

Protective effects of estradiol on TRAIL-induced apoptosis in a human oligodendrocytic cell line: evidence for multiple sites of interactions

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Abstract

Demyelinating diseases are high impact neurological disorders. Steroids are regarded as protective molecules in the susceptibility to these diseases. Here, we studied the interactions between tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), a potent proapoptotic molecule toxic to oligodendrocytes, and 17- β -estradiol (E-17- β), in human oligodendrocytic MO3.13 cells. Exposure of cells to TRAIL resulted in the upregulation of both death receptors DR4 and DR5 and apoptosis, as well as the activation of caspase-8 and -3, increased phosphorylation of Jun-N-terminal kinase and p38 kinase, and the reduction of bcl-2 and bcl-xL proteins. TRAIL-mediated MO3.13 cell apoptosis was abrogated by the dominant-negative form of the adaptor protein FADD and by caspase inhibitors. Preincubation with E-17- β completely prevented both TRAIL-induced DR4 and DR5 upregulation and apoptosis. Estrogen-induced cytoprotection was time and concentration dependent and reverted by antiestrogens. Estrogen treatment *per se* reduced kinase phosphorylation, and upregulated bcl-2 and bcl-xL proteins. In conclusion, our data show that the detrimental role of TRAIL on oligodendrocytes can be effectively counteracted by estrogens, thus suggesting that the underlying molecular interactions can be of potential relevance in characterizing novel targets for therapy of demyelinating disorders.

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Abbreviations: CNS, central nervous system; E-17- β , 17- β -estradiol; ER, estrogen receptor; FADD, Fas-associated death

domain; JNK1, Jun-N-terminal kinase; MS, multiple sclerosis; TNF, tumour necrosis factor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand

Introduction

Demyelinating diseases affect a wide percent of population today. Thus, unraveling the mechanisms related to these diseases as well as the role of potential protective agents have become priority tasks.

It is a commonly accepted notion that immunoinflammatory phenomena are involved in the pathogenesis of demyelinating diseases, including multiple sclerosis (MS).¹ A crucial role in the initial event of MS has been attributed to oligodendroglia, a cell type actively contributing to myelin formation.² Accordingly, although the precise mechanism(s) underlying oligodendrocyte death during MS *poussées* has not yet been elucidated, the loss of these cells is one of the major factors for progression of the disease.^{3,4}

Interestingly, proinflammatory/proapoptotic cytokines, particularly those belonging to the tumour necrosis factor (TNF) superfamily, have been suggested to be, at least in part, responsible for increased cell death rate in the central nervous system (CNS), involving both the neuronal⁵ and the glial⁶ component. In this line, recent observations suggest that the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) could play a role in the pathogenesis of MS, as its increased activity is associated with detrimental effects on oligodendrocytes.^{7,8}

TRAIL is a recently characterized member of the TNF superfamily that binds to five specific membrane receptors.^{9,10} Among these, receptors DR4 and DR5 are type I membrane proteins and belong to the TNF/NGF receptors superfamily.^{9,11,12} DR4 and DR5 also contain a death domain in their cytoplasmic region and are able to transduce a TRAIL-induced death signal.^{9,10} The DcRI, a glycopospholipid-anchored cell surface protein, and DcRII, a receptor containing a truncated death domain, are decoy receptors that block TRAIL-induced apoptosis.^{11–13} A fifth TRAIL receptor, osteoprotegerin, has been characterized as a mediator of bone remodeling effects of the cytokine.^{14,15} Both DR4 and DR5 are distributed in the CNS of animal species and human,¹⁶ and mediate detrimental effects of TRAIL in brain ischemia¹⁷ and in β -amyloid-dependent neurotoxicity.¹⁸ Upon binding to DR4 and DR5, TRAIL activates the caspase-8 and, eventually, the Jun-N-terminal kinase (JNK)/p38 pathway through the mitogen-activated protein kinase cascade.^{19,20}

The observation that hormonal factors may influence susceptibility to MS has drawn attention on the possible protective effects of estrogens,²¹ whose receptors are expressed in different glial cells.²² For example, the severity of clinical symptoms of MS is significantly diminished during

late pregnancy,²³ when estrogen levels are significantly high. Such gender dimorphism in MS appears similar in animals with experimental autoimmune encephalomyelitis (EAE), a useful animal model for MS.^{24,25}

Interestingly, it has been reported that estradiol interferes with the caspase activation²⁶ and with different molecular events mediating TRAIL-induced cell death, such as the phosphorylation of the effector molecules JNK1 and p38 kinase.²⁷ In support of these data, others have shown that estradiol is able to prevent TRAIL-induced HeLa cell death through the prevention of reactive oxygen species production and inhibition of the p38 kinase.²⁷

Matysiak *et al.*²⁸ recently showed that human primary oligodendrocytes from human brain surgery *ex vivo* expressed TRAIL receptors and their exposure to TRAIL *in vitro* eventually resulted in cell death.

Such reports on the destructive role of TRAIL in the brain, as well as of the protective effect of estrogens, prompted us to study molecular events governing TRAIL-induced oligodendrocyte death and the possible interference of estrogens with such mechanisms.

To accomplish this task, we first investigated the expression of TRAIL and estradiol receptors in the human oligodendrocytic cell line MO3.13, a cellular hybrid of adult human oligodendrocyte and rhabdomyosarcoma cells, which expresses some properties of both oligodendrocytes and astrocytes.²⁹ Subsequently, we assessed the reciprocal effects of TRAIL and 17- β -estradiol (E-17- β) on these cells. We found that estrogens protect oligodendrocytes against TRAIL-induced apoptosis acting at different levels of the intracellular TRAIL death pathway.

