BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 219–226 (1996)
ARTICLE NO. 0208

The Sigma Receptor Ligand, Reduced Haloperidol, Induces Apoptosis and Increases Intracellular-Free Calcium Levels $[Ca^{2+}]_i$ in Colon and Mammary Adenocarcinoma Cells

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Received December 22, 1995

The sigma receptor ligand reduced haloperidol (50 and 100μM), potently inhibited cell proliferation, and induced apoptosis in WIDr colon and MCF-7 adenocarcinoma cell lines. Apoptosis was confirmed after drug treatment of the cells by the presence of nuclear fragmentation after staining of the cells with Hoechst 33258 and cellular DNA fragmentation ELISA and by condensation of the heterochromatin using transmission electron microscopy. However, internucleosomal DNA cleavage was not detected using gel electrophoresis. Reduced haloperidol (100 μM) increased the intracellular free calcium levels $[Ca^{2+}]_i$ in both cell lines, which was independent of extracellular calcium, suggesting that the rise in $[Ca^{2+}]_i$ was from intracellular stores and that an increase in $[Ca^{2+}]_i$ may act as a 'trigger' for apoptosis in these cell lines.

Sigma (gks) binding sites are distributed in the brain, peripheral tissues (1), and in various neuronal and non-neuronal cell lines (2), and evidence suggests a role for a σ sites in maintenance of cell function (2,3,4). We recently demonstrated that σ site ligands dose-dependently inhibited cell proliferation in MCF-7 mammary carcinoma and colon carcinoma cells in culture (3). The molecular mechanisms whereby σ ligands inhibit cell growth are not known, however the gross morphological alterations produced by σ ligands in the cell lines examined previously, included rounding of cells, cell detachment and cell death. These changes resemble the process of programmed cell death or apoptosis, in which a series of events are initiated such that the cell participates in its own death.

Cell death by apoptosis is characterized by typical morphological alterations including condensation of the chromatin in tight apposition to the nuclear envelope and fragmentation of the nucleus, and can be demonstrated by various techniques such as transmission electron microscopy and staining of nuclear chromatin with Hoechst 33258 stain (5). Furthermore, this process has been described as the result of the activation of Ca^{2+}-dependent endonucleases which split double stranded DNA at exposed sites between nucleosomes, generating fragments of 180–200 base pairs or multiples thereof (5). Thus, an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels is considered to be a possible trigger for the apoptotic process in several tissues (6). Since a relationship between σ binding sites and cations such as Ca^{2+} has been demonstrated (7,8,9), in a recent study we suggested the possibility that σ ligands might produce their effects on cell growth via induction of apoptosis and/or modulation of $[Ca^{2+}]_i$ in certain cancer cell lines (3). The present study examined firstly whether apoptosis was induced by the selective σ ligand, reduced haloperidol, found to potently inhibit cell proliferation in colon (WIDr) and mammary (MCF-7) adenocarcinoma cell lines (3), and secondly, whether reduced haloperidol modulated $[Ca^{2+}]_i$ in these cell lines.

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0006-291X/96 $18.00

MATERIALS AND METHODS

Cell culture. Human mammary adenocarcinoma cells (MCF-7) and the colon carcinoma cell line (WIDr) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in complete Dulbecco's Modified Eagle's Medium with 10% foetal calf serum (CSL, Australia). MCF-7 and WIDr cells were cultured with or without reduced haloperidol (50 and 100 μM) as previously described (3).

Assessment of apoptotic cells. Cytocentrifuge MCF-7 and WIDr cell preparations treated with reduced haloperidol (100 μM) were stained with Hoechst 33258 and then examined under a Zeiss fluorescent microscope fitted with an UG-1 excitation filter and a UV-absorbing barrier filter for identification of apoptotic cells showing condensed and fragmented nuclei. DNA fragmentation was assessed by gel electrophoresis or quantitated in culture supernatants using an ELISA kit (Boehringer Mannheim, Germany).

