ELSEVIER

PII S0024-3205(96)00116-6

Life Sciences, Vol. 58, No. 17, pp. 1461-1467, 1996

0024-3205/96 $15.00 + .00

THE ENDOGENOUS ESTROGEN METABOLITE 2-METHOXYESTRADIOL INDUCES APOPTOTIC NEURONAL CELL DEATH *IN VITRO*

Yuzo Nakagawa-Yagi *, Nobuo Ogane, Yutaka Inoki and Naomi Kitoh

Laboratory of Neurochemistry, Research Institute of Life Science, Snow Brand Milk Products, 519 Shimoishibashi, Ishibashi, Shimotsuga-gun, Tochigi 329-05, Japan.

(Received in final form February 26, 1996)

Summary

We examined the effects of 2-methoxyestradiol, a metabolite of estradiol, on cell death in retinoic acid (RA)-differentiated neuroblastoma SH-SY5Y cell cultures. Cell death was induced by 2-methoxyestradiol in a concentration-dependent manner. Estradiol and 2-methoxyestriol failed to induce cell death. The cell death response to 2-methoxyestradiol was sensitive to the protein synthesis inhibitor cycloheximide and the apopain inhibitor Ac-Asp-Glu-Val-Asp-H(aldehyde). 2-Methoxyestradiol also induced internucleosomal DNA fragmentation. Hence we propose a role for an endogenous neuroactive steroid metabolite in the etiology of some neurodegenerative diseases.

Key Words: 2-methoxyestradiol, neuronal cell death, neuron-like cells, SH-SY5Y, DNA ladder, TUNEL method, *in situ* apoptosis, apopain inhibitor

The molecular mechanism responsible for neuronal death in neurodegenerative disorders such as Alzheimer's disease is not known. However, accumulating evidence suggests that apoptosis contributes to this neuronal death (1-4). We previously described the colchicine-induced changes in neuronal cell death and in the characteristic ladder pattern of DNA fragmentation in human neuroblastoma SH-SY5Y cells differentiated with retinoic acid (RA) (5-7). In addition, a similar finding was reported by Bonfoco et al., who studied the effect of colchicine on apoptosis in cultured rat cerebellar granule cells (8). Based on these findings, the notion of an impaired microtubule system was proposed for neuronal apoptosis. A recent study by D'Amato et al. showed that the endogenous estrogen metabolite 2-methoxyestradiol inhibits tubulin polymerization by interacting at the colchicine site (9). The observations of D'Amato et al. led us to study the effect of 2-methoxyestradiol on cell death in neuronal cells.

Methods

Materials 2-Methoxyestradiol (1,3,5[10]-estratriene-2,3,17-triol 2-methyl ether), 2-methoxyestrone, 2-methoxyestriol, RNase A and *all-trans* retinoic acid were obtained

* To whom correspondence should be addressed. Fax : 81-285-53-1314

from Sigma (St.Louis,MO,U.S.A.). ß-Estradiol and colchicine were from Wako Pure Chemical (Osaka,Japan). Ac-Asp-Glu-Val-Asp-H(aldehyde) and Ac-Tyr-Val-Ala-Asp-H (aldehyde) were from Peptide Institute Inc.(Osaka,Japan). Cycloheximide and ethidium bromide were from Nacalai Tesque Inc.(Kyoto,Japan). Agarose, proteinase K, FX174 RF DNA/Hae III fragments, DAB (3,3'-diaminobenzidine tetrahydrochloride), and DNase I were from Gibco-BRL (Gaithersburg,MD,U.S.A.). Triton X-100 was from Boehringer Mannheim (Mannheim, Germany).

Cell culture Human neuroblastoma SH-SY5Y cells obtained from Dr. Biedler (Sloan-Kettering Institute,NY,U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM,Gibco) with 10 % fetal calf serum (FCS, Gibco), penicillin (100 U/ml,Gibco), and streptomycin (100 µg/ml, Gibco) in a humidified atmosphere of 95 % air, 5 % CO_2 at 37 ^oC, as described (7,10).

LDH release Lactate dehydrogenase (LDH) release was measured as described (7). Briefly, the cells (3 X 10^5) were seeded in 60 mm culture dishes (Falcon-PRIMARIA 3802). After culturing for 24 hr, 10 µM retinoic acid was added and cultured for 5 days in DMEM with 10 % FCS and antibiotics. Five days later, the medium was changed to DMEM containing 10 % FCS, then 2-methoxyestradiol was added. Three days later, cell toxicity was determined by the LDH release assay. LDH activity was measured using a colorimetric kit (Shino-test,Tokyo,Japan). LDH values were obtained after subtracting the mean background values in DMEM with 10 % FCS. The percent of LDH release was calculated as the amount released divided by the sum of the amount released, plus that present in the cell lysates.

