOX indirectly, by inhibiting the 5-LOX-activating protein (FLAP; Ref. 20). Caffeic acid was also used to test for 15-LOX induction because this inhibitor is reported to inhibit 15-LOX with an IC₅₀ of 0.8 μM (21). A 15-LOX-specific inhibitor, PD146176 (22), was obtained from Parke-Davis Pharmaceutical

Research (Ann Arbor, MI) and used at a concentration of 0.3 μM with SH-SY5Y cells and 0.1 μM with HTLA230 cells. Baicalein (a specific inhibitor of 12-LOX) and ETI (Calbiochem) were used to inhibit 12-LOX at concentrations of 1 μM and 40 μM , respectively, in SH-SY5Y cells. For experiments with HTLA230 cells, ETI was diluted to a final concentration of 50 μM . All of the inhibitors were diluted in ethanol except diaphenylene iodonium, esculetin, and ketoconazole, which were diluted in DMSO with an equivalent amount of diluent added to control cells.

For experiments with 12-HETE and 12-HpETE (both from Sigma), 2×10^6 cells were seeded into 25-cm² 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 an additional 2 h prior to the addition of fenretinide \pm 12-HETE/12-HpETE for 22 h. Two h after the addition of fenretinide \pm 12-HETE/12-HpETE, 10% FCS was added back into cultures until cells were harvested.

Extraction of RNA, Size Fractionation, and Northern Blotting. Total cytoplasmic RNA was extracted as described previously (23). Northern blots were prepared and probed with [α -³²P]dATP-labeled cDNA probes (23); the GADD153 probe was a full-length cDNA (830-bp) from the I.M.A.G.E. consortium (Lawrence Livermore National Laboratory) expressed sequence tag clone 2262830 (24), confirmed by sequencing as comprising the complete 3' untranslated region, coding sequence and 165 bp of the 5' untranslated region. This cDNA was in the sense orientation in the pCMV-SPORT6 vector. The p21/WAF1 cDNA probe was a 593-bp insert originally generated by reverse transcription-PCR as an antisense construct in pCR3.1, and was a gift of Dr. John Lunec. The RAR β and rat GAPDH cDNA probes were as described previously (23).

Screening Atlas Human cDNA Expression Arrays. Polyadenylated RNA was prepared by treating total cytoplasmic RNA samples with RNase-free DNase I (Roche, Lewes, Sussex, United Kingdom), followed by oligo(dT)-latex-bead chromatography (Qiagen Ltd, Sussex, United Kingdom) according to the manufacturers' specifications. RNA integrity was verified by electrophoresis. [α -³²P]dATP-labeled cDNA probes were generated by reverse transcription of 1 μg of polyadenylated RNA from SH-SY5Y cells treated with fenretinide or ethanol control and hybridized to one of two identical Atlas human cDNA array filters for apoptosis-related genes (Atlas Human Apoptosis Array, 7743-1; Clontech Laboratories Inc) according to the user manual. After high-stringency washing, the hybridization pattern for each filter was visualized and quantified by phosphorimager analysis using ImageMaster software (Amersham Pharmacia Biotech).

Flow Cytometry. Apoptosis was evaluated by flow cytometry of PI-stained cells as described previously (6). Cells for immunofluorescence flow cytometry were detached by trypsinization and washed with 2 ml of PBS before fixation in 500 μl of 4% paraformaldehyde in PBS for 10 min at room temperature. After washing twice with PBS, cells were permeabilized with 500 μl of 0.5% Triton X (Sigma) in PBS for 2 min at room temperature before washing twice again with PBS before incubation for 1 h at room temperature in the presence or absence of a mouse antihuman monoclonal GADD153 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 2 $\mu\text{g}/\text{ml}$ in PBS containing 5% BSA in a volume of 200 μl . After an additional two washes in PBS, the binding of anti-GADD153 antibodies was detected by incubation for 40 min at room temperature with 10 μg of a secondary goat antimouse FITC-conjugated antibody (Molecular Probes, Leiden, the Netherlands) diluted in PBS with 5% BSA in a final volume of 200 μl . After a final wash in PBS, cells were resuspended in 300 μl of PBS and 20,000 events acquired for flow cytometry (6). ROS generation in response to fenretinide for 22 h in the presence or absence of 2-h pretreatment with inhibitors was detected by staining cells with DCFDA and was evaluated by flow cytometry as described previously (6, 7).

Western Blotting. Total protein was extracted from SH-SY5Y cells treated in the presence or absence of fenretinide for 6–48 h; 25 μg of total protein were separated by electrophoresis through 12.5% SDS-PAGE gels and blotted onto nitrocellulose (6). GADD153 was identified with the same antibody used for flow cytometry experiments, diluted 1:1000, and detected by chemiluminescence (6) using an affinity-purified goat antimouse peroxidase-conjugated IgG (Bio-Rad, Hemel Hempstead, United Kingdom). Expression of 5-LOX was detected with a rabbit anti 5-LOX antibody (Alexis Corporation Ltd, Nottingham, United Kingdom), diluted 1:2000, and expression of 12-LOX detected with a rabbit anti-12-LOX (Alexis) also diluted 1:2000. 15-LOX was

detected by a sheep anti-15-LOX antibody (kindly donated by Parke-Davis Pharmaceutical Research). For the expression of 15-LOX, results were calibrated against 15-LOX standards, a kind gift of Parke-Davis Pharmaceutical Research.

Stable Transfection of a Tet-inducible GADD153 cDNA into SH-SY5Y Neuroblastoma Cells. To prepare stably transfected clones for GADD153 sense and antisense constructs, GADD153 cDNA in pCMV-SPORT6 was digested with *EcoR*I and *Not*I (sense construct) or *EcoR*I and *Hind*III (antisense), subcloned into the Invitrogen pcDNA4/TO vector, and confirmed by sequencing. Tet-inducible expression of these constructs is dependent on expression of the Tet repressor in the recipients. Recipient cells were prepared by stable transfection of the pcDNA6/TR tet repressor plasmid (Invitrogen) into SH-SY5Y cells with LipofectAMINE and selected using blasticidin. A clone of cells, referred to as SH-SY5Y^{tet12}, were selected and grown in culture medium containing 5 μ g/ml blasticidin (Invitrogen). These cells were characterized by transient transfection with pcDNA- β -galactosidase and showed no or minimal β -galactosidase expression in the absence of Tet but marked induction in response to 1 μ g/ml Tet. Sense- and antisense-GADD153 constructs in pcDNA4/TO were transfected into SH-SY5Y^{tet12} cells using LipofectAMINE and selected with zeocin (250 μ g/ml) and blasticidin (5 μ g/ml). Antibiotic-resistant transfectants were selected and evaluated for GADD153 expression (Western blotting and flow cytometry) in response to Tet.

Incorporation and Release of Arachidonate (AA) and Assay of LOX. Incorporation and release of [³H]AA (specific activity, 98.6 Ci/mmol; from Amersham Pharmacia Biotech) was measured as reported earlier (25). SH-SY5Y cells were washed and resuspended in serum-free medium containing 1 μ Ci [³H]AA per flask in the presence or absence of 10 μ M fenretinide. After 2-h incubation at 37°C, cells were washed and resuspended in normal culture medium containing 10 μ M fenretinide or vehicle alone for an additional 22 h at 37°C. Membrane lipids were then extracted (26), and radioactivity was measured in both lipid extracts and culture media by liquid scintillation counting.