Transmission electron microscopy. Following incubation in the presence or absence of 50 and 100 μM reduced haloperidol for 24 and 48h, MCF-7 and WIDr cells were harvested and washed with 0.1M phosphate buffered saline (PBS), fixed for 1 h with 3% glutaraldehyde in PBS, washed and post-fixed for 1 h with 1% osmium tetroxide in PBS. The cells were dehydrated through a series of alcohols, cleared in acetone and embedded in Procure (Probing and Structure). Ultrathin sections were cut on a Reichert FC4E ultramicrotome, stained with lead citrate and uranyl acetate, and viewed with a JEOL 1200 EX11.

Measurement of $[Ca^{2+}]_i$. Fura-2/AM (the membrane permeable acetoxymethylester form of Fura-2) was prepared as a 1mM stock solution in dimethyl sulfoxide (DMSO) and (0.5%) pluronic acid. Colon (WIDr) and mammary adenocarcinoma (MCF-7) cells were cultured onto glass cover slips, incubated in a loading solution of 2μM Fura 2-AM in Locke's buffer for 45 min at room temperature to facilitate loading and hydrolysis of the Fura-2/AM to the free acid. Cells were then placed in a stage mount and perfused with Locke's buffer at 3 ml/min (36.5°C), either containing 2.5 mM Ca^{2+} or without Ca^{2+} and with 1 mM EGTA added. Dual excitation at 340nm and 380nm was followed by monitoring of emission at 510nm using an Oriel photomultiplier and Nikon inverted microscope. The basal $[Ca^{2+}]_i$ of resting Fura-2-loaded cells was recorded for 10 min, and further fluorescence measurements were made following addition of reduced haloperidol to the perfusion buffer. The data were collected as changes to 340/380 ratios which are indicative of changes to $[Ca^{2+}]_i$. The concentrations of reduced haloperidol used were chosen on the basis of our previous study demonstrating inhibition of cell proliferation in the same cell lines (4).

The paired t-test was used to test the significance of the effects of reduced haloperidol on changes to 340/380 ratios compared to baseline values in cells without drug. Data were expressed as % increase from baseline control levels.

RESULTS

Under phase-contrast microscopy, WIDr and MCF-7 cells appeared as rounded dying cells or in clumps which were no longer adherent (see Fig. 1C for WIDr and Fig. 1D for MCF-7 cells treated with reduced haloperidol, 100 μM, compared to untreated controls Fig. 1A and 1B).

Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in WIDr and MCF-7 cells treated with reduced haloperidol for 24 h (50 and 100 μM; see Fig. 2C for WIDr and Fig. 2D for MCF-7 cells treated with reduced haloperidol, 100 μM, compared to untreated controls Figs. 2A and 2B). Control cells exhibited a normal nuclear morphology characterized by a diffuse chromatin structure and therefore light staining. In addition, Cellular DNA Fragmentation ELISA analysis showed that cells treated with reduced haloperidol exhibited a marked increase in DNA fragmentation proportional to the number of cells cultured compared with controls (Fig. 3).

Ultrastructural examination (Fig. 4) demonstrated that reduced haloperidol-treated WIDr cells showed shrunken nuclei, nucleolar disintegration, extensive margination and condensation of the heterochromatin toward one pole of the cell. Similar apoptotic features were not observed in MCF-7 cells which showed extensive vacuolization following treatment with reduced haloperidol. However, both WIDr and MCF-7 cells did not undergo internucleosomal DNA cleavage using conventional gel electrophoresis techniques (data not shown).

The effect of reduced haloperidol (100 μM) after 24 h in WIDr colon cells on $[Ca^{2+}]_i$ levels (340/380 ratio) is shown in Table 1. For colon carcinoma cells, addition of reduced haloperidol (100 μM) to the perfusate increased 340/380 ratios above resting baseline levels (mean increase ± SEM; 0.296 ± 0.078 n = 5; P = 0.019, paired t-test; Table 1), indicative of an increase in $[Ca^{2+}]_i$ levels. When Locke's buffer containing no Ca^{2+} and 1mM EGTA was used, reduced haloperidol (100 μM) produced similar increases in 340/380 ratios (mean increase ± SEM; 0.263 ± 0.066, n = 5; P = 0.028, paired t-test; Table 1) to those obtained when Locke's buffer containing Ca^{2+} was

