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Inhibition of the Expression of Ornithine Decarboxylase by Haloperidol in Difluoromethylornithine-Resistant Leukemia Cells

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ABSTRACT. In difluoromethylornithine-resistant L1210 cells stimulated to grow from quiescence, haloperidol caused an early and dose-dependent inhibition of the induction of ornithine decarboxylase (ODC) activity, with an IC_{50} of 3.5 μM. This effect was accompanied by a reduction in the ODC mRNA level and inhibition of cell growth. Other σ ligands of different chemical classes inhibited the induction of ODC activity, whereas sulpiride, a dopamine antagonist devoid of σ-binding affinity, was ineffective. These results indicate that the inhibition of ODC expression may be an early event involved in the antiproliferative response of leukemia cells to haloperidol. BIOCHEM PHARMACOL 52;9:1393–1397, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. haloperidol; ornithine decarboxylase; gene expression; leukemia L1210 cells; cell proliferation; σ receptors

Recent studies have shown that haloperidol, a widely used neuroleptic, can exert antiproliferative and antitumoral activities *in vivo* and *in vitro*. Administration of haloperidol resulted in a decrease in the incidence of colon tumors induced by azoxymethane in rats [1] and in the number of tumor cells of mice bearing Ehrlich ascites carcinoma [2]. Micromolar concentrations of haloperidol and some related drugs can exert antiproliferative effects on several tumor-derived cell lines [3, 4] and mitogen-stimulated lymphocytes [5–7]. In addition, prenatal and early postnatal exposure to haloperidol or other neuroleptics can inhibit DNA synthesis and growth of rat brain [8, 9]. However, the molecular events related to these antiproliferative and anticancer actions are largely unknown.

Growing evidence suggests that polyamines are intimately involved in the control of cell proliferation and even in the development of cancer. ODC† (E.C. 4.1.1.17), the first and rate-limiting enzyme in polyamine biosynthesis, is essential for cell proliferation and is rapidly induced following growth stimuli [10–12]. The expression of the ODC gene is regulated by numerous hormones, growth factors, and cytokines through different signal transduction pathways. The ODC gene is a transcriptional target for the proto-oncogenes c-myc and c-fos [13, 14] and according to a recent report [15] should be recognized as a proto-oncogene itself. Studies with specific inhibitors of the enzyme and with transfected cells or transgenic animals overexpressing ODC have shown a critical role for ODC in malignant transformation and tumor promotion [15–17].

The present study reports the effects of haloperidol on cell growth and ODC expression in a line of leukemia L1210 cells selected for resistance to the ODC inhibitor DFMO (L1210-DFMO^r) [18]. Because of gene amplification, these cells can express ODC at high levels while maintaining the usual mechanisms of regulation of the enzyme. The effects of haloperidol on wild-type L1210 cells and mitogen-stimulated thymocytes were also examined.

MATERIALS AND METHODS

Materials

L1210-DFMO^r cells were a generous gift from Dr. L. Persson (University of Lund, Lund, Sweden). Haloperidol, (−)-cis-(1S, 2R)-U50488, carbetapentane, metaphit methanesulfonate, and 3-(4-fluorobenzoyl) propionic acid (carboxylic acid metabolite of haloperidol or haloperidol metabolite III) were from RBI (Natick, MA, USA). Oligonucleotide primers for reverse transcriptase/PCR were synthesized with an ABI 391 DNA synthesizer (Applied Biosystems, Cheshire, UK).

Cell Culture and Treatment

Mouse L1210-DFMO^r cells were routinely grown as previously described [18, 19]. For experiments, quiescent cells (cell density ≥2 × 10^6/mL) were seeded at 2 × 10^5/mL in

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†*Abbreviations:* ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; PCR, polymerase chain reaction.

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fresh medium (RPMI 1640) containing 10% fetal calf serum and 50 μM β-mercaptoethanol and incubated with drugs as indicated. Wild-type L1210 cells were treated for 16 hr under the same experimental conditions. Thymocytes were isolated from rat thymus and treated with 10 μg/mL concanavalin A for 4 hr [19, 20]. This treatment induced ODC activity from negligible levels [20] to 5.32 units/mg protein. Cell viability was verified by trypan blue exclusion.

Detection of ODC Activity

Cell extracts were prepared and assayed for ODC activity as previously described [19]. ODC activity is expressed as units/mg of protein, where 1 unit corresponds to 1 nmol CO_2/hr incubation. Haloperidol (10–50 μM) did not affect ODC activity when added *in vitro* directly to the assay mixture.