Results

TRAIL receptors are expressed by human oligodendrocytic cells and mediate TRAIL-induced apoptosis

As TRAIL represents a substantial cause of oligodendrocyte death,²⁸ in order to confirm specificity of such effect, we first studied whether the MO3.13 cell line expressed the two TRAIL receptors DR4 and DR5. In line with previous data,²⁸ Reverse transcriptase-PCR (RT-PCR) analysis for mRNAs and Western blot analysis for proteins showed that MO3.13 cells express both DR4 and DR5 receptors (Figure 1a and b). In the experiments, HeLa cell samples were used as internal positive controls.²⁸

In further experiments, we evaluated the cytotoxic effect of TRAIL by adding 100 ng/ml of the cytokine to MO3.13 cell cultures. This concentration of TRAIL is known to induce cell death in most *in vitro* systems.^{10,18} As shown in Figure 2a, TRAIL induced death of cultured MO3.13 cells after 24 h incubation. The apoptotic nature of the cell death was indicated by both the Hoechst (Figure 2b; upper pictures) and the propidium iodide (Figure 2b; lower pictures) staining. Normal MO3.13 cells are shown in the left section of the figure, whereas the right section shows typical features of apoptotic cells, such as blebbing and accentuated nuclear staining (arrows).

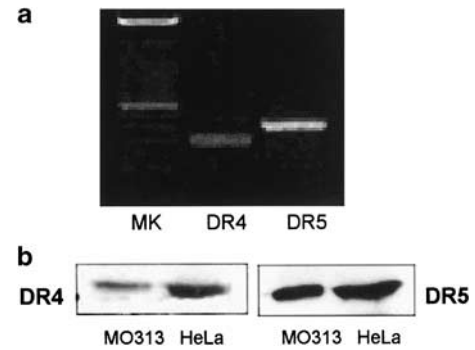


Figure 1 (a) RT-PCR analysis of the two TRAIL DR4 and DR5 receptor mRNAs in the human oligodendrocytic cell line MO3.13. Lane 1: 100 bp laddering (MK); lane 2: DR4; lane 3: DR5. (b) Western blot analysis of the TRAIL receptor DR4 in untreated human oligodendrocytic cells MO3.13 (lane 1); positive control for DR4 was run using HeLa cells (lane 2). (c) Western blot analysis of the TRAIL receptor DR5 in untreated human oligodendrocytic cells MO3.13 (lane 1); positive control for DR5 was run using HeLa cells (lane 2)

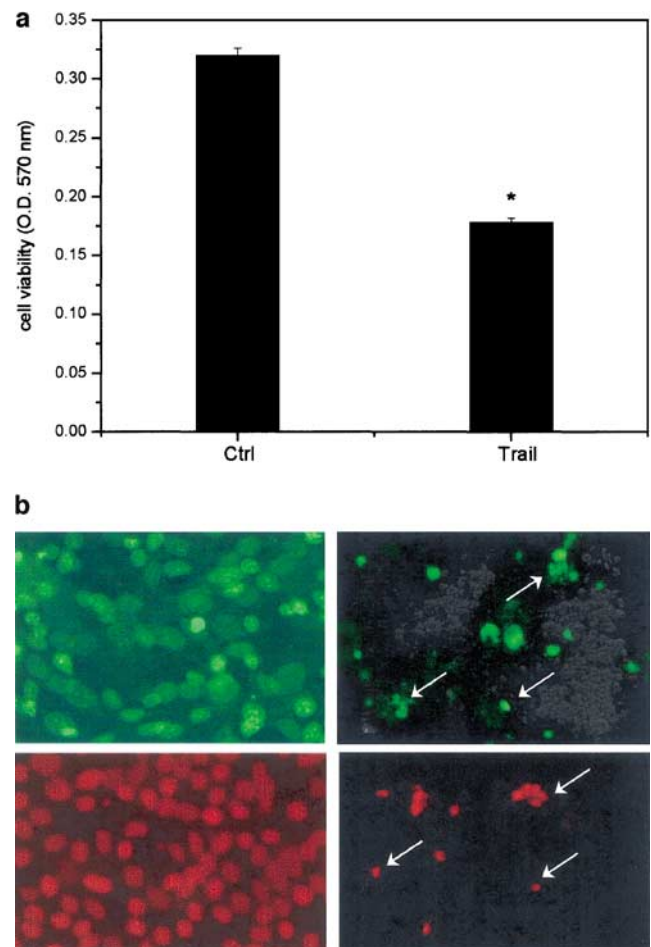


Figure 2 (a) Effects of TRAIL on cell death in the human oligodendrocytic cell line MO3.13. Cells were treated with 100 ng/ml of the cytokine for 24 h. Vertical bars are mean + S.E. of at least three separate experiments from two separate culture preparations; * $p < 0.05$ (Student's *t*-test). (b) Hoechst 33258 (upper section) and propidium iodide (lower section) staining performed upon the human oligodendrocytic cell line MO3.13 untreated (left pictures) or treated with TRAIL (100 ng/ml for 24 h; right pictures). Arrows point to cells showing features of apoptosis

E-17- β protects human oligodendrocytic cells from TRAIL-induced apoptosis

An additional set of experiments was carried out to evaluate the capacity of estrogens to counteract TRAIL-induced apoptotic cell death in MO3.13. To do so, we first assessed the presence of the two estrogen receptor (ER) ER α and ER β mRNAs by RT-PCR analysis. As shown in Figure 3a, both transcripts were present in MO3.13 cells. Samples from

MCF7 cells were used as experimental internal positive controls.³⁰

Secondly, we preincubated cells with graded concentrations of E-17- β (ranging from 0.2 to 30 nM) for 12, 24 and 48 h before TRAIL treatment. We found that E-17- β was significantly protected from TRAIL-induced cell death both in concentration- (data not shown) and time-dependent modes. The maximal effect of E-17- β occurred at a concentration of 2 nM for a 48 h incubation (Figure 3b).