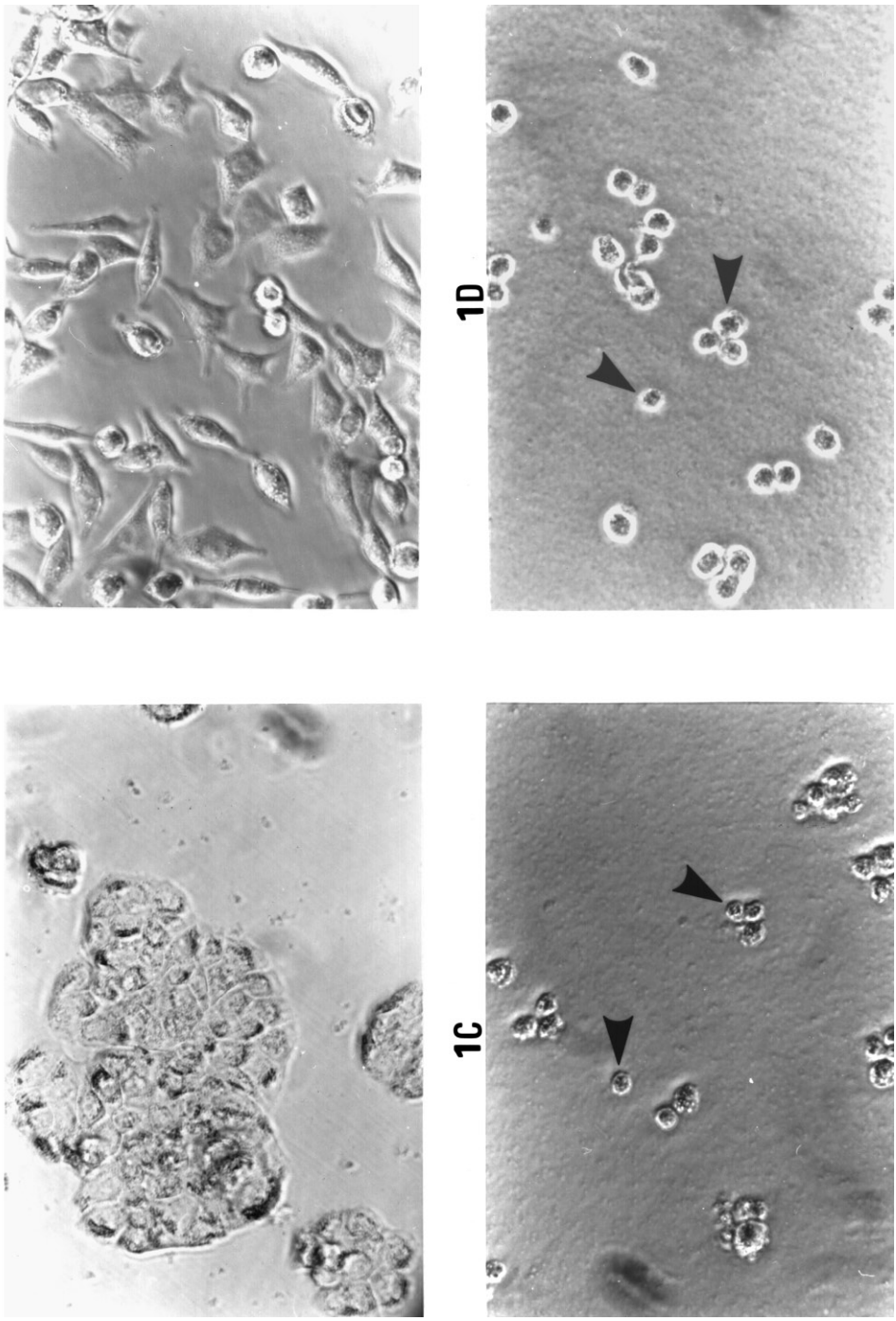

FIG. 1. Phase contrast microscopy of MCF-7 and WIDr cells exposed to reduced haloperidol. MCF-7 and WIDr cells (1×10^6 cells/mL) were cultured for 24 h in the absence or presence of reduced haloperidol (100 μM). (A and B) Control untreated cells showed adherent cells of cuboidal or spindle in shape (MCF-7) or growing in colonies (WIDr). (C and D) MCF-7 and WIDr cells treated with reduced haloperidol (100 μM) showing cell detachment and rounding (arrows) typical of dying cells (magnification ×100). Photograph is from a representative experiment repeated three times.