LOX activity of SH-SY5Y cells, treated with 3 μ M fenretinide and/or 1 μ M baicalein as reported above, was measured by incubating cell extracts for 10 min at 37°C in 0.1 M sodium phosphate buffer (pH 7.0), containing 40 μ M [³H]AA. The hydroperoxyeicosatetraenoic acids generated by LOX activity were then extracted on octadecyl-SPE columns (Baker, Deventer, the Netherlands) and radioactivity measured by liquid scintillation counting (Amersham Pharmacia Biotech; Ref. 27). The contribution of 5-LOX to the cellular LOX activity was ascertained by adding 1 mM ATP and 2 mM CaCl₂ to the assay buffer (20). LOX activity was expressed as pmol of hydroperoxyeicosatetraenoic acids formed/min/mg of protein.

Glutathione Assay. GSSG and GSH were measured with an assay kit using the kinetic method according to the manufacturer's specifications (Cayman Chemicals Inc.). Results were expressed as GSH content as a percentage of GSSG.

RESULTS

Identification of GADD153 as a Fenretinide-inducible Gene.

Previous evidence from RAR-specific antagonists suggests that RARs are involved in mediating the apoptotic effects of fenretinide (6). This implies that fenretinide treatment of neuroblastoma cells will result in changes in gene expression. To identify fenretinide-induced changes in gene expression, human cDNA expression arrays for apoptosis-related genes were probed with ³²P-cDNA complementary to mRNA from control SH-SY5Y cells and SH-SY5Y cells treated with 3 μ M fenretinide for 24 h (Fig. 1A). A number of genes were apparently up-regulated or down-regulated at least 5-fold in response to fenretinide (Table 1). The induction of *GADD153* was of particular interest because this gene is induced by chemotherapeutic drugs in other tumor types (28, 29) and may play a key role in drug-induced apoptosis. Therefore, we used a full-length cDNA clone for human *GADD153* to confirm the induction of *GADD153* in response to fenretinide.

Northern blots of RNA extracted from SH-SY5Y cells exposed to fenretinide at a range of doses and incubation periods were probed for

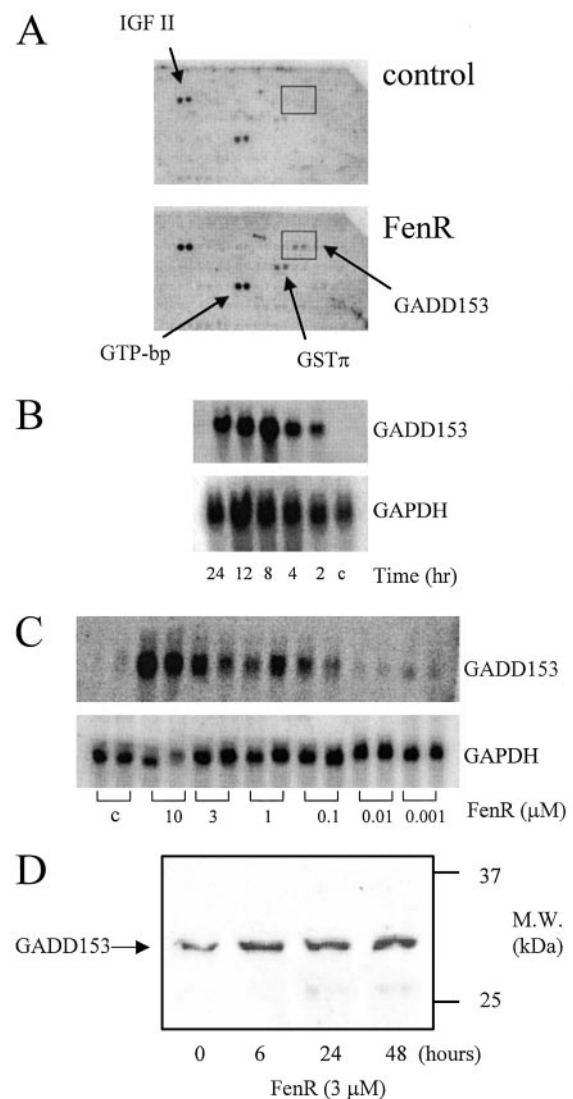


Fig. 1. Induction of GADD153 in SH-SY5Y cells in response to fenretinide. A, section of Atlas human cDNA array filters for apoptosis-related genes probed with cDNA from SH-SY5Y cells treated for 24 h with 3 μ M fenretinide (*FenR*) or ethanol control. Identified spots (in duplicate) are insulin-like growth factor II (*IGF II*), GTP-binding protein rho (*GTP-bp*), glutathione-S-transferase π (*GST\pi*) and GADD153 (boxed). Both GADD153 and *GST\pi* were induced by fenretinide (Table 1). B, Northern blot of RNA from SH-SY5Y cells exposed to 3 μ M fenretinide for 2–24 h (c, ethanol control), probed for GADD153 and GAPDH (loading control). C, Northern blot of RNA from SH-SY5Y cells exposed to fenretinide at concentrations ranging from 0.001 to 10 μ M for 8 h (samples in duplicate), probed for GADD153 and GAPDH (loading control). D, Western blot of SH-SY5Y cells treated with 3 μ M fenretinide for 0–48 h for GADD153; kDa, *M_r* in thousands.

GADD153 (Fig. 1, B and C). The results demonstrated that *GADD153* was induced within 2 h of exposure to 3 μ M fenretinide, peaking at 8 h (Fig. 1B). Induction was detectable with 0.1 μ M fenretinide, with 10 μ M giving the greatest induction (Fig. 1C). Western blotting and flow cytometry with a *GADD153*-specific antibody were then used to confirm that *GADD153* protein as well as mRNA was induced; both techniques demonstrated a clear increase in *GADD153* expression in response to fenretinide, reaching a maximum at around 6 h (Fig. 1D and Fig. 2). In contrast to this effect of fenretinide, no induction of *GADD153* mRNA or protein was observed in response to all-*trans*, 13-*cis*, or 9-*cis* retinoic acid (data not shown).

Fenretinide-induced GADD153 Was Not Blocked by RAR β / γ Antagonists. Because fenretinide-induced apoptosis is inhibited by antagonists to the RAR β / γ receptors (6), we asked whether these

Table 1 *cDNA array screening results genes for which the hybridization signal differed 5-fold between cDNA filters for apoptosis-related genes probed with cDNA from SH-SY5Y cells treated with ethanol (control) or 3 μ M fenretinide for 24 h*

Gene	Protein/gene type	Effect of fenretinide ^a
<i>Cell division protein kinase 4</i>	Cell cycle	+
<i>CDC 25 B</i>	Cell cycle	+
<i>c-jun</i>	Cell cycle	-
<i>CDC 27 HS protein</i>	Cell cycle	-
<i>Bak</i>	Bcl2 family	+
<i>glutathione S-transferase π</i>	Other regulator	+
<i>GADD153</i>	Other regulator	+
<i>CD27 ligand</i>	Other regulator	+
<i>Fas-activated serine kinase</i>	Other regulator	-

^a +, genes induced by fenretinide; -, genes repressed by fenretinide.

reagents also inhibited GADD153 induction. SH-SY5Y cells were treated with fenretinide in the presence or absence of the RAR antagonists CD2665 or CD2848; Northern blots of RNA from these cells were probed for GADD153 and showed that GADD153 induction was not inhibited by the RAR β / γ antagonists (Fig. 2A). Conversely, the induction of RAR β in response to all-*trans* retinoic acid was inhibited by both RAR β / γ antagonists (Fig. 2B). Fenretinide was ineffective at inducing RAR β by comparison with all-*trans* retinoic acid.

