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General Pharmacology 35 (2001) 1–9

General Pharmacology
The Vascular System

17β-Estradiol protects lymphocytes against dopamine and iron-induced apoptosis by a genomic-independent mechanism Implication in Parkinson's disease

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Received 8 November 2000; received in revised form 17 May 2001; accepted 17 May 2001

Abstract

Dopamine (DA) in combination with iron (Fe^{2+}) has been demonstrated to induce apoptosis in neuronal-like PC12 cells by an oxidative stress mechanism. To get a better insight of cell death and protective mechanisms in DA/Fe^{2+}-induced toxicity, we investigated the effects of DA/Fe^{2+} and the antioxidant action of 17β-estradiol (E2) in peripheral blood lymphocytes (PBL). We found that DA/Fe^{2+}-induces apoptosis in PBL via a hydrogen peroxide (H_2O_2)-mediated oxidative mechanism, which in turn triggers a cascade of molecular events requiring RNA and de novo protein synthesis. We have also demonstrated that E2 prevents significantly DA/Fe^{2+}-induced apoptosis in PBL by directly inhibiting the intracellular accumulation of peroxides generated by DA/Fe^{2+}-reaction. This protective activity is independent of the presence or activation of the estrogen receptors (ERs). These data further support and validate our previous hypothesis that DA/Fe^{2+}/H_2O_2 could be a general mediator of oxidative stress through a common cell death mechanism in both neuronal and nonneuronal cells. These findings may be particularly relevant to the potential approaches to rescue and prolong the survival of neurons by estrogens in patients with Parkinson's disease (PD). © 2001 Elsevier Science Inc. All rights reserved.

1. Introduction

Parkinson's disease (PD) is characterized by selective degeneration of the nigrostriatal containing the neurotransmitter dopamine (DA). Although the mechanism by which this neuronal loss occurs still remains unclear, oxidative stress has been proposed to play an important role in the pathogenesis (Fanh and Cohen, 1992; Jenner, 1998). One of the major contributors to oxidative stress in PD may be DA. Indeed, DA turnover is elevated in PD brain (Kopin, 1985) and it can form reactive metabolites through either DA metabolism by monoamine oxidase (MAO) or autoxidation of the catechol ring (Graham, 1978; Riederer and Youdim, 1986; Hastings et al., 1996). DA oxidation may produce an increase of hydrogen peroxide (H_2O_2), which in turn interacts with transition metals to form hydroxyl radicals ($OH^{\cdot}$), which can damage DNA, proteins and lipids (Halliwell, 1992) or may act as a second intermediate in intracellular signaling (Hampton and Orrenius, 1997; Lee and Um, 1999). Additionally, the autoxidation of DA produces semiquinones and quinones, which are capable of binding covalently to proteins (Ito et al., 1988; Jimenez Del Rio et al., 1993; Hastings and Zigmond, 1994; Velez-Pardo et al., 1995) and redox cycling (Velez-Pardo et al., 1996).

It has been demonstrated that, besides DA, iron could also play an important role in the pathogenesis of idiopathic PD (Gerlach et al., 1994; Hirsh and Faucheux, 1998). Indeed, the substantia nigra from parkinsonian patients contains high levels of total iron and reduced ferritin buffering (Dexter et al., 1991; Riederer et al., 1989; Hirsh and Faucheux, 1998) and it has been shown to produce parkinsonism in the rat when iron is injected intranigral (Ben-Shachar and Youdim, 1991). Several animal models as well as neuronal and nonneuronal cell lines have been used to investigate the toxic effect of iron and catecholamines independently (Michel and Hefti, 1990; Ben-Shachar and Youdim, 1991; Sengstock et al., 1992; Mytilineou et al., 1993; Burke et al., 1997; Walkinshaw and Waters, 1994, 1995; Tsao et al., 1996; Zilkhafalb et al., 1997; Blum et al., 1997). We have demonstrated that DA in the presence of iron (Fe^{2+}) was capable of inducing apoptosis in undifferentiated sympathetic neuron-like PC12

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PII: S0306-3623(01)00082-9

cells (Velez-Pardo et al., 1997). The apoptotic cell death process was consistent with compartmentalized cytotoxic events at the level of the nucleus (chromatin condensation and fragmentation), cytoplasm (high molecular-weight protein–DA conjugates) and membrane (lipid peroxidation). Moreover, we have also demonstrated that depletion of Ca^{2+} may contribute or even lead to apoptosis in PC12 cells (Velez-Pardo et al., 1998). Accordingly, we have recently postulated a molecular model wherein DA/Fe^{2+} may trigger the activation of an ordered cascade of molecular events of cell death machinery in PD (Jimenez Del Rio and Velez-Pardo, 2000). However, the precise mechanisms by which DA/Fe^{2+} induce apoptosis is not yet well established.