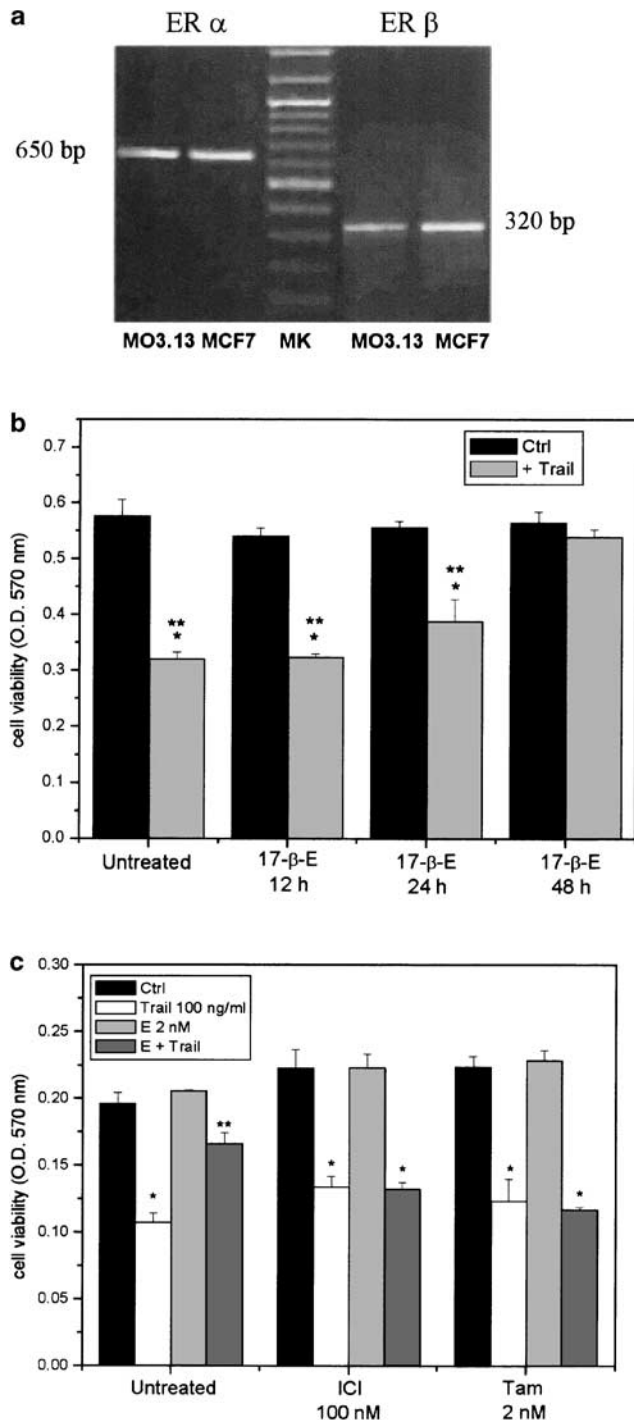
The specificity of the protective effects of E-17- β was proven by the ability of the two ER antagonists tamoxifene and ICI 187,580 (2 nM) to restore TRAIL-dependent cell death abrogating the protective effect of estradiol (Figure 3c) totally. The concentrations of the two ER antagonists used in these experiments were defined by *t* concentration–response analysis carried out previously (range: 10 pM–10 μ M; data not shown).

Protective effects of estradiol on TRAIL-induced cell death (Figure 4a) were also confirmed in experiments using specific staining for apoptosis (Figure 4b).

In addition, we attempted to provide evidence that estradiol could affect TRAIL receptor expression. Since we had previously demonstrated modulatory effects of TRAIL on its own receptors in neuronal-like cell line,¹⁸ we first investigated whether receptor expression could be modulated by the addition of the cytokine itself to MO3.13 cultures. Both DR4 and DR5 protein levels were increased by the addition of TRAIL (100 ng/ml) to the MO3.13 cell cultures after 24 h incubation (Figure 5).

DR4 and DR5 TRAIL-induced expression was effectively suppressed by treatment with E-17- β (2 nM) in a time-dependent manner, reaching maximum inhibition after 48 h (Figure 5).

Similar effect of estradiol on DR4 and DR5 expression was observed at the mRNA level in Northern blot experiments, suggesting that estrogens act at the transcriptional level (data not shown).



Mechanism of cytotoxic effects of TRAIL: role of caspases and intracellular interactions between estradiol and TRAIL

It has been suggested that the binding of TRAIL to its death-domain receptors leads to the activation of caspase-8, with

Figure 3 (a) RT-PCR analysis for the two ER ER α and ER β mRNAs in the human oligodendrocytic cell line MO3.13. Estradiol ER α and ER β receptors in the human oligodendrocytic cell line MO3.13 (respectively lanes 1 and 4) and in an ER-positive strain of the human breast cancer cell line MCF-7 (positive control, respectively, lanes 2 and 5); MK: 100 bp laddering (lane 3). (b) Time-dependent effect of E-17- β on TRAIL- (100 ng/ml) induced apoptosis in the human oligodendrocytic cell line MO3.13. Vertical bars are mean + S.E. of at least three separate experiments from two separate culture preparations; **p* < 0.05 (one-way ANOVA, followed by a Duncan test). (c) Abrogation of the protective effect of 2 nM E-17- β (48 h) in the human oligodendrocytic cell line MO3.13 treated for 24 h with TRAIL (100 ng/ml) by treatment with the two ER antagonists tamoxifen and ICI 187,580 (2 nM). The drugs were incubated together with or without E-17- β for 48 h. Vertical bars are mean + S.E. of at least three separate experiments from two separate culture preparations; **p* < 0.05 (one-way ANOVA, followed by a Duncan test)