Lack of Synergy or Additivity between Fenretinide and Chemotherapeutic Drugs in Relation to the Induction of GADD153 Expression. Treatment of SH-SY5Y cells with fenretinide and cisplatin, or etoposide, or carboplatin results in the synergistic induction of apoptosis (7). Studies in other tumor types have shown that GADD153 can also be induced by chemotherapeutic reagents (28, 30). Therefore, to investigate the possibility that chemotherapeutic drugs and fenretinide can induce GADD153 synergistically, SH-SY5Y cells were treated with fenretinide, cisplatin, etoposide or carboplatin or with both fenretinide and each chemotherapeutic drug in turn. Apoptosis induced by chemotherapeutic drugs is apparently p53-dependent (11, 31), and, therefore, we examined the expression of the p53-regulated gene, *p21/WAF1* in addition to that of *GADD153*. Northern blots demonstrated that GADD153 was induced only by fenretinide and that the response was not increased in the presence of increasing concentrations of cisplatin, etoposide, or carboplatin (Fig. 2C). In contrast, *p21/WAF1* was induced by cisplatin, etoposide, and carboplatin in a dose-dependent manner but not by fenretinide. Furthermore, the presence of fenretinide did not increase the induction of *p21/WAF1* in response to increasing concentrations of chemotherapeutic reagents (Fig. 2C).

Evidence for a Functional Role of GADD153 in Fenretinide-induced Apoptosis. The above data suggest that GADD153 induction has a functional role in fenretinide-induced apoptosis. In preliminary experiments, transient transfection of SH-SY5Y cells with the GADD153 cDNA (pCMV-SPORT6 vector) resulted in a marked increase in apoptosis, both in the absence and presence of fenretinide (data not shown). To confirm this, clones were derived by stable transfection of a Tet-inducible GADD153 sense construct into SH-SY5Y cells expressing the Tet-repressor protein (SH-SY5Y^{Tet12}); relative to control-uninduced cells, induction of GADD153 with Tet clones increased apoptosis in the absence of fenretinide and also increased the apoptotic response to fenretinide treatment (Fig. 3). To assess the effect of reducing GADD153 expression, SH-SY5Y^{Tet12} cells were stably transfected with a Tet-inducible antisense GADD153 expression construct; induction of the antisense GADD153 cDNA virtually abolished the apoptotic response to fenretinide (Fig. 3). Western blot analysis confirmed that GADD153 was induced by Tet in the stable sense-GADD153 transfectants and that Tet blocked the fenretinide-dependent induction of GADD153 in the stable antisense-GADD153 transfectants (Fig. 3).

Role of ROS in GADD153 Induction in Response to Fenretinide. Treating SH-SY5Y neuroblastoma cells with fenretinide results in the induction of ROS within 6 h, and both the induction of ROS and subsequent apoptosis are blocked by pretreating the cells with antioxidants (6). To determine whether GADD153 induction by fenretinide was also a result of free radical-mediated effects, SH-SY5Y cells were pretreated with the antioxidants vitamin C or NAC before exposure to 10 or 3 μ M fenretinide for 24 h. Northern blots and immunofluorescence flow cytometry showed that the induction of GADD153 mRNA and protein was blocked by both antioxidants, although to a lesser extent by NAC (Fig. 4, A and B). As an additional test for a link between GADD153 induction and ROS generation, we evaluated the expression of GADD153 mRNA and protein in three SH-SY5Y cell lines selected for resistance to fenretinide; compared with wild-type cells, SH-SY5Y cells resistant to 1, 2, or 3 μ M fenretinide show reduced levels of ROS in response to fenretinide (6), and these cells also showed no induction of GADD153 at either the protein or mRNA level when treated with 3 μ M fenretinide for 24 h (Fig. 4, C and D).

Enzyme Pathways Mediating the Generation of ROS and Apoptosis in Response to Fenretinide. Results from the preceding experiments suggest that fenretinide induces apoptosis via RAR-dependent and -independent pathways in which the RAR-independent pathway is characterized by the ROS-dependent induction of GADD153. Intracellular ROS in neuroblastoma cells accumulate rapidly within 2 h of treatment with fenretinide, peaking at 6 h (6) and

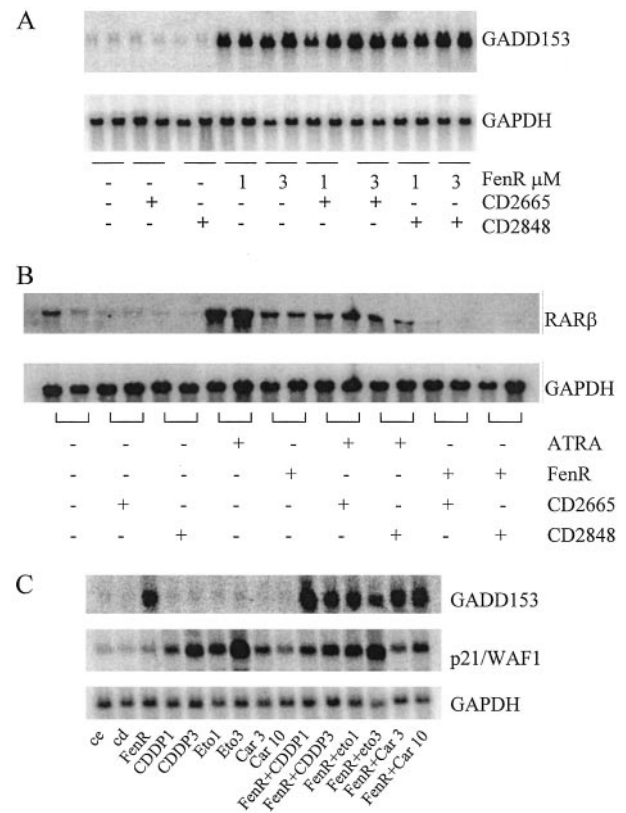


Fig. 2. GADD153 expression in SH-SY5Y cells is not inhibited by RAR β / γ antagonists nor induced by chemotherapeutic drugs. Northern Blots of RNA from SH-SY5Y cells treated with: A, 1 μ M or 3 μ M fenretinide (*FenR*) in the presence or absence of the RAR β / γ antagonists CD2665 or CD2848 (1 μ M) for 24 h and probed for GADD153 and GAPDH (loading control); B, 1 μ M all-*trans* retinoic acid (*ATRA*) or 3 μ M fenretinide (*FenR*) in the presence or absence of 1 μ M CD2665 or CD2848 for 24 h and probed for RAR β and GAPDH (loading control). C, 3 μ M fenretinide (*FenR*), 1 or 3 μ M cisplatin (*CDDP*), 1 or 3 μ M etoposide (*Eto*), 3 or 10 μ M carboplatin (*Car*), or with both fenretinide and each chemotherapeutic drug together for 24 h, and probed for GADD153, p21/WAF1, and GAPDH (loading control). *ce*, ethanol control; *cd*, DMSO control.