Detection of ODC mRNA

ODC mRNA was detected in L1210-DFMO^r cells by combined reverse transcriptase/PCR analysis [21]. Total RNA was isolated from approximately 10^7 cells by using the guanidinium thiocyanate method [22]. Any contaminating DNA was removed by DNAase treatment essentially as described by Hyttinen *et al.* [21]. This treatment was necessary because mouse genome contains many pseudogenes for ODC [11]. cDNA synthesis and PCR were performed by the same enzyme, r*Tth* DNA polymerase (Perkin Elmer) according to manufacturers' instructions. The reaction mixture contained 0.25 μg of total RNA extracted from cells, 5 units of r*Tth* DNA polymerase, 0.2 mM each dNTP, and 100 pmoles of each primer in a final volume of 50 μL. The PCR primers for mature ODC mRNA were designed to yield a PCR product of 224 nt. The 5′ primer (5′-TCATAGCTGAGCCAGGCAGATA-3′) was targeted to a sequence in exon 9 of mouse ODC mRNA, and the 3′ primer (5′-CTTGGGTCTCTTCTGCAGC-3′) recognized a sequence at the junction of exons 10 and 11. The reverse transcriptase/PCR consisted of a reverse transcriptase phase (60°C for 30 min, 94°C for 2 min) followed by amplification (94°C for 45 sec, 60°C for 75 sec). PCR products were either visualized by ethidium bromide fluorescence after separation on 1.5% agarose gel electrophoresis or quantitated by capillary electrophoresis coupled to laser-induced fluorescence. As for capillary electrophoresis, PCR products were directly separated at 35°C in a 27 cm-total length × 0.1-mm inner diameter deactivated fused-silica capillary (Beckman Instruments, Fullertone, CA). Before separation, the capillary was equilibrated for 30 min with a running solution containing 89 mM Tris/boric acid, 2 mM EDTA (pH 8.5), 0.5% hydroxy-propyl-methyl-cellulose (4000 cps), and 3 μL/mL of the fluorescent intercalator YOPRO (Molecular Probes, Eugene, OR, USA). The samples were injected by pressure for 5 sec and separated within 20 min at a constant voltage of 4 KV. Detection was performed by a sensitive laser-induced fluorescence detector equipped with an argon lamp. Values were corrected by comparison with a 525-bp internal standard (Bio Ventures). The high sensitivity of this technique allowed detection of samples not visualized on agarose gels. For the determination of the relative amounts of ODC mRNA, the number of PCR cycles and the amount of total RNA added were chosen in the range of proportionality of PCR amplification.

RESULTS AND DISCUSSION

We reported that dilution of quiescent L1210-DFMO^r cells in fresh medium containing serum leads to induction of ODC activity and immunoreactive ODC protein, supported by an accumulation of ODC mRNA [19]. Fig. 1 shows that addition of haloperidol to the cell medium resulted in a concentration-dependent inhibition of the induction of ODC activity, with an IC_{50} of 3.5 μM and a reduction of approximately 90% at 10 μM. The time course of the induction of ODC activity in the absence or presence of 10 μM haloperidol is shown in Fig. 2. ODC induction was significantly inhibited after 4 hr of treatment and remained low thereafter. ODC may be regulated at multiple levels of gene expression, and ODC mRNA content can be elevated by several agents including serum, growth factors, tumor promoters, cAMP elevating agents, and steroid hormones [10–12]. To investigate whether the reduction of ODC activity may result from a suppression of ODC mRNA, the messenger was detected by reverse transcriptase/PCR analysis (Fig. 3). The level of ODC mRNA, very low in quiescent cells, increased after dilution of the cells,

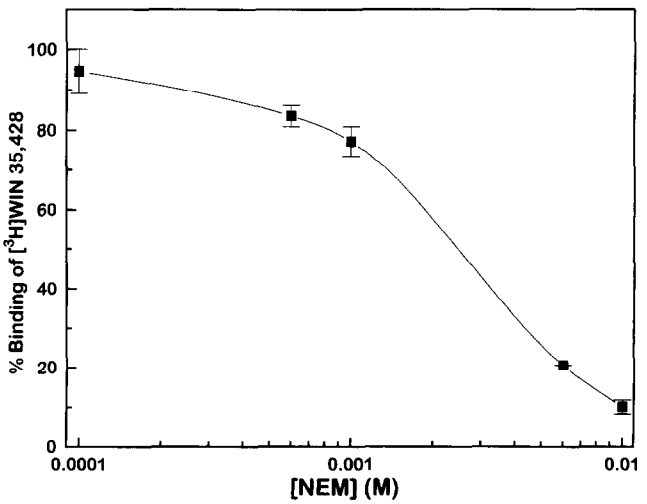

FIG. 1. Effect of increasing concentrations of haloperidol on ODC induction in L1210-DFMO^r cells. Quiescent cells were diluted in fresh medium containing serum and incubated in the presence of the indicated concentration of haloperidol for 16 hr. Results are means ± SD of three separate determinations.