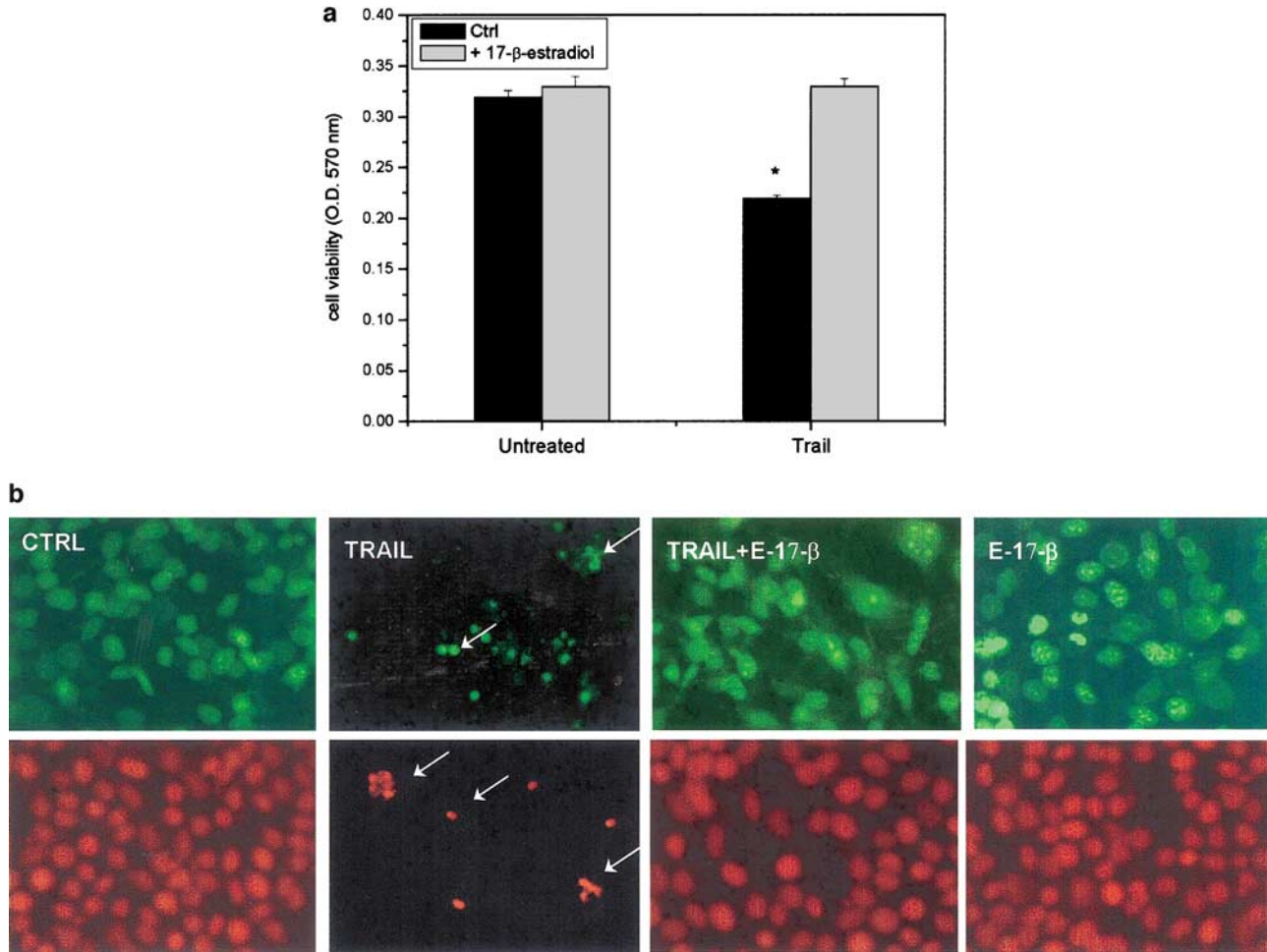


Figure 4 (a) Effect of 48 h pretreatment with E-17- β (2 nM) in the human oligodendrocytic cell line MO3.13, then treated for 24 h with TRAIL (100 ng/ml). Vertical bars are mean + S.E. of at least three separate experiments from two separate culture preparations; * $p < 0.05$ (one-way ANOVA, followed by a Duncan test). (b) Hoechst 33258 (upper section) and propidium iodide (lower section) staining performed upon the human oligodendrocytic cell line MO3.13 treated as indicated. TRAIL: 100 ng/ml for 24 h; E-17- β : 2 nM, added 48 h prior to TRAIL. Arrows point to cells showing features of apoptosis

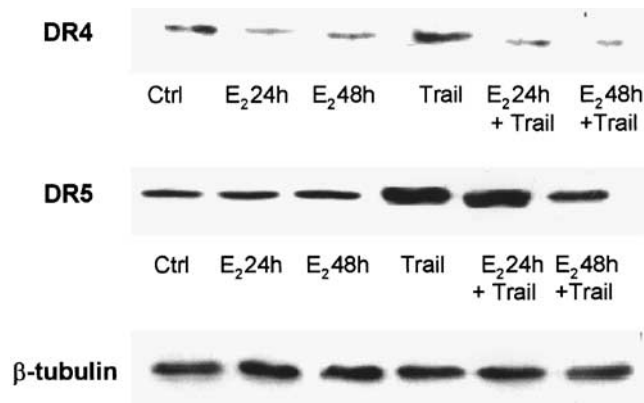


Figure 5 Effects of 17- β -E upon DR4 and DR5 TRAIL receptor and β -tubulin expression in the human oligodendrocytic cell line MO3.13. Western blot analysis was performed as follows: lane 1, untreated cells; lane 2, cells treated 24 h with E-17- β (2 nM); lane 3, cells treated 48 h with E-17- β (2 nM); lane 4, cells treated 24 h with TRAIL (100 ng/ml); lane 5, cells preincubated 24 h with E-17- β and then treated with TRAIL for additional 24 h; lane 6, cells preincubated 48 h with E-17- β and then treated with TRAIL for additional 24 h. Data represent results from at least two different experiments in different cultures

the subsequent activation of caspase-3, finally resulting in apoptosis. Caspase-8 activation via TRAIL may occur, in turn, through the recruitment of the adaptor protein FADD. The involvement of FADD in apoptosis of MO3.13 cells was examined by overexpressing FADD or its dominant-negative form (FADD-DN) in these cells. As shown in Figure 6a, overexpression of FADD caused cytotoxic effects leading to about 50% of cell viability 48 h after transfection. The extent of cell loss was comparable to that found after the exposure of cells to TRAIL. The cytotoxic effects induced by FADD and TRAIL was not observed in cells overexpressing the FADD-DN.

The involvement of caspase-8 was also investigated by studying cleavage of procaspase-8 by Western blot analysis. We found that TRAIL induced cleavage of caspase-8 in MO3.13 cells and this effect was completely prevented when cells were preincubated with E-17- β for 48 h (Figure 6b).