The growing understanding of the importance of oxidative stress in PD has allowed a direct search for new neuroprotective strategies. Research has focused on ways to block metabolic pathways that lead to free radical generation, such as MAO B inhibitors, scavengers of free radicals, enhancer of antioxidants defenses, and DA agonists (Simon and Standaert, 1999; Dunnett and Bjorklund, 1999; Hubble, 1999). Recently, estrogens have been implicated in the regulation of neuronal cell death and survival in neurodegenerative disease. In fact, 17β-estradiol (E2) protects dopaminergic neurons from oxidative stress-induced cell death by glutamate, superoxide anions and hydrogen peroxide (Sawada et al., 1998) and decreased striatum neurotoxicity induced by infusion of 6-hydroxydopamine (6OHDA; Deluzen, 1997). Although E2 has been shown to have antioxidant activity, the neuroprotective molecular mechanisms are largely unknown.

To get insight into these issues, we were interested to investigate the protective effect of E2 against DA and iron cytotoxicity in peripheral blood lymphocytes (PBL). Our first goal was to understand the response of PBL to oxidative stress induced by DA and Fe^{2+}. Thus, we began to characterize the molecular mechanism underlying oxidative DA and Fe^{2+} toxicity. Recent studies suggest that E2 protects against oxidative stress either by a genomic (Pike, 1999) or by a nongenomic mechanism (Deluzen, 1997; Sawada et al., 1998; Behl and Lezoualc'h, 1998; Behl et al., 1997). Therefore, our second goal was to establish the cytoprotective mechanism of E2 in DA/Fe^{2+}-induced cell death in this cell model. The present data may give some clues into the molecular mechanism(s) of DA/Fe^{2+} toxicity and estrogen action and, thereby, open new avenues in the design of strategies in the prevention and treatment of PD.

2. Materials and methods

2.1. Materials

If not otherwise specified, substances were purchased from Sigma (St. Louis, MO, USA) and were of analytical grade or better. Dihydrorhodamine (DHR) was purchased from Molecular Probes (Eugene, OR, USA). Iron sulfate ($FeSO_4$) was from Aldrich Chemie (Belgium).

2.2. Isolation of lymphocytes

PBL from venous blood of healthy adult male (age range 30–40 years old) were obtained by Ficoll-Hypaque gradient centrifugation. Isolated PBL was washed three times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH = 7.4) and finally suspended in RPMI 1640 (Gibco Laboratories, New York) plus 10% fetal calf serum (FCS, Gibco Laboratories). The PBL in suspension was cultured in RPMI 1640 supplemented with 10% fetal calf, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The PBL was plated in 24 wells (1×106 cells/ml/well).

2.3. Experiments with PBL

2.3.1. Assessment of apoptotic indexes

PBL was preincubated for 30 min at 37°C in culture medium containing 1 mM pargyline and E2 and then incubated with 1 mM DA in the absence or presence of 50 μM Fe^{2+} and other products of interest for 24 h. PBL was then used for parallel microscopic examinations and biochemical assays. For viability studies, 95 μl either untreated (control) or treated cells were mixed with 5 μl (0.1 mg/ml) acridine orange/ethidium bromide (AO/EB) and 5 μl of the suspension was placed onto a slide and examined under fluorescence on a confocal microscope (Nikon, Japan). Based on the differential uptake of the fluorescent DNA binding dyes acridine orange and ethidium bromide, normal PBL cells (bright green chromatin) can be discriminated from early apoptotic cells (bright green highly condensed or fragmented chromatin), late apoptotic cells (LA, bright orange highly condensed or fragmented chromatin) and necrotic cells (N, bright orange chromatin) (McGahon et al., 1995). Quantification of apoptosis was done by counting a minimum of 250 total cells as follows: % apoptotic cells = 100 × (total number of early and late apoptotic cells/total number of cells counted). Necrotic cells were not detected under the present experimental conditions. Assessment of apoptotic indexes was repeated three times in independent experiments and quantification of apoptotic cells were recorded blind by two researchers (MJRio and CVP).