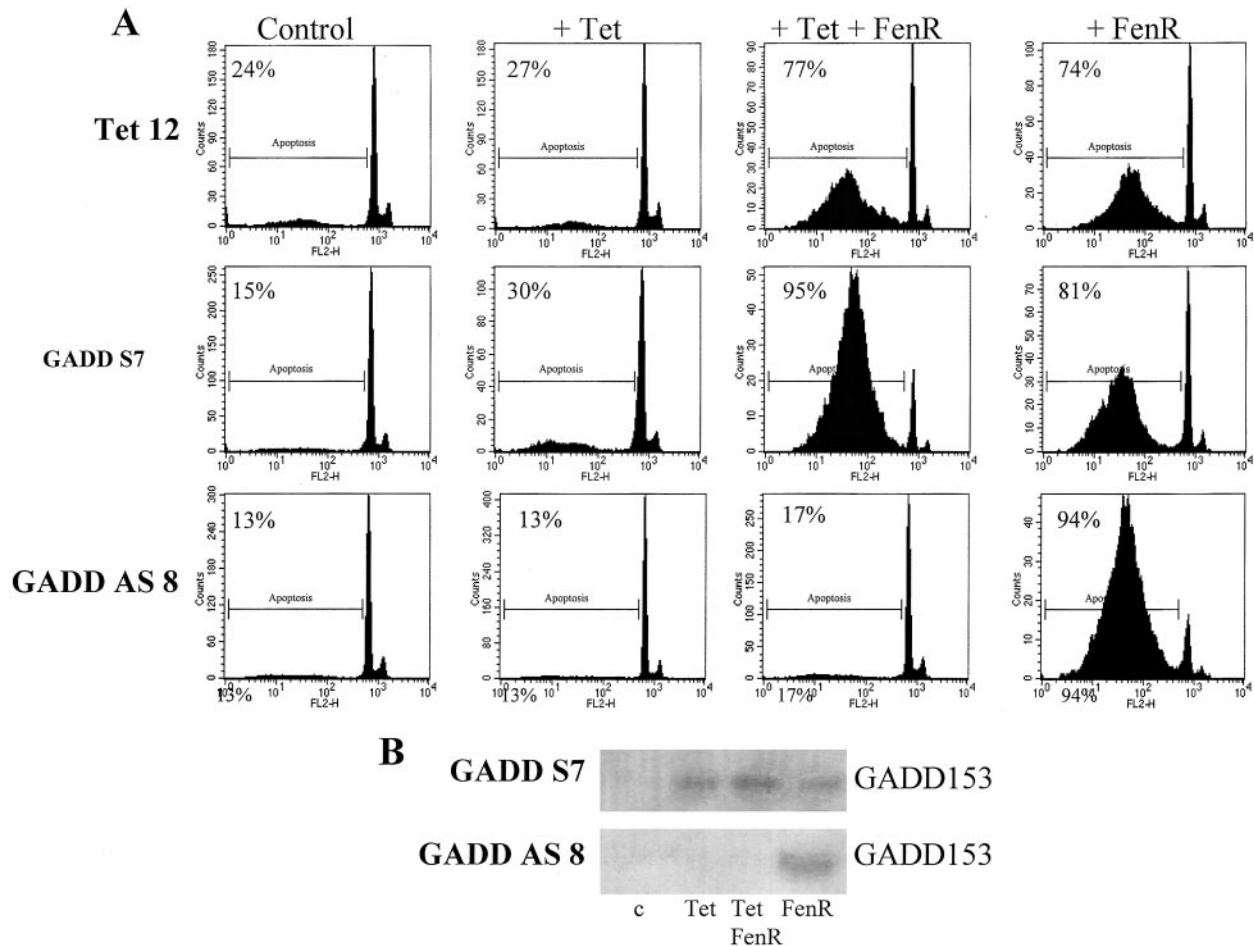


Fig. 3. A, flow cytometry profiles of PI-stained SH-SY5Y^{tet12} cells (*Tet 12*, top panel), and SH-SY5Y^{tet12} cells stably transfected with a tet-inducible sense-GADD153 (*S7*, middle panel) or antisense-GADD153 (*AS8*, bottom panel) cDNA construct. The horizontal line in each profile represents the gating limits for apoptotic cells, summarized as a percentage in each graph. Cells were treated with 1 μ g/ml tet (+*Tet*) or ethanol control (*Control*) for 72 h, or for 24 h with 1 μ g/ml tet followed by an additional 48 h with 10 μ M fenretinide (+*Tet + FenR*), or with 10 μ M fenretinide alone for 48 h (+*FenR*). Cells were then fixed and stained with PI and analyzed by flow cytometry. The data shown are for one sense-GADD153 and one antisense-GADD153 clone of two of each type that were studied in detail. Similar data were obtained for the other clones. B, Western blot of protein from SH-SY5Y^{tet12} cells stably transfected with a tet-inducible sense-GADD153 (*S7*) or antisense-GADD153 (*AS8*) cDNA construct and probed with an antibody to GADD153, as for Fig. 1. Cells were treated with 1 μ g/ml tet (*Tet*) or ethanol control (*c*) for 48 h, or for 24 h with 1 μ g/ml tet followed by an additional 24 h with 10 μ M fenretinide (*Tet FenR*), or with 10 μ M fenretinide alone for 24 h (*FenR*).

may 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 including NOS, NADPH oxidase (32), cytochrome P450s (33), LOXs, COXs, and enzymes mediating AA metabolism (34, 35). To investigate the contributions of these metabolic pathways to fenretinide-dependent ROS and apoptosis, SH-SY5Y cells were treated with 3 μ M fenretinide in the presence or absence of various inhibitors. Inhibitors of NOS, NADPH oxidase, and other flavoprotein-dependent superoxide-producing enzymes, cytochrome P450s, and COX did not block fenretinide-induced apoptosis or ROS (data not shown). Conversely, treatment of SH-SY5Y cells with AACOCF₃, the trifluoromethylketone analogue of AA and a selective inhibitor of PLA₂ (36), before treatment with fenretinide blocked fenretinide-induced ROS and apoptosis (Fig. 5). Similar results with respect to apoptosis (ROS was not measured) were obtained with HTLA230 neuroblastoma cells (data not shown). These results imply that the release of free AA is necessary for the apoptotic effects of fenretinide on neuroblastoma cells. However, fenretinide up to 10 μ M did not increase the availability of free AA, because it did not increase the ability of SH-SY5Y cells to incorporate AA into membrane lipids or to release it into the culture medium; incorporation and release of AA in cells treated with

10 μ M fenretinide were 98 and 94% of the control values, respectively. Under our experimental conditions, control cells incorporated $45 \pm 5\%$ (mean \pm SD) of the [³H]AA supplied and released $\sim 25\%$ of incorporated AA during the 22 h after incubation with [³H]AA.