To better characterize caspase-8 activation and the subsequent involvement of caspase-3 in MO3.13 cell death induced by TRAIL, cells were preincubated with either the specific caspase-8 inhibitor z-IETD-FMK (2 μ M) or the specific

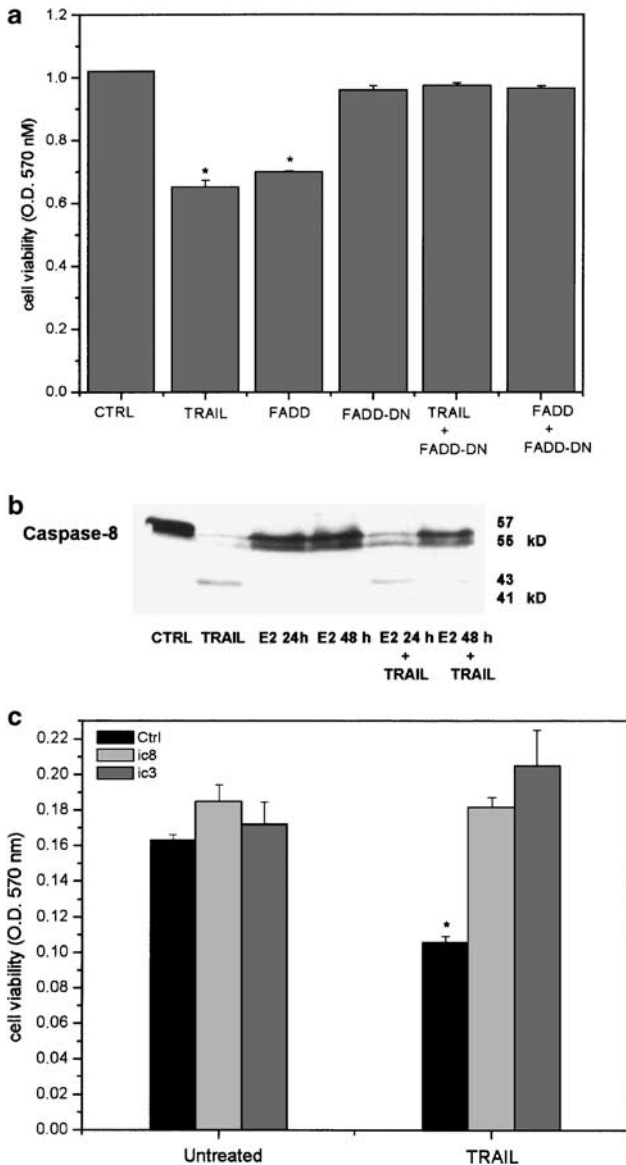


Figure 6 (a) Loss of FADD activity abrogates TRAIL toxicity in the human oligodendrocytic cell line MO3.13. Cells were transfected with plasmid with no insert (pcDNA), FADD (pc FADD), or FADD dominant negative (pc FADD DN) and, 48 h later, incubated, where indicated, for 24 h with TRAIL. Cell viability was determined by the crystal violet staining. Vertical bars represent the mean \pm S.E. of at least three separate experiments from two separate culture preparations. * $p < 0.05$ versus other experimental groups (one-way ANOVA followed by a Duncan test). (b) Inhibitory effects of E-17- β on caspase-8 activation in the human oligodendrocytic cell line MO3.13. Western blot analysis was performed as follows: lane 1, untreated cells; lane 2, cells treated 24 h with TRAIL (100 ng/ml); lane 3, cells treated 24 h with E-17- β (2 nM); lane 4, cells treated 48 h with E-17- β (2 nM); lane 5, cells preincubated 24 h with E-17- β and then treated with TRAIL for additional 24 h; lane 6, cells preincubated 48 h with E-17- β and then treated with TRAIL for additional 24 h. Caspase-8 is identified in its proenzyme and cleaved forms, respectively, 57–55 and 43–41 kDa. (c) Effects of the specific caspase-8 inhibitor z-IETD-FMK (2 μ M) or the specific the caspase-3 inhibitor Z-DEVD-FMK (2 μ M) in the human oligodendrocytic cell line MO3.13 exposed to 100 ng/ml TRAIL for 24 h. Cell viability was determined by the crystal violet staining. Vertical bars represent the mean \pm S.E. of at least three separate experiments from two separate culture preparations. * $p < 0.05$ versus control values (one-way ANOVA followed by a Duncan test)