Analysis of DNA ladder formation Analysis of DNA ladder formation was analyzed by agarose gel electrophoresis as described (7).

In situ apoptosis detection An *in situ* cell death detection kit (Boehringer Mannheim,Mannheim,Germany) was used according to vendor's protocol.

Results

Retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells were exposed to 2-methoxyestradiol for 3 days and photomicrographed (Fig.1). 2-Methoxyestradiol caused a loss of cell viability. In contrast to 2-methoxyestradiol, estradiol had no effect on cell viability (data not shown).

We next estimated cell damage by the release of the cytosolic enzyme lactate dehydrogenase (LDH), an index of cell mortality. As shown in Fig.2A, 2-methoxyestradiol produced an increase in the amount of LDH release into the culture media. The stimulatory effect upon LDH release was dose-dependent for 2-methoxyestradiol concentrations from 0.3 to 10 µM. The EC_{50} for stimulation was about 1 µM. In contrast to 2-methoxyestradiol, estradiol was quite inactive at concentrations up to 10 µM in stimulating LDH release. The results of the time course of the LDH release in response to 2-methoxyestradiol indicated that stimulation occurred after 2 days (Fig.2B). To examine whether LDH release could be similarly stimulated by 2-methoxylated steroid analogues, we tested the effects of 2-methoxyestrone and 2-methoxyestriol in differentiating neuroblastoma SH-SY5Y cells. As shown in Fig.2C, 2-methoxyestrone and 2-methoxyestriol were not as effective as 2-methoxyestradiol in stimulating LDH release.

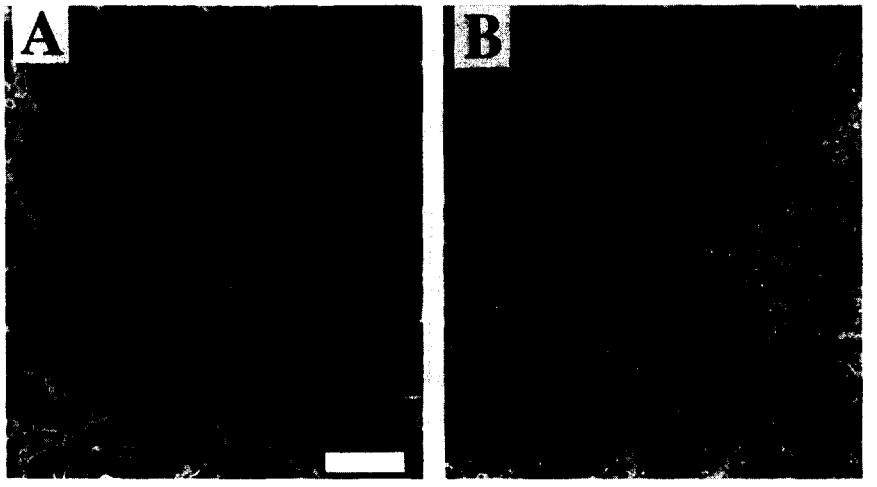

Fig.1
Phase-contrast photomicrographs of differentiating human neuroblastoma SH-SY5Y cell cultures. RA-differentiated cultures 3 days after exposure to vehicle (0.1% ethanol;A) or 2-methoxyestradiol (10 μM;B). Scale bar=100 μm.