Fenretinide-dependent Increases in 12-LOX Activity. The pan-LOX inhibitors nordihydroguaiaretic acid (NDGA; Ref. 37; data not shown), esculetin (38), and ETYA (Ref. 39; Fig. 5) were effective in blocking fenretinide-induced ROS and apoptosis in SH-SY5Y cells. To confirm these results and to identify specific LOX enzymes that mediate the effects of fenretinide in neuroblastoma cells, we used a range of LOX inhibitors with differing specificities to treat SH-SY5Y cells 2 h prior to the addition of fenretinide and at appropriate concentrations to avoid or minimize cross-specificity.

Compounds used to inhibit specific LOX enzymes were MK886, a specific inhibitor of 5-LOX (20); baicalein, a specific inhibitor of 12-LOX (40); and PD146176, a highly specific inhibitor of 15-LOX (22). Other reagents used were caffeic acid, an inhibitor of 5-LOX (19), which may also inhibit 15-LOX (21), and ETI, which inhibits both 5-LOX and 12-LOX at an IC₅₀ of 20 μ M (39). Of these inhibitors, MK886, caffeic acid, and PD146176 were unable to block the induction of ROS and apoptosis in response to fenretinide (Fig. 5). Conversely, the 12-LOX inhibitor baicalein, and the 5- and 12-LOX

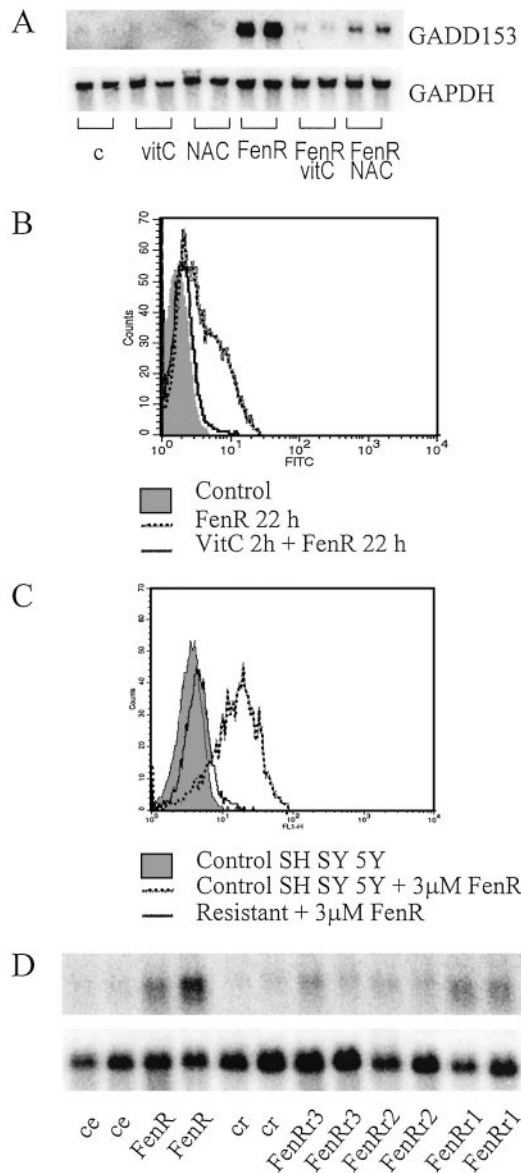


Fig. 4. GADD153 expression is inhibited by antioxidants and reduced in fenretinide-resistant SH-SY5Y cells. **A**, Northern blot, probed for GADD153 and GAPDH (loading control), of RNA from cells treated with 1 μ M fenretinide (*FenR*) for 24 h with and without pretreatment for 2 h with 100 μ M vitamin C (*vitC*) or 10 mM NAC. **B**, flow cytometry immunofluorescence profiles for GADD153-antibody staining of SH-SY5Y cells treated for 24 h with 3 μ M fenretinide (*FenR*) with (—) or without (---) pretreatment for 2 h with 100 μ M vitamin C (*vitC*); filled gray trace, vehicle (ethanol)-treated control cells. **C**, flow cytometry immunofluorescence profiles for GADD153 antibody staining of control SH-SY5Y cells (---) or 3 μ M-fenretinide-resistant SH-SY5Y (SH-SY5Y-R3) cells (—) treated for 24 h with 3 μ M fenretinide; filled gray profile, vehicle-treated control SH-SY5Y cells. **D**, Northern blot, probed for GADD153 and GAPDH (loading control), of RNA from SH-SY5Y cells, SH-SY5Y cells resistant to 1, 2, or 3 μ M fenretinide (*r1*, *r2*, or *r3*, respectively) and control resistant SH-SY5Y-R3 cells (*cr*), ethanol control SH-SY5Y-R3 cells) for 24 h.

inhibitor ETI, blocked fenretinide-induced ROS and apoptosis of SH-SY5Y cells (Fig. 5). Similar results with respect to apoptosis were obtained using HTLA230 cells (data not shown). These results clearly implicate 12-LOX as being the mediator of ROS and apoptosis signaling in response to fenretinide in neuroblastoma cells.

To evaluate the expression of 5-, 12-, and 15-LOX, total protein was extracted from SH-SY5Y cells treated for 2 h with specific LOX inhibitors with or without treatment with 3 μ M fenretinide for a subsequent 22 h. Western blotting showed that 12-LOX and 5-LOX

cross-reactivity was expressed in SH-SY5Y cells, whereas 15-LOX protein was undetectable, but levels of 12-LOX and 5-LOX protein did not change in response to fenretinide treatment (data not shown). Conversely, 3 μ M fenretinide increased LOX activity of SH-SY5Y