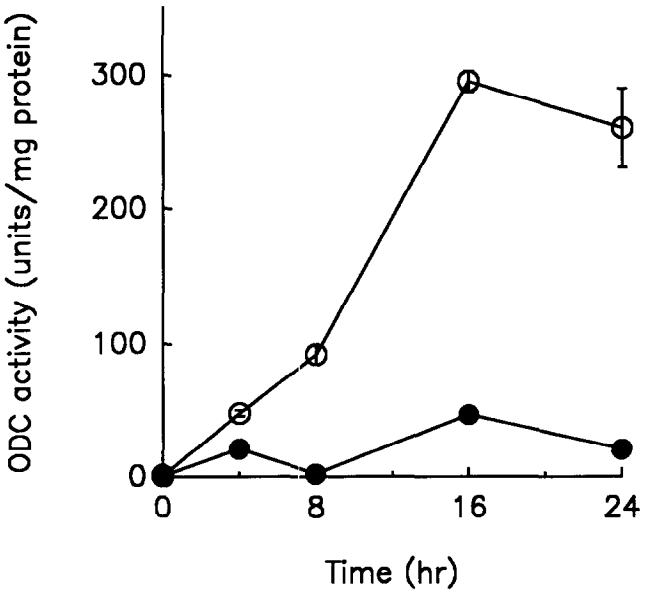

FIG. 2. Effect of haloperidol on the time course of ODC activity in L1210-DFMO^r cells. Quiescent cells were diluted in fresh medium containing serum in the absence (open circles) or presence (solid circles) of 10 μM haloperidol. Results are means ± SD of three separate determinations.

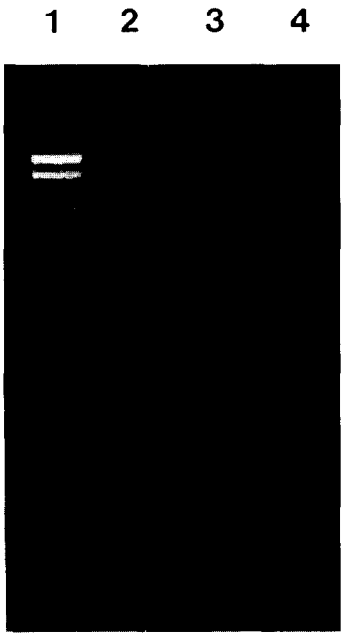

FIG. 3. Effect of haloperidol on the expression of ODC mRNA. L1210-DFMO^r cells were analyzed for ODC mRNA by reverse transcriptase/PCR (21 cycles). PCR products (224 bp) were visualized by ethidium bromide fluorescence after separation on gel agarose electrophoresis. Lane 1: Molecular size markers (Bgl I and Hinf I pBR328 DNA fragments of 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, and 154 bp). Lane 2: Quiescent cells. Lane 3: Cells diluted and treated with 10 μM haloperidol for 16 hr. Lane 4: Control cells (16 hr after dilution).

and haloperidol treatment reduced this increase markedly. Quantitation of ODC mRNA content by reverse transcriptase/PCR (18 cycles) followed by capillary electrophoresis showed that the level of the messenger in haloperidol-treated cells was approximately 20% that of control, indicating that the inhibition of the expression of the message may represent the main mechanism by which the drug reduces ODC induction. Treatment with 10 μM haloperidol for 16 hr as in the previous experiment did not affect cell viability significantly. After a longer treatment (40 hr), cell viability was reduced from 87% (control cells) to 63% (haloperidol-treated cells). However, in the presence of 10 μM haloperidol, a marked inhibition of cell growth was observed (Fig. 4).

Haloperidol is a dopamine antagonist and also binds σ receptors with high affinity; however, its antiproliferative effects on tumor cells do not seem to be related to dopaminergic receptor blockade [2–4]. On the contrary, Vilner *et al.* [4] reported that haloperidol and other neuroleptics exert antiproliferative and cytotoxic effects on several tumor cells of neuronal and non-neuronal origin in a manner correlated with the binding affinity at σ receptors. Table 1 shows that various σ ligands of different chemical classes [23] were able to inhibit ODC induction in L1210-DFMO^r cells, whereas sulpiride, a dopamine antagonist devoid of significant σ affinity [4, 23], was ineffective. Interestingly, the σ-receptor-inactive carboxylic acid metabolite of haloperidol [24] was also without effect (Table 1). A high density of σ-binding sites occurs in solid human tumors [25, 26] and in a wide variety of human and rodent tumor cell lines [27], suggesting important cellular functions for σ sites in