2.3.2. Assessment of DNA fragmentation

PBL was incubated as described above. The DNA was obtained according to manufactures' protocol (Wizard Genomic DNA purification kit, Promega, Cat #A1120). Briefly, PBL was centrifuged at $1200 \times g$ for 10 min at 20°C and resuspended in 300 μl nuclei lysis solution, 1.5 μl RNase I (4 mg/ml) and incubated at 37°C for 15 min. A 100-μl protein precipitation solution was added and vigorously vortexed. Samples were centrifuged at $16000 \times g$ for

3 min at room temperature. Gently, 300 μl of supernatant was mixed with 300 μl room temperature isopropanol and centrifuged at 16000 × *g* for 1 min. The supernatant was decanted and 300 μl of room temperature 70% ethanol was added and centrifuged at 16000 × g for 1 min. Ethanol is removed and pellet was dried for 20–30 min. Then, the DNA pellet was rehydrated with 100 μl of rehydration solution and supplemented with glycerol–0.05% bromophenol blue. Electrophoresis was performed for 5 h at 20 mA in 1% agarose slab gels containing ethidium bromide at a final concentration of 0.1 mg/ml in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA at pH 8.0). A DNA 123 ladder (Sigma) was applied to each gel to provide molecular size markers. DNA banding was evidenced with a UV transilluminator.

2.3.3. Determination of ROS

Detection of H_2O_2 in cellular systems can be obtained by the use of the sensitive uncharged and nonfluorescent dihydrorhodamine 123 (DHR) (Rothe and Valet, 1994). DHR is oxidized to the cationic green fluorescent dye rhodamine 123, which accumulated intracellularly owing to the electrically negative cytoplasmic and mitochondrial membrane potential. PBL (1 × 106 cells/ml) was preincubated 30 min with E2 alone or in combination with 1 mM DA/50 μM iron. Untreated and 250 μM 6OHDA (DA-related toxin) were used as negative and positive controls, respectively. Untreated and treated PBL was incubated at 37°C in the presence of 2 mM DHR (20 mM stock solution in DMSO) for 15 min and aliquots of 20 μl were taken at 3, 12 and 24 h to quantify H_2O_2 fluorescing cells by a fluorescence microscope (Nikon). The quantification of fluorescing cells was done by counting a minimum of 250 total cells as follows: % fluorescing cells = 100 × (total number of green fluorescent cells/total number of cells counted) compared with untreated control. In parallel, the apoptotic index was assessed to correlate H_2O_2 generation with DA/iron toxicity and/or protection by E2 and fluorescing cells were counted blind by two researchers (MJRio and CVP). Experiments were performed in three separate and independent settings.

3. Results

3.1. E2 protects lymphocytes from DA and iron-induced apoptosis

It has previously been demonstrated that DA in the presence of iron induces apoptosis in undifferentiated PC12 cells (Velez-Pardo et al., 1997, 1998). We therefore assess whether such phenomenon also takes place in PBL. As a first approach, we challenged PBL at the optimal concentration of 1 mM DA and 50 μM Fe^{2+} either alone or in combination for 24 h and the apoptotic index by AO/EB uptake was analyzed as described elsewhere (Velez-Pardo et al., 1997). Fe^{2+} alone did not affect PBL (1.6 ± 0.4% AO/EB index) but 1 mM DA alone produced a slight amount of cell death (13 ± 2.8%). However, when DA and Fe^{2+} were incubated together the apoptotic index increased dramatically (i.e., 38 ± 4%) as compared with iron alone and control (1 ± 0%) but increased threefold as compared with DA alone (Fig. 1). Upon close examination at the fluorescent microscope, untreated cells showed normal chromatin and round shape (Fig. 2A) whereas the PBL treated with DA/Fe^{2+} evidenced the morphological hallmarks of apoptosis such as chromatin condensation and fragmentation (Fig. 2B). To establish the cytoprotective mechanism(s) of E2 in DA/Fe^{2+}-induced apoptosis, we conducted estrogen protection experiments. We first tested increasing concentrations of E2 (0.5–10 μM) to determine the optimal concentration at which they do not promote cell death by themselves. Concentrations of E2 from 0.5 to 5 μM showed no cytotoxic effect by themselves, i.e., <2% apoptotic index, whereas 10 μM E2 yielded 37 ± 5% of apoptosis (data not shown). When lymphocytes were incubated with 0.5–3 and 5 μM E2 and then exposed to DA/Fe^{2+}, no significant protection was observed (mean 24 ± 3% and 32 ± 2% AO/EB, respectively) (data not shown). In contrast, 4 μM E2 produced a marked decrease in cell death induced by DA/Fe^{2+} as evidenced by AO/EB index assay: 10 ± 2% (Fig. 1) and morphological observations (Fig. 2C). Consequently, 4 μM E2 was chosen as the optimal protective concentration for further experiments.