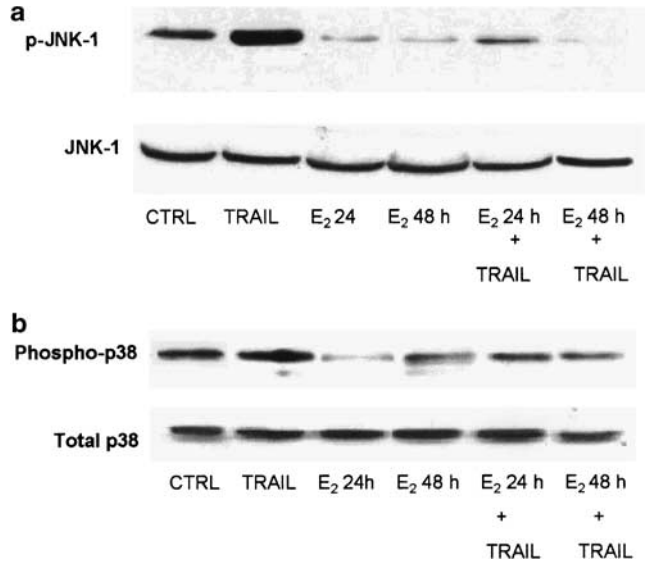


Figure 7 (a) Inhibitory effects of 17- β -E on TRAIL-dependent JNK1 phosphorylation in the human oligodendrocytic cell line MO3.13. Western blot analysis was performed as follows: lane 1, untreated cells; lane 2, cells treated 2 h with TRAIL (100 ng/ml); lane 3, cells treated 24 h with E-17- β (2 nM); lane 4, cells treated 48 h with E-17- β (2 nM); lane 5, cells preincubated 24 h with E-17- β and then treated with TRAIL for additional 2 h; lane 6, cells preincubated 48 h with E-17- β and then treated with TRAIL for additional 2 h. (b) Inhibitory effects of E-17- β on TRAIL-dependent p38 kinase phosphorylation in the human oligodendrocytic cell line MO3.13. Western blot analysis was performed as follows: lane 1, untreated cells; lane 2, cells treated 2 h with TRAIL (100 ng/ml); lane 3, cells treated 24 h with E-17- β (2 nM); lane 4, cells treated 48 h with E-17- β (2 nM); lane 5, cells preincubated 24 h with E-17- β and then treated with TRAIL for additional 2 h; lane 6, cells preincubated 48 h with E-17- β and then treated with TRAIL for additional 2 h. In both panels, the upper part of the blot represents the phosphorylated form of the protein; the lower part its unphosphorylated form. Data represent results from at least two different experiments in different cultures

caspase-3 inhibitor Z-DEVD-FMK (2 μ M). Both compounds were able to prevent death of MO3.13 cells incubated 24 h with TRAIL (Figure 6c).

As it has been shown that TRAIL induces apoptosis through the JNK/p38 kinase pathway and that such activation requires cleavage of caspase-8,^{19,20} we investigated the involvement of these kinases in our system.

Indeed, Western blot analysis showed that, whereas basal phosphorylation of JNK1 was significantly increased by TRAIL, it was reduced by E-17- β alone. Addition of TRAIL in cultures pretreated with E-17- β for 24 h partially restored phosphorylation of JNK1, but failed to restore phosphorylation after 48 h incubation with the estrogen (Figure 7a). A similar pattern was observed for p38 kinase (Figure 7b).

E-17- β prevention of TRAIL-induced apoptosis is associated with an increase in the antiapoptotic genes bcl-2 and bcl-xL expression

Based on reports that E-17- β protects glial cells from apoptotic death by inducing the expression of antiapoptotic proteins of the bcl family, we investigated the role of bcl-2 and bcl-xL expression in MO3.13 cells treated with TRAIL, by Western blot analysis.

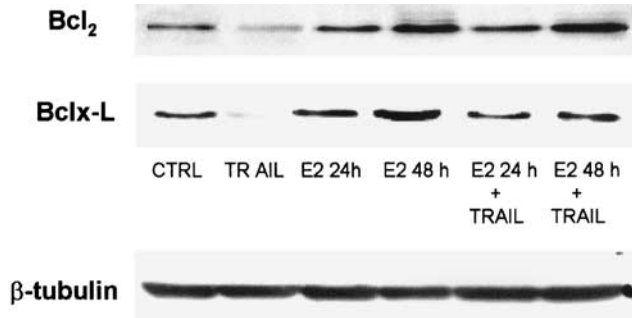


Figure 8 Effects of E-17- β upon bcl-2 and bcl-x-L proteins, and β -tubulin expression in the human oligodendrocytic cell line MO3.13. Western blot analysis was performed as follows: lane 1, untreated cells; lane 2, cells treated 24 h with TRAIL (100 ng/ml); lane 3, cells treated 24 h with E-17- β (2 nM); lane 4, cells treated 48 h with E-17- β (2 nM); lane 5, cells preincubated 24 h with E-17- β and then treated with TRAIL for additional 24 h; lane 6, cells preincubated 48 h with E-17- β and then treated with TRAIL for additional 24 h. Data represent results from at least two different experiments in different cultures

Both bcl-2 and bcl-xL were found to be expressed at significant levels in untreated cells. Treatment of cells with TRAIL resulted in the downregulation of both bcl-2 and bcl-xL, while treatment with estrogen for 24 or 48 h resulted in the upregulation of both proteins. When combined, TRAIL was unable to abolish the effect of E-17- β (Figure 8).

Discussion

It has been hypothesized that estradiol may play a neuroprotective role.^{31,32} However, the molecular mechanisms of interaction of female steroids with noxious stimuli remain, so far, largely unclarified. For the first time, in the present paper, we provide evidence for protective effects of estradiol on oligodendrocyte death caused by the powerful proapoptotic cytokine TRAIL.

First, by using different methods, we have shown that TRAIL caused death of the human oligodendrocyte cell line MO3.13, which expresses both functional TRAIL receptors DR4 and DR5, supporting the previously described effects of the cytokine upon primary human oligodendrocytes.²⁸ Accordingly, such TRAIL-induced oligodendrocyte death was of apoptotic nature.

Then, in the attempt to characterize the TRAIL death pathway, we found that TRAIL upregulates its receptors in MO3.13 cells. This confirms our previous observation that DR5 receptor expression on human neuronal cell lines exposed to β -amyloid is upregulated also by TRAIL.¹⁸ Thus, the subsequent activation of TRAIL surface death receptors DR4 and DR5 may finally trigger an apoptosis program in oligodendrocytic cells. Our data are in accordance with the general concept that cytokines can regulate their own receptors,^{33–36} in either a paracrine or an autocrine manner, and with the observation that oligodendrocytes remain sensitive to TRAIL-induced apoptosis, in conditions where other cells, such as microglia, become insensitive to it. In fact, TRAIL upregulates its own decoy receptors in the latter cell type, thus neutralizing the effects of the molecule.²⁸

It is known that, after stimulation by TRAIL, the apoptosis-transducing receptors DR4 and DR5 homo- or heterotrimerize and then bind to downstream death-domain proteins, such as FADD, TRADD and RIP, depending upon the cellular system.^{37,38} The recruitment of FADD seems crucial since it allows the activation of caspase-8 leading in turn to cell death.^{37,38} Our results show that TRAIL activates caspase-8 through FADD and the lack of FADD function in cells transfected with its DN form rescues them from TRAIL-induced cell death. We also demonstrated that TRAIL effects are mediated both by caspase-8 and -3, since the addition of the respective specific inhibitors of the two enzymes to cultures resulted in the complete suppression of TRAIL-induced cell death.