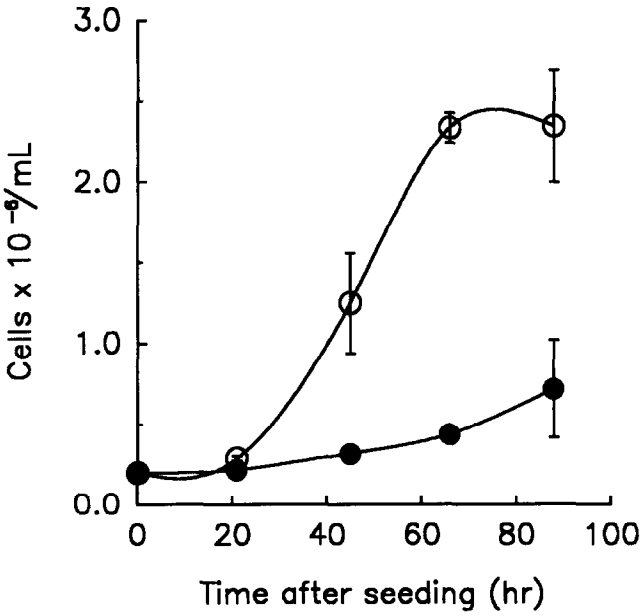

FIG. 4. Effect of haloperidol on the growth curve of L1210-DFMO^r cells. Quiescent cells were diluted in fresh medium containing serum in the absence (open circles) or in the presence (solid circles) of 10 μM haloperidol. Results are means ± SD of three separate flasks.

TABLE 1. Effect of some pharmacological agents on ODC induction

Drug	ODC activity (% of control)
Haloperidol	11.4 ± 4.1
Carbetapentane	42.9 ± 3.6
(−)-cis-(1S,2R)-U50488	31.9 ± 3.3
Metaphit	29.5 ± 2.2
Sulpiride	100.9 ± 2.3
Haloperidol metabolite (carboxylic acid)	98.2 ± 12.3

Quiescent L1210-DFMO^r cells were diluted and incubated with the indicated drugs at 10 μM for 16 hr. Results are means ± SD of at least three determinations.

cancer. Sigma sites have been identified even in lymphocytes [7, 28], whose growth can also be affected by haloperidol [5–7]. Despite nanomolar binding affinities at σ receptors, micromolar concentrations of σ ligands are generally required for physiological effects [23], including inhibition of agonist-stimulated phosphoinositide turnover, which may be involved in the antiproliferative response. In the study by Vilner *et al.* [4], the morphological and antiproliferative effects of haloperidol and other ligands on C6 glioma cells required concentrations of 30–100 μM. Despite this, Vilner *et al.* concluded that these effects showed a remarkable specificity for compounds exhibiting σ-receptor-binding affinity. Similar results were obtained by Brent and Pang [29] by using various carcinoma and melanoma cell lines and concentrations of σ ligands, including haloperidol, ranging from 6.25 to 100 μM. In another study on σ-receptor-mediated neuroprotection against glutamate toxicity in primary rat neuronal cultures [30], a positive correlation was found between neuroprotective potency and σ_1, but not σ_2, site affinity. Even in this case, the concentration of σ ligands at which 50% protection occurred was in the micromolar range and that of haloperidol in particular was 3.7 μM, a value very close to that effective in the present work. Thus, the involvement of σ receptors in the action of haloperidol on ODC expression cannot be excluded.

Administration of haloperidol (10 μM for 16 hr) reduced the induction of ODC activity in wild-type L1210 cells (ODC activity, negligible in quiescent cells, was 5.74 ± 0.09 units/mg protein in haloperidol-treated cells vs 10.84 ± 0.27 of control cells). Thus, the ODC-inhibiting effect of haloperidol is not limited to a particular ODC overproducing cell line, even if it appeared less evident in the wild-type L1210 cells. Although the reason for this difference is unknown, L1210-DFMO^r cells contain higher levels of polyamines [18], which may modulate receptor activity [31] and signal transduction pathways [32] and ODC itself [11]. Besides, this effect was associated with a reduction of ^{3}H-thymidine incorporation (not shown). ODC activity induced in isolated thymocytes by concanavalin A for 4 hr [19, 20] was hardly sensitive to haloperidol (only an 18% reduction at 20 μM), in accordance with the reported lack of efficacy of the drug (10 μM) in inhibiting DNA synthesis of thymocytes [7].

In conclusion, the present research indicates that inhibition of ODC expression may be an early event involved in the antiproliferative response of leukemia cells to haloperidol and other σ ligands.

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