To ascertain that the DA/Fe^{2+}-induced apoptosis was related with DA oxidation, we tested DA antioxidant ascorbic acid (AA) (Velez-Pardo et al., 1997) and reactive oxygen species (ROS) scavenger *N*-acetylcysteine (NAC) (Offen et al., 1996) and the monoamine uptake inhibitor, desipramine (DES). We observed that 1 mM AA or NAC block DA

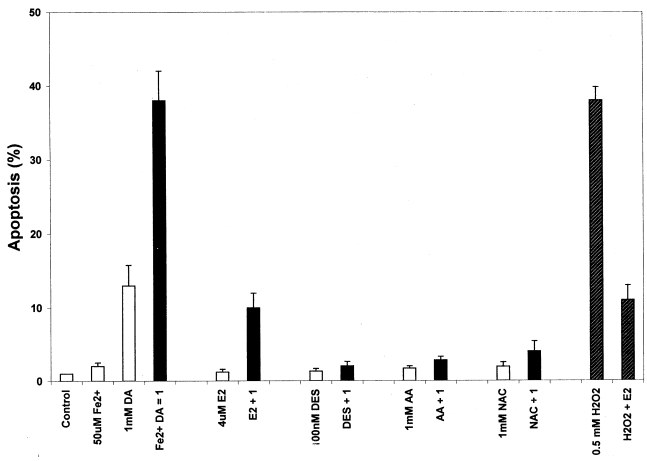

Fig. 1. Percentage of apoptotic cells in PBL treated with DA and Fe^{2+} with or without antioxidants. After 24-h treatment with the listed additives, the evaluation of apoptosis was performed as described in Section 2. E2 prevents the increase of apoptotic cells at the concentration of DA/Fe^{2+} and H_2O_2 used. The amount of apoptosis is expressed as a mean of percentage ± S.D. from three independent experiments.