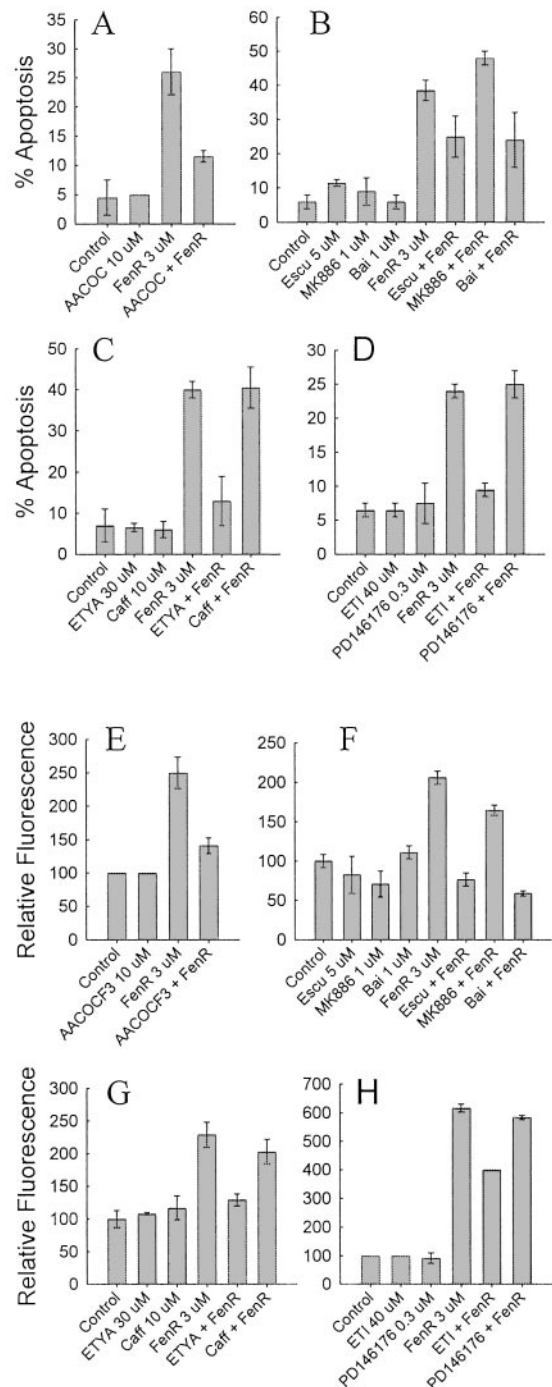


Fig. 5. Inhibition of fenretinide-induced apoptosis (**A–D**) and ROS (**E–H**) in SH-SY5Y cells by inhibitors of PLA₂ and LOX. Percentage (%) apoptosis or relative DCFDA fluorescence (*ordinate*) is indicated for SH-SY5Y cells treated with inhibitors or 3 μ M fenretinide (*FenR*) alone for 24 h or after pretreatment with inhibitors for 2 h with subsequent addition of fenretinide (3 μ M) for 22 h. Results are for treatment with ethanol vehicle control (*Control*), ETYA (30 μ M, **C** and **G**), DMSO vehicle control (*Control*), AACOCF3 (10 μ M, **A** and **E**), caffeic acid (*caff*, 10 μ M, **C** and **G**), MK886 (1 μ M, **B** and **F**), baicalein (*Bai*, 1 μ M, **B** and **F**), ETI (40 μ M, **D** and **H**), PD146176 (0.3 μ M, **D** and **H**). Each column, the mean \pm range of duplicate treatments. The absolute values for relative DCFDA fluorescence and apoptosis vary between experiments, and each graph presents the results of separate experiments, each with appropriate vehicle controls. *Escu*, esculetin.

cells from 300 ± 25 (mean \pm SD) pmol/min per mg protein to 750 ± 60 pmol/min per mg protein, and $1 \mu\text{M}$ baicalein reduced LOX activity of fenretinide-treated cells to 80 ± 10 pmol/min/mg of protein. The addition of 1 mM ATP and 2 mM CaCl_2 to the assay buffer led to a LOX activity of 350 ± 30 pmol/min/mg of protein, close to that of control SH-SY5Y cells; and $1 \mu\text{M}$ baicalein reduced LOX activity of these control cells to 30 ± 5 pmol/min/mg of protein. These data suggest that SH-SY5Y cells contain an active 12-LOX and that 5-LOX does not contribute to cellular LOX activity (20).

12-LOX catalyzes the stereospecific oxygenation of AA to form 12-HpETE and 12-HETE (41). The production of 12-HpETE by 12-LOX should lead to the production of ROS via glutathione peroxidase and a concomitant depletion of GSH. Measurement of GSH in SH-SY5Y cells treated with fenretinide showed a reduction, relative to control, untreated cells (Fig. 6), that was consistent with increased 12-LOX activity; conversely, baicalein, on its own, increased GSH levels, consistent with the blocking of 12-LOX activity, whereas the combination of fenretinide and baicalein gave GSH levels similar to

control, untreated cells (Fig. 6). These results provide further evidence for the involvement of 12-LOX in fenretinide-induced ROS and apoptosis of neuroblastoma.

Products of 12-LOX Reverse the Inhibition of Fenretinide-induced Apoptosis by Baicalein and ETI. The addition of 12-HETE can reverse the inhibition of 12-LOX-induced apoptosis of epidermal carcinoma cells (42). Because fenretinide-induced apoptosis of neuroblastoma cells appeared to be mediated by 12-LOX, the addition of the 12-LOX products 12-HpETE or 12-HETE should reverse the inhibition of fenretinide-induced ROS and apoptosis by baicalein or ETI. To test this, SH-SY5Y cells were treated for 2 h with either baicalein or ETI before washout and incubation in serum-free medium in the presence of these inhibitors for an additional 2 h prior to treatment with either 12-HETE or 12-HpETE in the presence or absence of fenretinide. Inhibition of 12-LOX by baicalein or ETI appeared to be irreversible because their inhibitory effects on fenretinide-induced apoptosis or ROS were the same whether cells were treated briefly (2 h) with the inhibitors or cultured in their continued presence (data not shown). The addition of either 12-HETE or 12-HpETE to cells pretreated with baicalein (Fig. 6) or ETI (data not shown) in the presence of fenretinide reversed the inhibition of fenretinide-induced apoptosis by the 12-LOX inhibitors. In addition, 12-HETE or 12-HpETE did not induce apoptosis on their own when added to SH-SY5Y cells (Fig. 6). These results provide further evidence for the involvement of 12-LOX in fenretinide-induced ROS and apoptosis of neuroblastoma.

12-LOX Mediates the Induction of GADD153 in Response to Fenretinide in SH-SY5Y Neuroblastoma Cells. To investigate the link between ROS, apoptosis, and GADD153, flow cytometry of cells stained with a GADD153 antibody was used to determine whether the induction of GADD153 in response to fenretinide was also mediated by 12-LOX. As was the case with the induction of ROS and apoptosis, inhibitors of NADPH oxidase, COX, and cytochrome P450 did not block the induction of GADD153 in response to $3 \mu\text{M}$ fenretinide; the PLA_2 inhibitor ETYA produced a partial inhibition, and the pan-LOX inhibitor esculetin effectively inhibited the GADD153 response (data not shown). Of the specific LOX inhibitors, only baicalein and ETI inhibited GADD153 induction, whereas there was no detectable response to caffeic acid, MK886, or PD146176 (Fig. 7). These results indicate that GADD153 was induced by fenretinide as a result of increased 12-LOX activity.

DISCUSSION

GADD153 Induction and Apoptosis. Originally isolated as a gene that is rapidly induced by alkylating agents and UV light, GADD153 is now known to be induced by other forms of stress as well, including nutrient stress, the generation of ROS, and endoplasmic reticulum stress (43–45). The results reported in this present study suggest that the rapid induction of GADD153, at both the mRNA and protein level, is a key event in response to fenretinide in SH-SY5Y cells, and results from a fenretinide-dependent increase in ROS. $\text{RAR}\beta/\gamma$ antagonists block fenretinide-induced apoptosis in SH-SY5Y cells (6) but do not block GADD153 expression. This suggests that the induction of apoptosis in these cells in response to fenretinide requires both RAR-dependent and RAR-independent events, with GADD153 induction representing RAR-independent effects of fenretinide necessary for apoptosis. The transfection results clearly demonstrate that GADD153 is a key control point in fenretinide-induced apoptosis, and, in this respect, SH-SY5Y neuroblastoma cells are similar to other cell types (28, 46, 47). Because GADD153 is itself a transcription factor, this suggests that fenretinide-induced