Moreover, as TRAIL-activated caspases also imply the activation of the downstream molecules JNK1 and p38 kinase, we have observed TRAIL-induced phosphorylation of both proteins during an incubation of 1–2 h, a time considered as optimal to observe TRAIL effects.³⁹ Finally, the exposure of cells to TRAIL also resulted in the downregulation of two antiapoptotic proteins belonging to the bcl family, namely bcl-2 and bcl-xL.

Oligodendrocytic MO3.13 cells also expressed both ER subtypes α and β , thus suggesting that these cells could be responsive to estradiol. In fact, when the steroid was added to the culture media prior to TRAIL, we observed a significant, concentration-dependent decrease in the number of dead oligodendrocyte. Nuclear staining data supported cell viability experiments, suggesting that estradiol prevents TRAIL-induced apoptosis of MO3.13 cells. The beneficial effect of estradiol was specific, as the addition to the cultures of the two ER antagonists tamoxifen or ICI 187,580 at concentrations not influencing protein synthesis and known to antagonize estradiol effects in most *in vitro* systems,^{40,41} resulted in restored cell death rate after addition of TRAIL.

Interestingly, estradiol substantially prevented TRAIL-induced DR4 and DR5 expression. It appears reasonable to hypothesize that protective effects of estradiol could be mediated, at least in part, by its ability to downregulate TRAIL receptor molecules at the transcriptional level.

Interestingly, literature reports that estradiol also inhibits caspase-3 in rat mesencephalic neurons *in vitro*,⁴² and, in addition, inhibits caspases through a receptor-mediated, nongenomic induction of a specific inhibitor, CIF, in primary human neurons.²⁶ In accordance with these data, our present findings show that the treatment of MO3.13 cells with estradiol resulted in the inhibition of TRAIL-induced caspase-8 activation.

There are controversial hypotheses on the mechanism underlying neuroprotective effects of estradiol, as literature suggests that it might make cells more resistant to injuries,⁴³ or rather attenuate toxicity phenomena mediated by non-neuronal cells,^{44,45} including, for example, glial production of proinflammatory cytokines, such as IL-1, TNF- α ⁴⁶ and others. In this regard, our data indicated that estrogen treatment *per se* causes a reduction in the JNK1 and p38 kinase phosphorylation levels and upregulation of bcl-2 and bcl-xL protein contents, supporting the first theory.

To summarize, we have found that estradiol efficaciously prevents TRAIL-induced oligodendrocytic cell death. Such

estradiol-dependent inhibition of TRAIL-induced apoptosis appears mediated by (1) direct effects on the cells, including decreased phosphorylation state of the caspase-related JNK1 and p38 kinases and increased levels of the antiapoptotic proteins of the bcl family, and (2) specific action on the intracellular TRAIL death pathway, such as the prevention of TRAIL-induced DR5 expression. These effects may contribute together to generate oligodendrocytes more resistant to different apoptotic stimuli, including TRAIL. Along with the involvement of oligodendrocytes in demyelinating processes,^{47,48} and with the protective properties of estrogens in murine EAE,^{49,50} our findings suggest novel potential targets for estrogen therapy of MS, and provide further rationale for therapeutic intervention.

Materials and Methods

Cell cultures and reagents

All materials and media were from Invitrogen Srl, San Giuliano Milanese, Italy, unless otherwise specified. MO3.13 cells were a kind gift from Professor Catherine Waters, Division of Neuroscience, University of Manchester, Manchester, UK.

MCF-7, HeLa and MO3.13 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin (100 U/ml) and streptomycin (100 µg/ml), and kept at 37°C in humidified 5% CO₂. MO3.13 were differentiated in DMEM without serum and containing 100 nM 4-β-phorbol 12 myristate 13-acetate (PMA) for 3 days prior to an experiment as described.⁵¹

MO3.13 viability assay

A total of 1.5×10^3 cells/well were plated in 96-multiwell plates in DMEM without serum containing 100 nM PMA to be differentiated. After 72 h, differentiated cultures were incubated for 48 h with 17-β-E (2 nM) alone or with tamoxifene (2 nM) (Sigma-Aldrich, St. Louis, MO, USA) or ICI 182.780 (100 nM) (Tocris, UK) in phenol red-free DMEM with 1% FCS. Then, the medium was replaced with fresh medium containing TRAIL (100 ng/ml) and its potentiator (1.5 µg/ml; potentiator-enhanced TRAIL-induced cell death; Upstate Biotechnology, Lake Placid, NY, USA) and incubated for other 24 h.

In the other experiment, after the differentiation cells were incubated with 100 ng/ml TRAIL and its potentiator (1.5 µg/ml) either alone or, respectively, with the caspase-8 inhibitor z-IETD-FMK (2 µM; Alexis Biochemicals, San Diego, CA, USA), and the caspase-3 inhibitor DEVD-FMK (2 µM; Alexis Biochemicals, San Diego, CA, USA) in serum-free DMEM at 37°C for 24 h. The caspase inhibitors were added 30 min before the TRAIL addition.

At the end of the experiments, cells were stained with 0.5% crystal violet solution for 30 min, washed with bidistilled water and lysed in 10% acetic acid for 15 min. Optical density was read at 570 nm.

Transfection

A total of 5×10^3 cells/well were plated in 96-well plates in DMEM without serum containing 100 nM PMA to be differentiated. 24 h before transfection, the medium was replaced by 10% FBS DMEM without antibiotics. The expression vectors for pcDNA Fas-associated death domain (FADD) and its dominant negative (pcDNA FADD-DN) were kindly provided by Dr. Marcus Schuchmann (Klinikum der Johannes Gutenberg,

Universitat, Mainz, Germany). The plasmid DNA (300 ng) was diluted in 25 µl of serum and antibiotic-free DMEM/well, and 0.5 µl of LF2000 reagent were diluted into 25 µl/well in the same media in a different tube and incubated for 5 min at room temperature. The diluted DNA and LF2000 reagent were then combined and incubated at room temperature for 30 min. The resulting complexes were directly added to cultures (50 µl/well) and incubated for 5 h. The transfection medium was then replaced with the growth medium, containing 10% FBS without antibiotics. Cells were cultured for additional 48 h before undergoing specific treatments. Cell viability was evaluated by the crystal violet method.