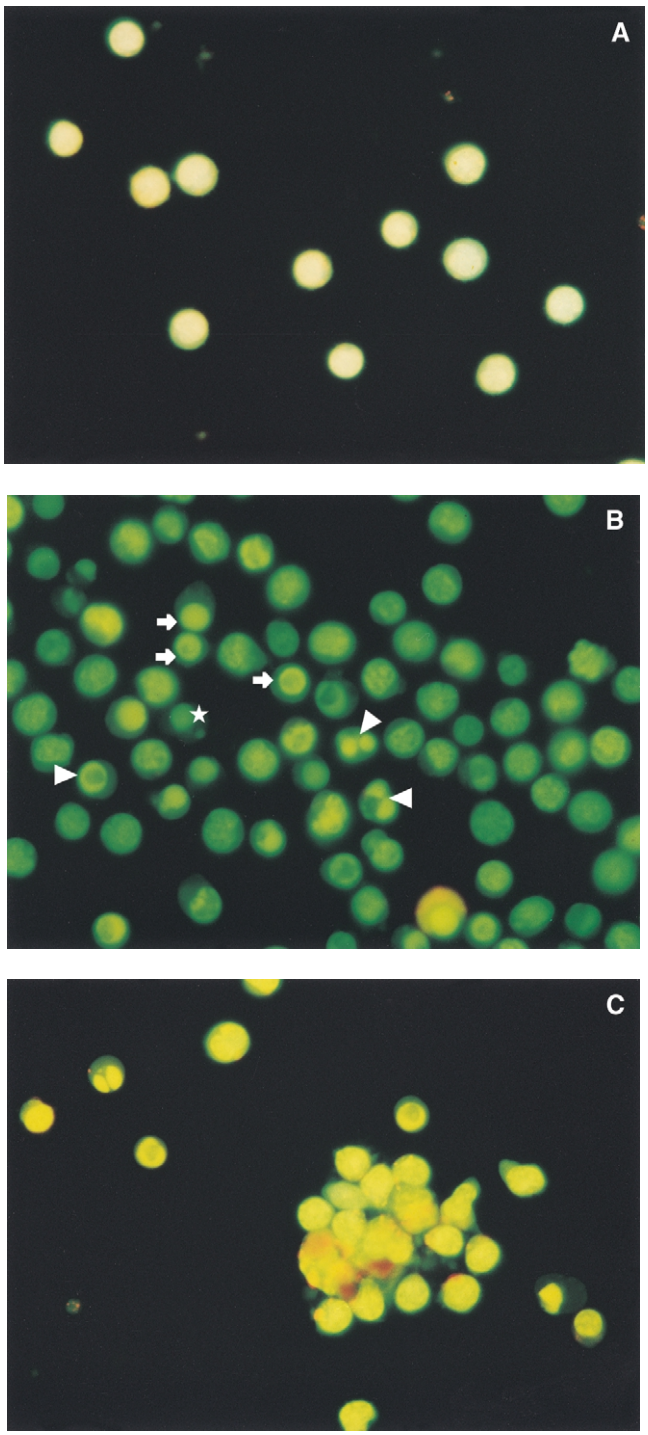

Fig. 2. Morphological changes of PBL treated with DA and Fe^{2+} with or without E2 and stained with 1 μl AO/EB (0.1 mg/ml) after 24 h. (A) Untreated control. (B) With 1 mM DA/50 μM Fe^{2+}. Notice that these apoptotic cells show highly condensed chromatin (arrow) or nuclear fragmentation (arrowhead), and apoptotic bodies (star). (C) Pretreatment with 4 μM E2 30 min before addition of DA/Fe^{2+}. Notice that most of the cells show bright green chromatin as control. Pictures represent one out of three independent observations. Magnifications: A, B, C × 400.

oxidation toxicity (2.8 ± 0.5% and 4 ± 1.4% AO/EB, respectively) (Fig. 1). Similar effect was observed with DES, which has been shown to block the uptake of catecholamines and related toxins (Tsao et al., 1996; Walkinshaw and Waters, 1994). Within 30 min prior DA/Fe^{2+} exposure, 100 nM DES greatly suppressed the amount of apoptosis induced by DA/Fe^{2+} (2 ± 0.6%).

3.2. E2 reduced H_2O_2-induced cell death generated by DA/Fe^{2+} reaction

Recent data suggest that the estrogens act as a neuroprotective by preventing the toxic effect of H_2O_2 (Behl et al., 1995; Sawada et al., 1998). To evaluate this possibility and to investigate whether estrogen treatment affects cell death, we assessed the generation of H_2O_2 by DA/Fe^{2+} (reflected as fluorescing cells) in parallel with apoptotic index determination. PBL treated with 4 μM E2 followed by 3, 12 and 24 h exposure to DA/Fe^{2+} toxic dose were protected from both H_2O_2-accumulative insult (5 ± 1.4%, 2.33 ± 0.57% and 1.83 ± 0.28% fluorescing index, respectively) and apoptosis at the different time intervals tested: 0%, 2.6 ± 0.3% and 10 ± 2% AO/EB as compared to DA/Fe^{2+} alone (Fig. 3). We also observed that E2 had similar cytoprotection effects on 250 μM DA-related 6OHDA that is a very well established H_2O_2 generating compound (Cohen and Heikkila, 1974). Additionally, to establish that this cytoprotection was due to its antioxidant activity, lymphocytes were incubated with 0.5 mM H_2O_2 alone or in combination with 4 μM E2. We observed that the estrogen effectively inhibited 3.5-fold PBL apoptosis (11 ± 2% AO/EB, Fig. 1) as compared with H_2O_2 alone (38 ± 1.83%).