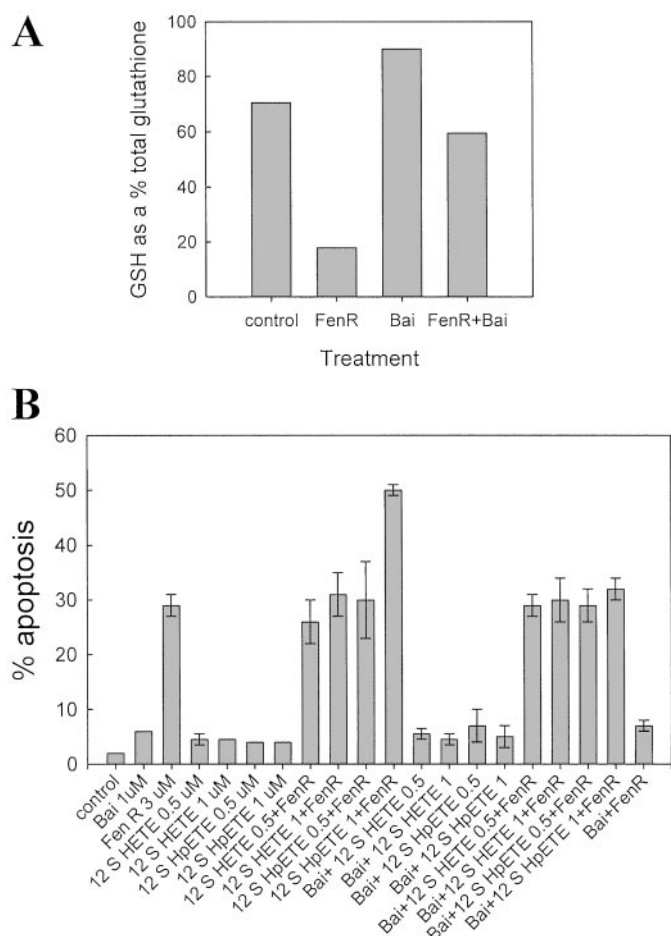


Fig. 6. A, changes in GSH content of SH-SY5Y cells treated with $10 \mu\text{M}$ fenretinide (FenR) for 22 h, baicalein (Bai) for 24 h, or pretreated with baicalein for 2 h and then treated with fenretinide and baicalein together for 22 h (FenR+Bai); control, vehicle-treated cells. GSH is expressed as a percentage of GSSG levels. B, reversibility of baicalein inhibition of fenretinide-induced apoptosis of SH-SY5Y cells by 12-HETE (12 S HETE) and 12-HpETE (12 S HpETE). % apoptosis, apoptosis after treatment: with baicalein (Bai 1 μM) alone for 24 h, or with $3 \mu\text{M}$ fenretinide (FenR 3 μM) alone for 22 h; or with 0.5 or 1 μM 12-HETE (12 S HETE 0.5 μM or 1 μM) or 12-HpETE (12 S HpETE 0.5 μM or 1 μM) alone for 22 h or with 0.5 or 1 μM 12-HETE or 12-HpETE and $3 \mu\text{M}$ fenretinide (12 S HETE, 0.5 or 1, or 12 S HpETE, 0.5 or 1, + FenR) together for 22 h. The % apoptosis is also shown for pretreatment with baicalein for 4 h prior to the addition of 0.5 or 1 μM 12-HETE (Bai + 12 S HETE 0.5 or 1) or 12-HpETE (Bai + 12 S HpETE 0.5 or 1) \pm $3 \mu\text{M}$ fenretinide (FenR) for 22 h, or with just the addition of $3 \mu\text{M}$ fenretinide (Bai + FenR) for 22 h. Each column, the mean \pm range of duplicate treatments.

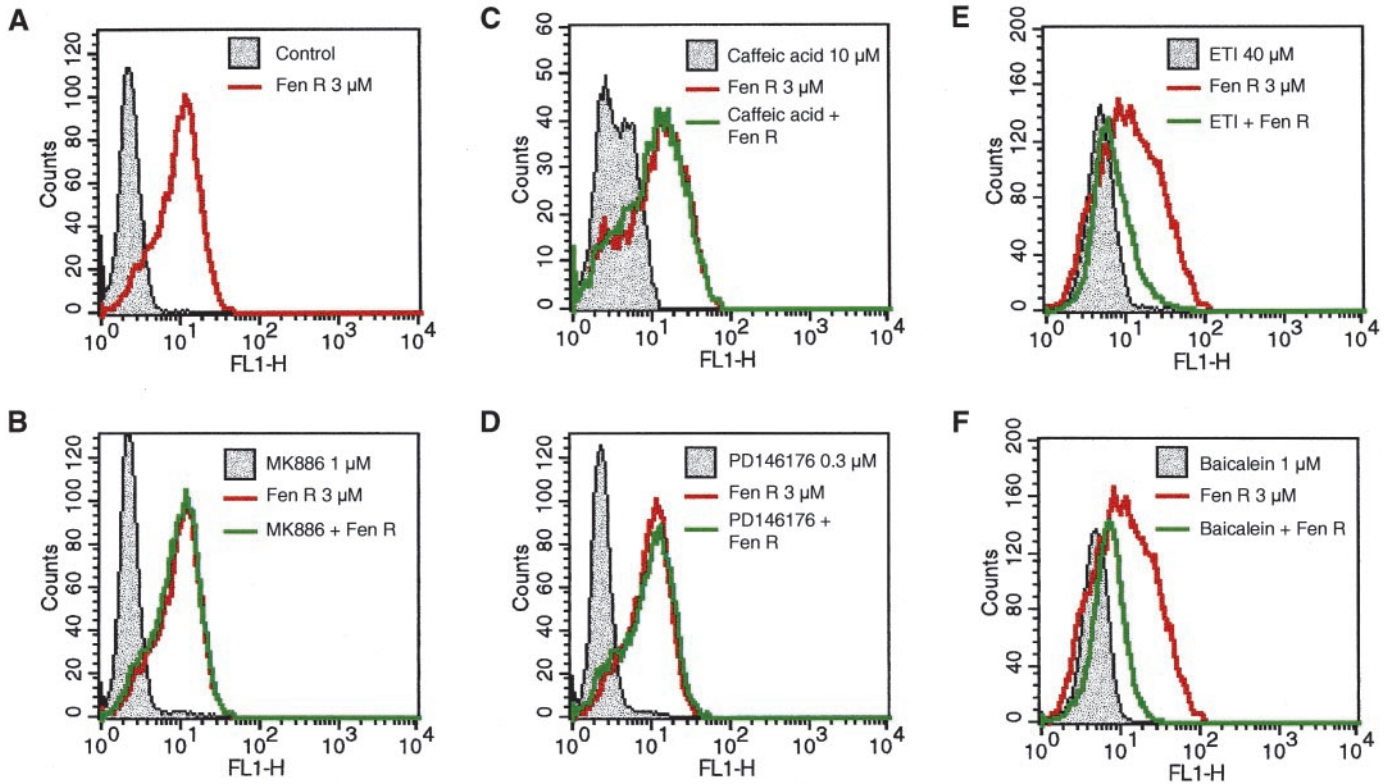


Fig. 7. Inhibition of fenretinide-induced GADD 153 by LOX inhibitors. Flow cytometry immunofluorescence profiles for GADD153 antibody staining of (A) control SH-SY5Y cells treated with vehicle-control (filled gray trace) or 3 μM fenretinide (Fen R, red) for 24 h or (in B–F) with inhibitor (filled gray trace), 3 μM fenretinide (red trace) for 24 h or with 2-h pretreatment with inhibitor prior to the addition of 3 μM fenretinide for 22 h (green trace). Results for each inhibitor: B, MK886 (1 μM); C, caffeic acid (10 μM); D, PD146176 (0.3 μM); E, ETI (40 μM); and F, baicalein (1 μM).

apoptosis in these cells requires transcriptional activation of other key genes, as yet unidentified.