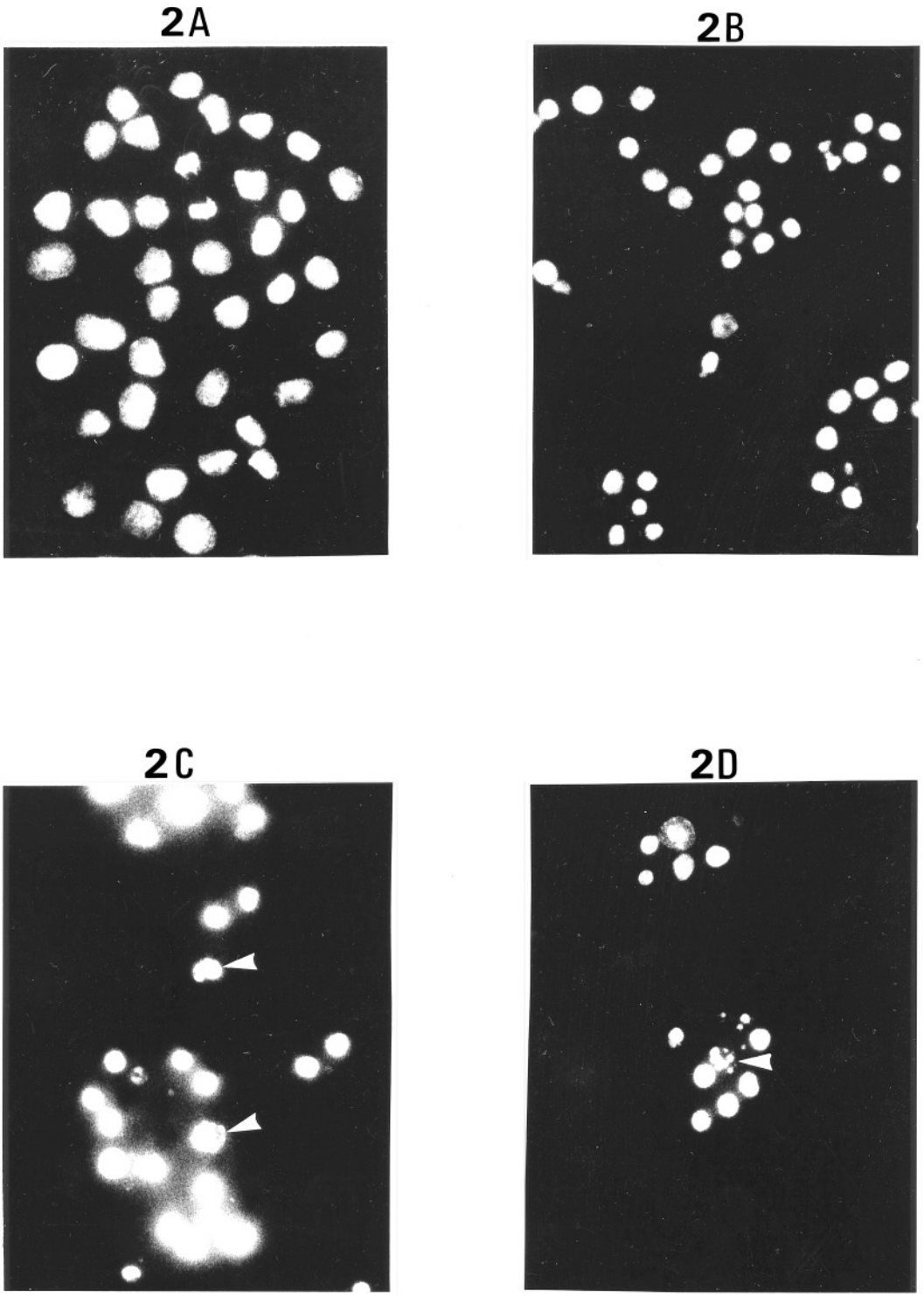

FIG. 2. Morphologic features of apoptosis in MCF-7 and WIDr cells treated with reduced haloperidol. Cells (1×10^6/mL) were cultured in the absence or presence of reduced haloperidol (100 μM) for 24 h and then stained with Hoechst 33258. (A and B) Untreated cells showing intact nuclei staining; (C and D) Treated cells showing condensed and fragmented nuclei typical of apoptosis. Photograph is a representative experiment repeated three times.