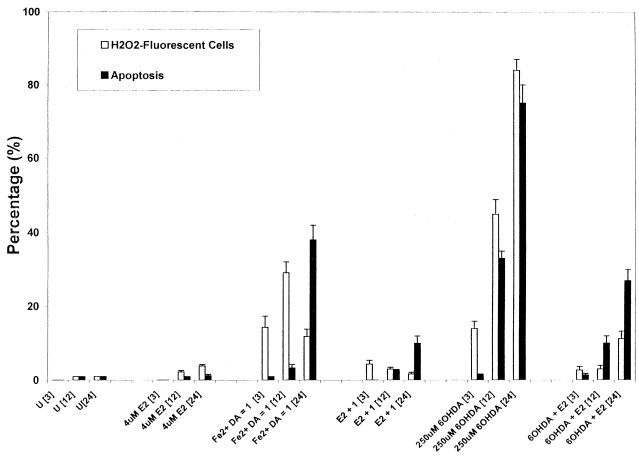

Fig. 3. H_2O_2 generation and apoptosis induced by DA and Fe^{2+} with or without E2 pretreatment in PBL cells. The cells were exposed to the insults listed in the presence or absence of E2 for the time indicated and left with fresh medium for 24 h. After 3, 12 and 24 h of incubation, intracellular peroxide production and apoptosis was determined using DHR fluorescence and AO/EB staining, respectively. Values are the mean of three separate experiments with error bars representing standard deviations. Abbreviation: U = untreated.

3.3. DA/Fe^{2+}-induced apoptosis is dependent on de novo proteins expression but E2 protection is independent

In order to investigate whether new gene expression is a prerequisite for DA/Fe^{2+}-induced death and E2 protection, PBL cells were treated with DA alone or in combination with Fe^{2+} in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, or actinomycin D (AMD), an inhibitor of RNA synthesis. As shown in Fig. 4, treatment with 5 pM CHX or AMD provided significant protection from apoptosis either when DA alone was present ($2 \pm 0.4\%$ and $3.8 \pm 1.5\%$, respectively) or when DA/Fe^{2+} was added in combination ($13 \pm 3\%$ and $9 \pm 2.5\%$). To evaluate whether H_2O_2 was involved in PBL death process and that that process was dependent on de novo protein expression, we treated PBL with 0.5 mM H_2O_2 alone or in the presence of CHX or AMD. We found that H_2O_2 provoked cell death similar to DA/Fe^{2+} condition but death was inhibited almost completely by CHX ($3 \pm 0.5\%$) and AMD ($3.8 \pm 1\%$) (Fig. 4). Similarly, apoptosis was diminished to the same extent when E2 was included in the mixture (Fig. 4).

Recently, it has been suggested that E2 protects against oxidative stress by a genomic-independent pathway and consistent with the notion that lymphocytes do not contain functional estrogen receptors (ERs) (Neifeld et al., 1977; King et al., 1996; Stewart et al., 1998), we would predict that ER antagonist should not inhibit the protection by E2. To evaluate this assumption, we examined the anti-ER tamoxifen (TAM) and raloxifene (RAL) activity. We noted that 20 μM RAL alone did not promote cell death ($1.6 \pm 0.6\%$) whereas 20 μM TAM produced a small effect, i.e., $4.5 \pm 1\%$. Surprisingly, TAM and RAL yielded a substantial increase in the extent of apoptosis in the presence of DA/Fe^{2+} ($65 \pm 5\%$ and $61 \pm 2\%$, AO/EB apoptotic index, respectively) as well as in the presence of E2: $56 \pm 6\%$ and $54 \pm 5\%$ AO/EB index (Table 1).

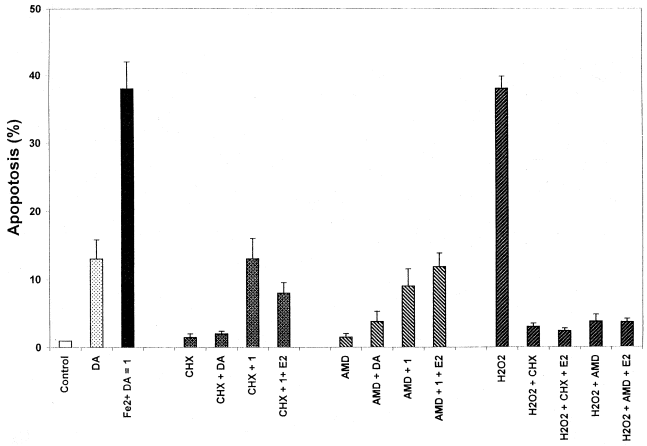

Fig. 4. Effects of CHX and AMD on DA/Fe^{2+}- and H_2O_2-induced apoptosis in PBL in the presence or absence of E2. The cells were pretreated with 5 pM CHX or AMD alone or in combination with E2 and incubated with the listed additives for 24 h. Values are the mean of three separate experiments with error bars representing standard deviations.