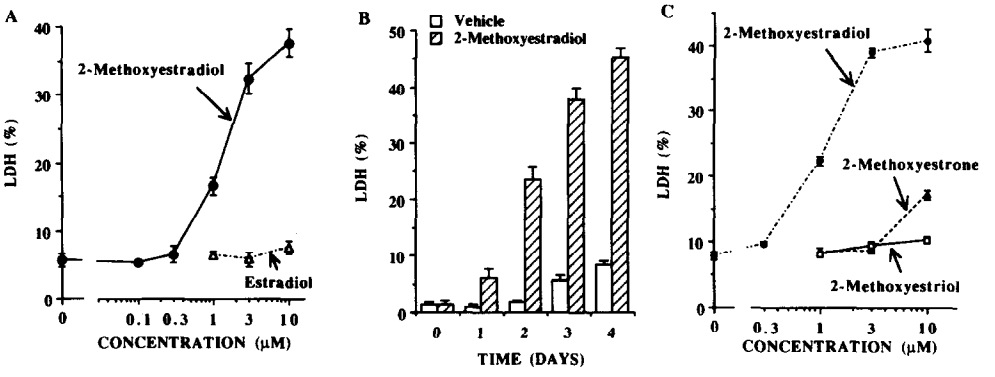

Fig.2
A) Dose-response curve of LDH release in differentiating SH-SY5Y cells stimulated by 2-methoxyestradiol. The cells were incubated with 2-methoxyestradiol or estradiol for 3 days. B) The time course of 2-methoxyestradiol neurotoxicity in differentiating SH-SY5Y cells. C) The effects of 2-methoxylated steroid analogues on cell death in differentiating SH-SY5Y cells. The cells were incubated for 3 days with 2-methoxyestradiol or 2-methoxylated steroid analogues. Values are the means ± SE on 4 replicate cultures.

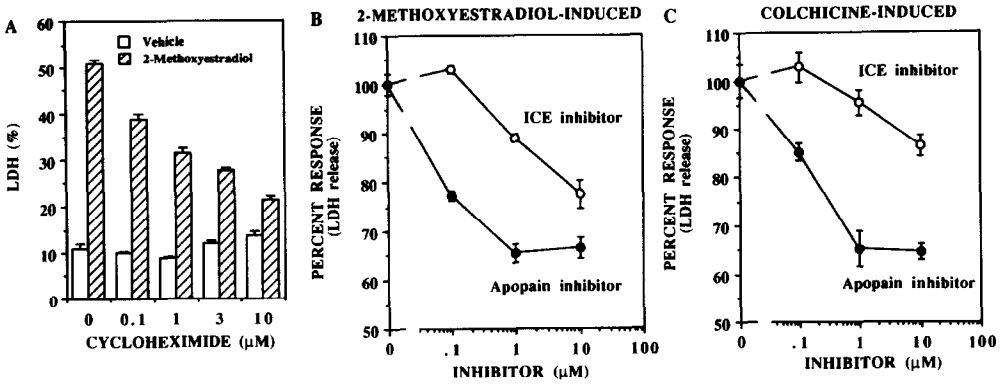

Fig.3

A) The effect of cycloheximide upon 2-methoxyestradiol-induced cell death in differentiating SH-SY5Y cells. The cells were incubated with cycloheximide for 15 hr before 2-methoxyestradiol (10 μM). After 3 days, the cell toxicity was quantified by the release of LDH. Values are the means ± SE on 3-4 replicate cultures. The effects of the apopain or ICE inhibitor upon 2-methoxyestradiol (B)- or colchicine (C)-induced cell death in differentiating SH-SY5Y cells. Inhibitor was added to cells 30 min prior to and during 2-methoxyestradiol (10 μM) or colchicine (1 μM) incubation (3 days). Values are the means ± SE on 4 replicate cultures.

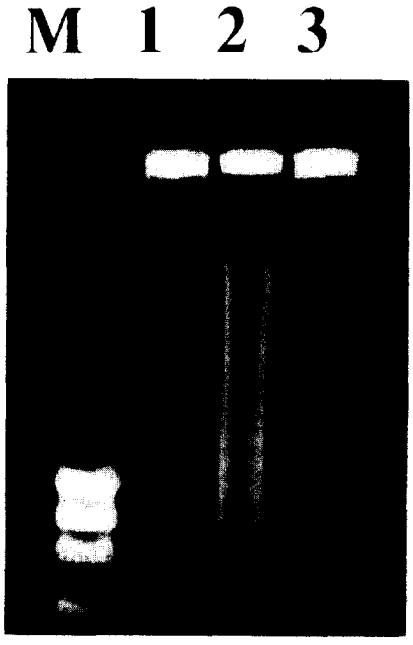

Fig.4

The effect of 2-methoxyestradiol on DNA fragmentation in differentiating SH-SY5Y cells. The cells were incubated for 3 days with vehicle (lane 1), 2-methoxyestradiol 10 μM (lane 2), or estradiol 10 μM (lane 3). M; molecular weight marker DNA (HaeIII digested FX174 DNA).