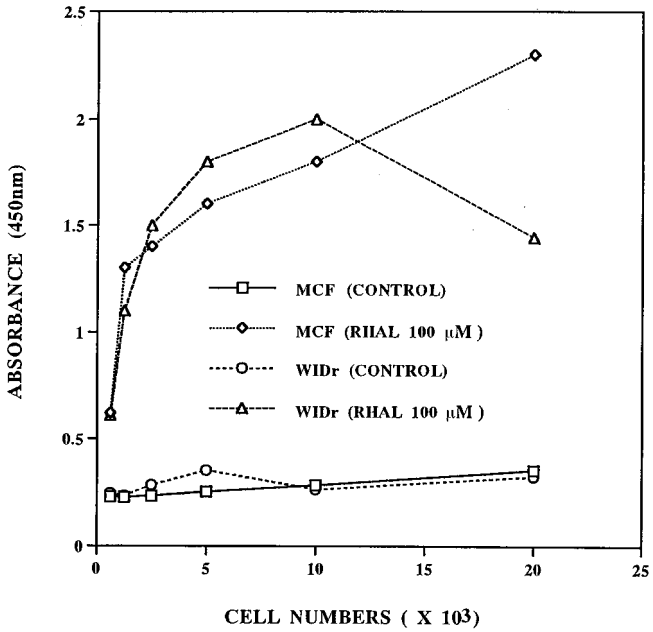

FIG. 3. Quantitation of DNA fragmentation in WIDr and MCF-7 cells treated with reduced haloperidol (100 μM). Cells were cultured at various concentrations for 24 h before the addition of reduced haloperidol (100 μM) and cultured for a further 24 h. DNA fragmentation in supernatants from cell cultures and lysates was measured by ELISA. The results shown are means (absolute absorbance at 450 nm) from duplicate cultures.

used. Decreasing the concentration of reduced haloperidol to 25μM produced similar increases in ratios compared to 100 μM (mean increase ± SEM; 0.274 ± 0.036, n = 8; P = 0.024, paired t-test).

For mammary adenocarcinoma cells, addition of reduced haloperidol (100 μM) to the perfusate increased 340/380 ratios above resting background levels (mean increase ± SEM; 0.500 ± 0.109, n = 7; P = 0.0038, paired t-test; Table 1). When Locke's buffer containing no Ca^{2+} and 1mM EGTA was used, reduced haloperidol (100 μM) produced similar increases in 340/380 ratios (mean increase±SEM; 0.70 ± 0.152, n = 5; P = 0.010, paired t-test; Table 1).

DISCUSSION

There is a growing body of evidence that σ receptors are expressed in a wide variety of human and rodent tumour cell lines, that σ-active drugs have profound effects on cell morphology and viability (2,4,10,11), and may have a role in diagnosis of cancers (12,13). Inhibition of cell proliferation in cultured colon (WIDr) and mammary adenocarcinoma (MCF-7) cells exposed to several σ receptor ligands has been recently shown (3), and induction of apoptosis and/or modulation of $[Ca^{2+}]_i$ was suggested as a possible molecular mechanism. In these latter studies, the metabolite of the antipsychotic drug haloperidol, reduced haloperidol, a selective σ receptor ligand (1), was particularly potent, whereas agents which bind to dopamine, 5-HT, NMDA, opioid receptors and β-adrenoceptors had no effect. The present study shows for the first time that WIDr colon and MCF-7 adenocarcinoma cells were stimulated to undergo apoptosis after treatment with reduced haloperidol for 24–48 h at the same concentrations previously demonstrated to inhibit cell proliferation. To demonstrate this phenomenon, we combined phase-contrast microscopy, nuclear chromatin staining with the fluorescent dye Hoechst 33258, Cellular DNA Fragmentation ELISA, transmission electron microscopy and gel electrophoresis/DNA fragmentation. In both WIDr and MCF-7 cell lines examined after treatment with reduced haloperidol (100 μM), light microscopy showed cell rounding, cell-cell detachment and cell death, whereas Hoechst staining and the