Table 1
Effects of TAM and RAL antagonists of ERs on the E2 protection against DA/iron-induced apoptosis

Treatments	Apoptotic index (%)
Untreated cells (control)	1 ± 0
DA (1 mM) + Fe^{2+} (50 μM)	38 ± 4
TAM (20 μM)	4.5 ± 1
TAM + DA + Fe^{2+}	65 ± 5
TAM + E2 (4 μM) + DA + Fe^{2+}	56 ± 6
RAL (20 μM)	1.6 ± 0.6
RAL + DA + Fe^{2+}	61 ± 2
RAL + E2 + DA + Fe^{2+}	54 ± 5

3.4. Analysis of DNA fragmentation by agarose gel electrophoresis

Although the bulk of DNA extracted from control, 0.5 mM H_2O_2, 1 mM DA, 50 μM Fe^{2+} alone, or DA/Fe^{2+} in combination treatments was largely preserved, none of the DNA samples exhibited visible ladder pattern of DNA fragmentation (data not shown).

4. Discussion

PD is a devastating neurodegenerative disorder that occurs throughout the world with increasing prevalence — 1.4% among 55-year-olds and 3.4% among 75-year-olds (Wood, 1997). In recent years, it became increasingly clear that nerve cell loss in the dopaminergic region pars compacta of the substantia nigra (SNpc) die by an apoptotic cell death mechanism (Tompkins et al., 1997; Tatton et al., 1998) and the degree of depletion of DA in that region is correlated closely with the neurological deterioration (Forno, 1996). At present, the best currently available hypothesis of the degeneration of the SNpc is the oxidant stress (reviewed by Jenner, 1998; Olanow and Tatton, 1999) which is supported by depletion of reducing substances, high concentration of iron and elevated DA turnover in SNpc (Olanow and Arendash, 1994; Jenner and Olanow, 1996; Olanow and Youdim, 1996). Thus, it is of major importance to clarify the mechanisms leading to nerve cell death to conceive potential therapeutic targets (Jimenez Del Rio and Velez-Pardo, 2000). Therefore, DA and iron-induced apoptosis appear to be a valuable experimental model to study oxidative stress and its regulation. Indeed, we have been able to demonstrate that DA in combination with iron-induced apoptosis in neuronal-like PC12 cells by an oxidative stress mechanism (Velez-Pardo et al., 1997, 1998). Additionally, several studies have demonstrated that E2 is a potent neuroprotective agent in neuronal cells (reviewed by Dubal et al., 1999).

In this study, we examine for the first time the E2 protective effects on DA and Fe^{2+} oxidative stress in