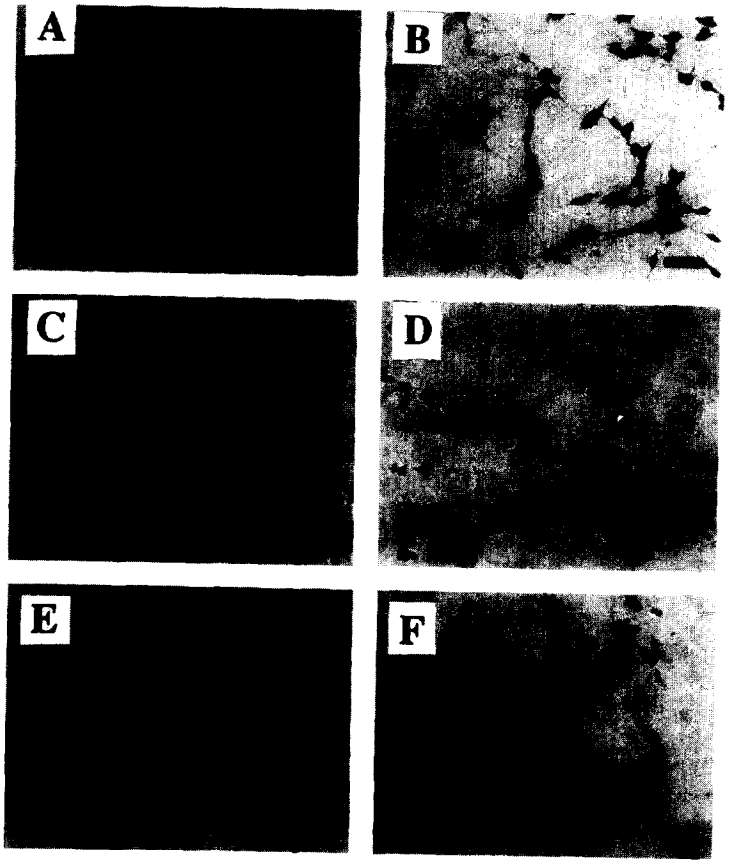

Fig.5
In situ apoptosis detection by TUNEL method in differentiating SH-SY5Y cell cultures. Negative control (without terminal transferase) showing no staining of cells (A). Positive staining control where DNA breaks have been introduced by DNase I treatment (B). RA-differentiated cultures 38 hr after exposure to vehicle (0.1% ethanol; C), 2-methoxyestradiol (10 μM, D), vehicle (E) or colchicine (1 μM; F). Scale bar = 50 μm.

Since the protein synthesis inhibitor cycloheximide blocks apoptosis in a variety of cells, we examined the effect of cycloheximide on 2-methoxyestradiol-induced cell death in differentiating neuroblastoma SH-SY5Y cells (Fig.3A). Exposing the cells to cycloheximide for 15 hours inhibited 2-methoxyestradiol-induced cell death. The inhibitory effect of cycloheximide upon 2-methoxyestradiol neurotoxicity was dose-dependent at concentrations from 0.1 to 10 μM. Thus 2-methoxyestradiol induction of cell death required cell protein synthesis. To understand the interleukin-1b-converting enzyme (ICE)-like proteases responsible for the 2-methoxyestradiol-induced cell death, we tested the effect of incubating the cells with the apopain inhibitor Ac-Asp-Glu-Val-Asp-H(aldehyde) (11). As shown in Fig.3B, adding apopain inhibitor to differentiating neuroblastoma SH-SY5Y cells inhibited the cell death elicited by 2-methoxyestradiol.

The inhibitory effect of apopain inhibitor upon 2-methoxyestradiol neurotoxicity was dose-dependent at concentrations from 0.1 to 10 μM. Also, there was slight inhibition with the ICE inhibitor Ac-Tyr-Val-Ala-Asp-H(aldehyde). Similar results were obtained using colchicine instead of 2-methoxyestradiol (Fig.3C). Thus 2-methoxyestradiol induction of cell death required apopain responsible for the cleavage of poly(ADP-ribose)polymerase.

We showed that changes occur in the characteristic ladder pattern of DNA fragmentation following colchicine exposure (7). We therefore tested the effect of 2-methoxyestradiol on DNA ladder formation in differentiating neuroblastoma SH-SY5Y cells by agarose gel electrophoresis. As shown in Fig.4, a DNA ladder of 180-200 base pairs was induced by exposing the cells to 2-methoxyestradiol (10 μM). Under these conditions, exposing the cells to estradiol (10 μM) did not lead to DNA fragmentation.

Since the ladder-like DNA fragments of nucleosomal sizes in agarose gel electrophoresis were observed, we next examined the effect of 2-methoxyestradiol on in situ apoptosis at single cell level, based on labeling of DNA strand breaks. Using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method, we observed typical positive staining in differentiating neuroblastoma SH-SY5Y cell cultures exposed to 2-methoxyestradiol for 38 hours (Fig.5D). In contrast, the cells exposed to vehicle for 38 hours had no staining (Fig.5C). Similar results were obtained using colchicine instead of 2-methoxyestradiol (Fig.5F).