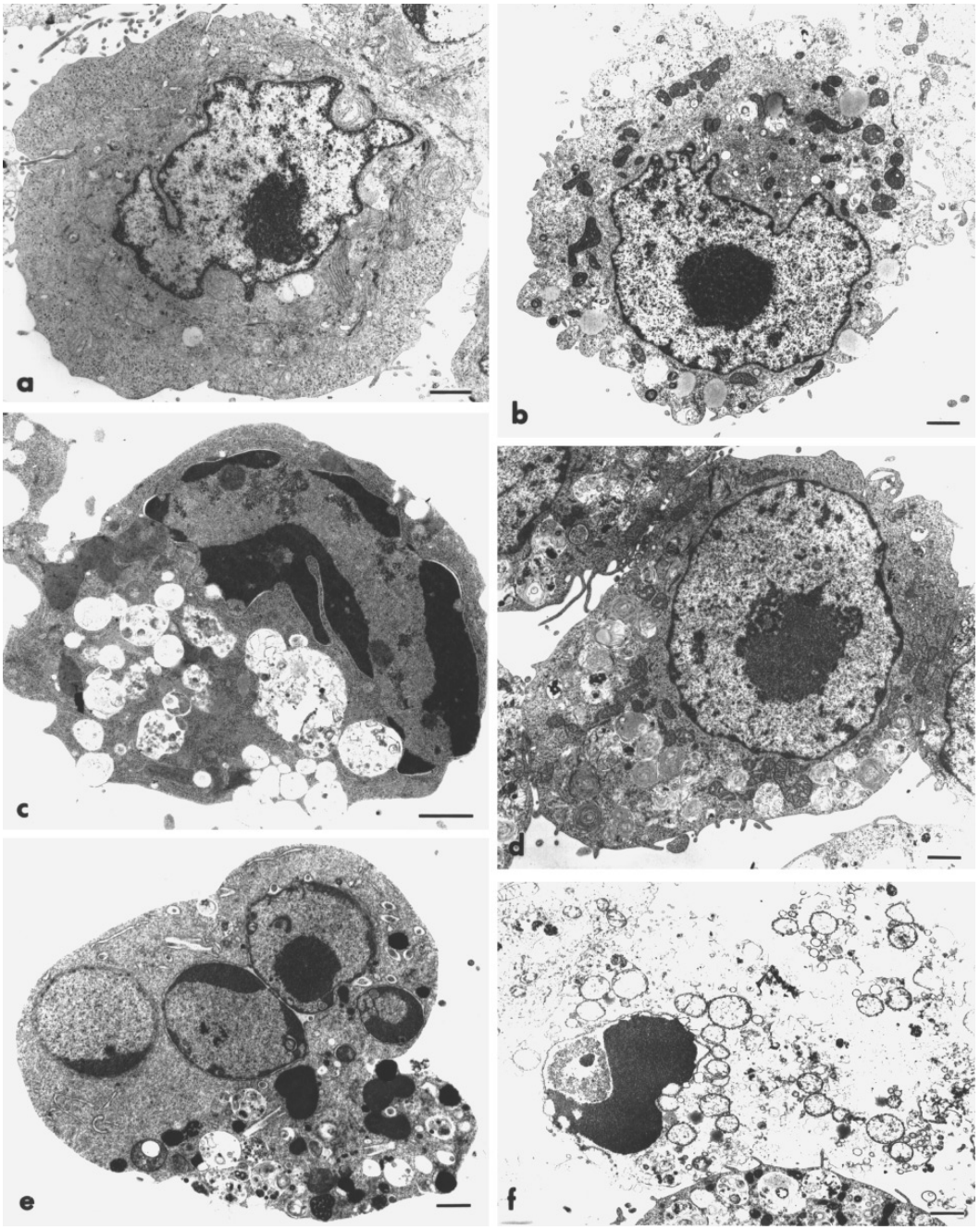

FIG. 4. Ultrastructural features of apoptosis in MCF-7 and WIDr cells treated with reduced haloperidol. Cells (2×10^6/mL) were cultured in the absence or presence of reduced haloperidol (100 μM) for 24 or 48 h. (a and b) Untreated WIDr and MCF-7 cells showing normal morphology. (c and e) WIDr cells displaying features of apoptosis characterized by margination and chromatin condensation (c) and nuclear fragmentation (e). MCF-7 cells showing extensive vacuolization (d), although infrequently cells showing apoptotic morphology (f) were observed. Scale bar, 1 mm.