Western blot analysis

A total of 6×10^4 cells/well were plated in 6 cm Petri dishes and grown to 50% confluence in DMEM without serum containing 100 nM PMA as a differentiating agent.⁵¹ After 72 h, differentiated cells were incubated for 24 or 48 h with E-17-β (2 nM) in phenol red-free DMEM with 1% FCS. Then, medium was replaced with fresh medium containing TRAIL (100 ng/ml) and incubated, respectively, for 2 additional hours, in the JNK/p38 kinase phosphorylation experiments, or 24 additional hours in all other cases.

Cells were harvested in 100 µl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, 0.5 µg/µl leupeptin, 5 µg/µl aprotinin and 1 µg/ml pepstatin. The samples were sonicated and centrifuged at $15000 \times g$ for 30 min at 4°C. The resulting supernatants were isolated and the protein content determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL, USA). Protein extracts (15 µg) underwent electrophoresis on 12% SDS-PAGE, and were transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Filters were incubated at room temperature overnight with polyclonal anti-DR5, anti-DR4 (Alexis Biochemicals, San Diego, CA, USA), polyclonal anti-JNK-1 and monoclonal pJNK-1, polyclonal anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal antiphospho p38 (Promega Italia, Milan, Italy), monoclonal anticaspase-8 (Cell Signaling Technology Inc., USA), monoclonal anti-Bcl₂ and polyclonal anti-Bcl-xL (Upstate Biotechnology, NY, USA) or anti-tubulin (Ab3 Neo Markers) antibody in 5% nonfat dried milk (Sigma). Secondary antibodies (Amersham Italia S.r.l., Milan, Italy) and a chemiluminescence blotting substrate kit (Amersham Italia S.r.l., Milan, Italy) were used for immunodetection.

Evaluation of apoptosis

A total of 6×10^4 cells were plated onto glass coverslips in 6 cm Petri dishes and cultured as described above. For quantitation of apoptosis, cells were stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA)⁵² or propidium iodide⁵³ (Sigma-Aldrich, St. Louis, MO, USA) and nuclear morphological changes, such as chromatin condensation and fragmentation, were examined under a fluorescent microscope (Leica).

RT-PCR

Total RNA from cells grown to 80% confluence was isolated after solubilization in guanidinium thiocyanate by phenol-chloroform extraction and precipitation.⁵⁴ For first-strand cDNA synthesis, 1 µg of total RNA was reverse-transcribed using 25 µg/ml oligo(dT)₁₂₋₁₈ primer in a final volume of 20 µl, in the presence of 200 U of M-MLV reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy). The reaction was carried out at 37°C for 1 h and heated at 95°C for 10 min, and subsequently for 5 min at 4°C. PCR was performed in a total volume of 100 µl, containing 5 µl of the cDNA, 5 pmol of each upstream and downstream primer and 1.8–U of

Taq polymerase (Invitrogen, San Giuliano Milanese, Italy). The cycle program for (a) human DR4 and human DR5 primers consisted of 35 runs of denaturation at 95°C for 45 s, annealing at 60°C for 1 min and elongation at 72°C for 1 min; (b) human ER α primers consisted of 35 runs of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min; (c) human ER β primers consisted of 30 runs of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min. PCR products were analyzed by 1.0% agarose-gel electrophoresis and visualized with ethidium bromide. The following RNA transcripts were detected via the amplification of the corresponding cDNAs: the human DR4 using a primer pair composed of the sense primer 5'-ACTCGCTGTCCACTTTCGTCTCTGA-3' and the antisense primer 5'-CATCCCCTGGCCCTGCTGTGTA-3'; the human DR5 using a primer pair composed of the sense primer 5'-GGGAGCCGCTCATGAGGAAGTT-3' and the antisense primer 5'-CTGGGTGATGTTGGATGGGAGAGT-3'; the human ER α using the primer set composed of the sense 5'-TACTGCATCAGATCCAAGGG-3' and antisense 5'-ATCAATGGTGCCTGTTGG-3'; the human ER β using the primer pair composed of the sense 5'-GATGAGGG-GAAATGCGTAGA-3' and the antisense 5'-CTTGTTACTCCATGCCTGA-3'; the human GADPH using the primer set composed of the sense 5'-CCACCCATGGCAAATCCATG-3' and antisense 5'-TCTAGACGG-CAGGTCAGGTCCACC-3'.

Northern blot analysis

A total of 3×10^6 cells/well were plated in 10 cm Petri dishes and grown to 50% confluence in DMEM without serum containing 100 nM PMA as a differentiating agent.⁵¹ After 72 h, differentiated cells were incubated for 24 or 48 h with E-17- β (2 nM) in phenol red-free DMEM with 1% FCS. Then, medium was replaced with fresh medium containing TRAIL (100 ng/ml) and its potentiator (1.5 μ g/ml) and incubated for 6 additional hours.

Total RNA from cells grown to 80% confluence was isolated after solubilization in guanidinium thiocyanate by phenol-chloroform extraction and precipitation.⁵⁴

Northern blot analysis was performed according to standard protocols. Total RNA (20 μ g) was electrophoresed on a formaldehyde-containing 1.2% agarose-gel. The RNA was transferred to a nylon membrane for hybridization with ³²P-labeled cDNA probes. The hybridized RNA was detected by autoradiography.

The cDNA probes of DR4, DR5 receptors and the GADPH were synthesized by RT-PCR as described above. The resulting cDNAs were purified by High Pure PCR product (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. Then, the probes were labeled with [³²P]dCTP by random primer labeling (Invitrogen s.r.l., San Giuliano Milanese, Italy).

Statistical analysis of results

Results were analyzed by either one- or two-way analysis of variance (ANOVA), followed by Duncan's least significant difference test. Where appropriate, the Student's *t*-test was applied for *in vitro* data. A *p*-value < 0.05 was considered significant.

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