Discussion

As far as we understand, this is the first report of an endogenous metabolite of estradiol inducing apoptotic neuronal cell death. The role of 2-methoxyestradiol in angiogenesis on endothelial cells has been documented (12). The present results indicated that 2-methoxyestradiol induces protein synthesis inhibitor- and apopain inhibitor-sensitive neuronal cell death, accompanied by the typical ladder pattern of DNA fragmentation found in apoptotic cells. Moreover, 2-methoxyestradiol caused an increase in TUNEL staining of free 3'-OH ends of fragmented DNA in situ. In this study, estradiol receptor stimulation is not likely to be involved since estradiol had no effect on cell death. In addition, the induction of cell death by 2-methoxyestradiol exposure appeared to be specific to 2-methoxyestradiol, since the closely related 2-methoxylated steroid analogue 2-methoxyestriol also had no effect on cell death.

Interestingly, our result obtained for apopain inhibitor Ac-Asp-Glu-Val-Asp-H(aldehyde) is in agreement with camptothecin-induced apoptosis of osteosarcoma cells recently presented by Nicholson et al. (11). However, this apoptosis by camptothecin was not inhibited by ICE inhibitor Ac-Tyr-Val-Ala-Asp-H(aldehyde). This apparent discrepancy may result from one or more of the following. (i) Differences in experimental condition : e.g., 2-methoxyestradiol- or colchicine-induced cell death in neuroblastoma cell cultures versus camptothecin-induced cell death in osteosarcoma cell cultures. (ii) Differences in detection system used for the estimation of cell death. The pharmacological modulation of neuron-selective apopain activity may be important to lead to therapeutic approaches for some types of degenerative diseases in human nervous system.

It is unclear at present whether similar neurotoxicity in differentiating neuroblastoma cells occur in vivo. In addition, the relevance of comparing the effectiveness of 2-methoxyestradiol levels in culture studies in vitro with that in plasma in vivo is questionable. However, levels of 2-methoxyestradiol in synaptic sites of brain are unknown. Whether the induction of apoptotic neuronal cell death observed here is of pathophysiological significance in human brain remains to be determined.

Acknowledgements

This work was supported in part by Japan Health Sciences Foundation.

References

1. D.E.BREDESEN, *Apoptosis II - The Molecular Basis of Apoptosis in Disease*, L.D.TOMEI and F.O.COPE (Eds), 397-421, Cold Spring Harbor Press, New York (1994).
2. J.H.SU, A.J.ANDERSON, B.J.CUMMINGS and C.W.COTMAN, NeuroReport 5 2529-2533 (1994).
3. H.LASSMANN, C.BANCHER, H.BREITSCHOPF, J.WEGIEL, M.BOBINSKI, K.JELLINGER and H.M.WISNIEWSKI, Acta Neuropathol. 89 35-41 (1995).
4. C.B.THOMPSON, Science 267 1456-1462 (1995).
5. Y.NAKAGAWA-YAGI, Bull. Jap. Neurochem. Soc. 32 84-85 (1993).
6. Y.YAGI, J. Neurochem. 61 suppl.S32 (1993).
7. Y.NAKAGAWA-YAGI, Biochem. Biophys. Res. Commun. 199 807-817 (1994).
8. E.BONFOCO, S.CECCATELLI, L.MANZO and P.NICOTERA, Exp. Cell Res. 218 189-200 (1995).
9. R.J.D'AMATO, C.M.LIN, E.FLYNN, J.FOLKMAN and E.HAMEL, Proc. Natl. Acad. Sci. USA 91 3964-3968 (1994).
10. Y.NAKAGAWA-YAGI, Y.SAITO, Y.TAKADA and M.TAKAYAMA, Biochem. Biophys. Res. Commun. 178 116-123 (1991).
11. D.W.NICHOLSON, A.ALI, N.A.THORNBERRY, J.P.VAILLANCOURT, C.K.DING, M.GALLANT, Y.GAREAU, P.R.GRIFFIN, M.LABELLE, Y.A.LAZEBNIK, N.A.MUNDAY, S.M.RAJU, M.E.SMULSON, T.T.YAMIN, V.L.YU and D.K.MILLER, Nature 376 37-43 (1995).
12. T.FOTSIS, Y.ZHANG, M.S.PEPPER, H.ADLERCREUTZ, R.MONTESANO, P.P.NAWROTH and L.SCHWEIGERER, Nature 368 237-239 (1994).