The DNA-damaging agents etoposide, cisplatin, and carboplatin are important components of the chemotherapeutic regime used to treat advanced-stage neuroblastoma. Although the treatment of some cell types with chemotherapeutic drugs results in GADD153 induction (28, 30, 48), this is clearly not the case in SH-SY5Y neuroblastoma cells. Conversely, the p53-regulated gene, *p21/WAF1*, was induced by etoposide, cisplatin, and carboplatin in SH-SY5Y cells but not by fenretinide. Although p21/WAF1 can be regulated independently of p53, p53 plays a critical role in p21/WAF1 induction in response to DNA damage (49). DNA damaging agents induce apoptosis in a p53-dependent manner (11, 31), and recent studies indicate that etoposide and perhaps other chemotherapeutic drugs induce apoptosis in neuroblastoma cells via the translocation of p53 from the cytoplasm to the nucleus (50). The expression of p53 is not modulated by fenretinide and is unlikely to be involved in fenretinide-induced apoptosis of neuroblastoma (13). Therefore, the induction of p21/WAF1 by chemotherapeutic drugs in the present study, coupled with other evidence for SH-SY5Y cells that p53 mediates apoptosis in response to these drugs (50), suggests that fenretinide and chemotherapeutic drugs induce apoptosis by p53-independent and -dependent mechanisms, respectively.

12-LOX and ROS Generation. The ability of antioxidants to block GADD153 induction, the association between reduced ROS (6) and low GADD153 expression in fenretinide-resistant cells, and the similar time course of GADD153 induction compared with that of ROS (6) suggested that GADD153 is induced by ROS resulting from fenretinide treatment. Of the enzyme systems frequently implicated in ROS generation, only PLA₂ and LOXs, and specifically 12-LOX, were apparently involved in ROS generation and apoptosis of SH-

SY5Y cells in response to fenretinide. Because there was no increase in the amount of 12-LOX protein or its AA substrate (released from membrane phospholipids by PLA₂), the generation of ROS and subsequent apoptosis induced by fenretinide must have resulted from increased activity of the enzyme. Inhibitors of 12-LOX, but not the other enzyme pathways, also inhibited GADD153 induction in response to fenretinide, and this provides clear evidence of a link between ROS and GADD153 induction. The nature of this link remains to be elucidated but may involve the kinase-dependent activation of other nuclear transcription factors.

The role of LOX enzymes in carcinogenesis and tumor biology is complex and varies according to cell type. Individually, 5-, 12-, and 15-LOX enzymes have been implicated in modulating apoptosis in several cellular systems, either through inhibition (5- and 12-LOX) or activation, and it has been suggested that LOX pathways exist with a dynamic balance of procarcinogenic (5-LOX and 12-LOX) and anticarcinogenic (15-LOX) forms (51). We were unable to find evidence for 15-LOX expression in SH-SY5Y cells, and the data suggest that SH-SY5Y cells contain an active 12-LOX with little contribution of 5-LOX to cellular LOX activity. Although inhibiting 12-LOX activity in some cell types induces apoptosis (52), 12-LOX activation leads to apoptosis in fibroblasts (53). Furthermore, for CHP100 neuroblastoma cells, accumulated evidence shows a proapoptotic effect of cellular LOX activity (54, 55), and, in the present study, increased 12-LOX activity was clearly necessary for fenretinide-induced apoptosis in SH-SY5Y and HTLA230 neuroblastoma cells. Evidence from other studies also points to the importance of 12-LOX in the biology of neuronal cells; 12-HETE and its derivatives may be involved in aspects of synaptic transmission (56) and nerve cell death (57) and are the main LOX products in the mammalian brain (58).

Pathways of Fenretinide-induced Apoptosis. It is important to stress that, in SH-SY5Y neuroblastoma cells, 12-LOX activity is important for apoptosis in the context of fenretinide treatment. Evidence from other studies on these cells suggests that RAR activation via a conventional retinoid activity of fenretinide is also required, with RAR-dependent and ROS-dependent pathways acting in concert to induce apoptosis in neuroblastoma cells (6). A requirement for fenretinide-mediated RAR effects would explain why 12-HETE and 12-HpETE do not induce apoptosis on their own when added to SH-SY5Y cells. The nature of RAR-dependent effects of fenretinide are unknown. It is becoming increasingly apparent that LOX products are potent ligands for peroxisome proliferator-activated receptors (PPARs; Ref. 59), and because these work as heterodimers with retinoid x receptors, as do RARs, it is not inconceivable that interactions between retinoid x receptor-dependent signaling pathways underlie a requirement (6) for fenretinide to have RAR-dependent and ROS-dependent activities to induce apoptosis of neuroblastoma cells.

Other studies in neuroblastoma cells have shown that ceramide levels increase in response to treatment with high (10- μ M) doses of fenretinide (8, 13). Because both AA and ROS can induce ceramide production (60, 61), the PLA₂/12-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. The role of ceramide in GADD153 induction and subsequent events leading to apoptosis is uncertain. Additional studies are required to elucidate the link between 12-LOX products and the mechanism of GADD153 induction.

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 (6). In prostrate cancer cells, activation of GADD153 results in the dephosphorylation of the proapoptotic protein Bad, a BH3-domain-containing member of the Bcl2 family and its subsequent translocation to the nucleus and the mitochondria (62). Translocation of Bad to the mitochondria triggers the release of cytochrome c and the subsequent activation of caspase 9 resulting in apoptosis (62). In other cells, GADD153 down-regulates the antiapoptotic protein Bcl2, thereby sensitizing the cells to endoplasmic-reticulum stress (63). Because cytochrome c may catalyze the ability of 12-LOX to metabolize linoleic acid, which increases hydroperoxide production (64), cytochrome c release, facilitated by BH3-domain proteins, may increase cellular stress in the manner of a positive feedback loop. In this context, a LOX-mediated alteration of membrane fluidity and permeability may dissipate membrane potential and also increase cytochrome c release from mitochondria (55). Therefore, 12-LOX activation and subsequent GADD153 induction may represent key steps in initiating and amplifying cytochrome c release from mitochondria culminating in apoptosis.

Parallel Activation of Apoptotic Pathways and Drug Therapy. The activation of different apoptotic pathways by fenretinide and chemotherapeutic drugs may be responsible for the synergistic effects of these reagents when added to SH-SY5Y cells in combination (7). Such synergistic effects may occur at an organelle or mitochondrial level, or with respect to enhanced expression or repression of pro- and antiapoptotic genes. GADD153 may play a key role leading to these synergistic interactions; overexpression of GADD153 in gastric cancer cells increases their sensitivity to cisplatin-induced apoptosis (29), and GADD153 synergistically inhibits cell growth when overexpressed with the (unrelated) p53-regulated gene *GADD45* (65). Clearly, the regulation of 12-LOX and GADD153 by fenretinide may be elements of an alternative apoptotic pathway to explore with respect to designing or modifying therapeutic strategies for neuroblas-

toma treatment. LOX pathways may be important targets for the development of new drugs to arrest cancer progression (51), and in neuroblastoma cells, 12-LOX may act as a new target for 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 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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