TABLE 1
Effect of Reduced Haloperidol (10 μM) on $[Ca^{2+}]_i$ in Colon (WIDr) and Mammary Adenocarcinoma (MCF-7) Cells Using Fura-2 Assay

Cell line		% Increase from control	P value
WIDr	(+)Ca^{2+}	24.2 ± 4.3 (5)	0.019
	(−)Ca^{2+}	29.5 ± 5.7 (5)	0.028
MCF-7	(+)Ca^{2+}	15.3 ± 2.7 (7)	0.0038
	(−)Ca^{2+}	16.3 ± 3.3 (5)	0.010

For experimental conditions see Materials and Methods. Data are 340/380 ratios expressed as % increased from baseline control levels (mean ± SEM (n)).

Cellular DNA Fragmentation ELISA assay clearly demonstrated nuclear fragmentation in both cell lines compared to control cultures. Furthermore, using transmission electron microscopy, typical and unequivocal signs of apoptosis, such as shrunken nuclei, nucleolar disintegration, and extensive margination and condensation of chromatin were evident in WIDr cells after 24 h incubation with 100 μ M reduced haloperidol. However, ''typical'' apoptotic bodies were not evident in MCF-7 cells by 48 h after treatment, but cells showed evidence of extensive vacuolation of intracellular processes and a pattern of condensed chromatin which was different to that produced in the WIDr cell line. This difference may be due to the timing of the experiments, since only measures of apoptosis in cells collected 24–48 h after reduced haloperidol treatment are presented here, which may correspond to late time points in the kinetics of reduced haloperidol-induced apoptosis in MCF-7 cells. Taken together, these data indicate that reduced haloperidol produced differential effects on ultrastructural features and cell morphology in WIDr cells compared to MCF-7 cells.

By contrast, analysis of DNA integrity by conventional gel electrophoresis failed to detect internucleosomal DNA cleavage. Non-random cleavage of DNA, the principal biochemical feature that characterizes apoptosis (visualized as the characteristic ''ladder'' pattern when separated by conventional gel electrophoresis), was thought to be associated with one of the most highly conserved morphological features of apoptosis, the condensation of chromatin (5). However, the present data are consistent with a number of recent reports suggesting that the appearance of an apoptotic morphology, characterized by chromatin condensation, is not necessarily accompanied by internucleosomal DNA cleavage, including studies on prostatic carcinoma cells (14), neuroblastoma cells (15) and MOLT-4 human T lymphoblastoid cells (16).

The fluorescent calcium indicator Fura-2 was used in the present study to measure intracellular free Ca^{2+} levels in WIDr and MCF-7 cell lines. The effect of reduced haloperidol on $[Ca^{2+}]_i$ was investigated because of the growing evidence that changes to $[Ca^{2+}]_i$ may act as a ' trigger' for apoptosis (see 6 for review). The results demonstrate that reduced haloperidol, at a concentration which inhibited cell proliferation (3), and which caused morphological changes indicative of the induction of apoptosis (present study), produced consistent increases in $[Ca^{2+}]_i$ in both colon carcinoma and mammary adenocarcinoma cells, increases which were not dependent on the presence of extracellular Ca^{2+}. The present data are consistent with recent studies demonstrating that σ ligands increase $[Ca^{2+}]_i$ levels in neuroblastoma cells (17). It is therefore possible that the increased $[Ca^{2+}]_i$ may be an important step in the commitment of the particular cells to apoptosis.

In conclusion, we have shown that the σ ligand, reduced haloperidol, inhibits cell proliferation, induces apoptosis and increases $[Ca^{2+}]_i$ in colon and mammary cell lines. Elucidation of the mechanisms of the induction of apoptosis and the increase in $[Ca^{2+}]_i$ is the subject of our ongoing studies.

ACKNOWLEDGMENT

This study was supported by research funds from the Research Management Committee of the University of Newcastle

to Drs. P. J. Brent and G. Pang and from the National Health and Medical Research Council to P. J. Dosen and D. F. Van Helden. We thank Professor G. F. Burns for his suggestion that reduced haloperidol might inhibit cell proliferation by induction of apoptosis.

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