PBL. The results presented here are consistent with previous reports showing antioxidant protection by E2 of mesencephalic dopaminergic neurons from oxidative stress-induced neuronal death (Sawada et al., 1998; Behl and Lezoualc'h, 1998). The rationale to select PBL as a model system is based on the fact that lymphocytes have been shown to be devoid of functional ERs (Neifeld et al., 1977; King et al., 1996; Stewart et al., 1998). Moreover, lymphocytes have been demonstrated to express several dopaminergic neuroreceptors such as DA D2–D5 subtypes (Amenta et al., 1999; Ricci and Amenta, 1994; Ricci et al., 1995, 1997) and have also been shown to express de novo D1 and D2 like-receptors in PD patients compared with other patients affected by other neurodegenerative diseases and healthy control subjects (Barbanti et al., 1999). Therefore, it has been suggested that the expression of neuroreceptors on circulating immune cells may reflect the status of the homologous brain receptors (Shenkman et al., 1991). Additionally, catecholamines are actively produced by lymphocytes and have the capacity to act as auto and/or paracrine regulators of lymphocyte activity through induction of apoptosis (Josefsson et al., 1996). Thus, lymphocytes represent a remarkable example of nonneuronal model that can provide insight into the biological processes of response to oxidative stress stimulus by displaying different cell death pathways (reviewed by Lenardo et al., 1999). In this context, we demonstrated that DA/Fe^{2+} promote apoptosis in PBL (Fig. 1) showing clearly by fluorescence microscopic examination the morphological apoptotic features such as cell shrinkage, chromatin condensation, break up of the nucleus followed by fragmentation of the chromatin into discrete apoptotic bodies (Fig. 2A,B) whereas iron and DA provoked either none or little apoptosis by themselves. On the other hand, we found that E2 (4 μM), the uptake blocker DES, ROS scavenger NAC and the antioxidant AA reduce significantly DA/Fe^{2+}-promoted apoptosis in PBL (Figs. 1 and 2C). These findings suggest that DA uptake and DA oxidation is a prerequisite to induce cell death in PBL and also comply with the notion that Fe^{2+} catalyzed DA oxidation to produce quinones (Jimenez Del Rio et al., 1993; Velez-Pardo et al., 1996) and oxyradicals (Halliwell and Gutterrridge, 1989). Although AA is a well-known compound in vitro to reduce quinones and semiquinones back to catechols, there is no clear evidence for health benefits to be obtained by megadoses of AA and it cannot yet prove that it is not harmful over a lifetime (Halliwell, 1999). In contrast, E2 (a natural estrogen) can act as an antioxidant by scavenging free radicals due to its phenolic structure (Behl and Lezoualc'h, 1998). Unexpectedly, higher concentrations of E2 (5–10 μM) showed a proapoptotic effect when cotreated with DA/Fe^{2+} on PBL. Therefore, under the present experimental conditions, these results may suggest that E2 cytoprotection in vivo requires a very careful titration essay since it may act as a pro- or antioxidant agent.

We have previously postulated that iron promotes the oxidation of DA producing H_2O_2 (Jimenez Del Rio and Velez-Pardo, 2000), and based on previous observations, E2 is expected to block H_2O_2-mediated cytotoxicity (Behl et al., 1997; Sawada et al., 1998). Here, we have demonstrated that DA/Fe^{2+} generate H_2O_2 and concomitantly provoke apoptosis in a time-dependent fashion (Fig. 3). These results suggest that both phenomena occur dependently from each other and that DA oxidation by iron and its metabolite (H_2O_2) play an important role in the apoptotic process. This notion is further supported by the following observations: (1) the ability of 0.5 mM H_2O_2 alone to induce apoptotic death as much as DA/Fe^{2+} do (Fig. 1); (2) by the well-known H_2O_2-generator 6OHDA (250 μM) to provoke death (Fig. 3). It has been established that the apoptotic cell process occurs following sequential steps, which takes about 16–24 h to display its typical morphological features (Lenardo et al., 1999). Accordingly, we observed that the morphological changes of apoptosis induced by DA/Fe^{2+} in PBL require at least 24 h to be detected. In contrast, 6OHDA treatment showed apoptotic morphology at 12 h (Fig. 3). These differences of cell response to the stressors can be explained by the intrinsic oxidation properties (e.g., 6OHDA rate of oxidation is faster than DA/Fe^{2+}) of the related catechol moieties rather than the PBL cell death machinery itself (Velez-Pardo et al., 1996; Rathmell and Thompson, 1999). Alternatively, DA can exacerbate flux of H_2O_2 derived as a consequence of the natural metabolic mechanism of MAO activity. However, in this study this metabolic pathway has been blocked using MAO inhibitor pargyline in all treatment conditions. Thus, our data show that iron is capable of increasing the cytotoxicity of DA by increasing its rate of oxidation generating H_2O_2, which in turn induces apoptosis in PBL.

It has been demonstrated that E2 prevents intracellular H_2O_2 accumulation and thereby the degeneration of neurons (Behl et al., 1997). Here, we found that E2 in cotreatment with DA/Fe^{2+} condition protects PBL from DA/Fe^{2+}/H_2O_2-induced apoptosis by blocking directly the H_2O_2-mediated signaling of cell death. This result is supported by the fact that E2 reduces the production of H_2O_2 concomitantly with a reduction in the apoptotic index in a time-dependent fashion in both DA/Fe^{2+} and 6OHDA treatments (Fig. 3); E2 also reduces apoptosis when PBL was treated with 0.5 mM H_2O_2 alone (Fig. 1). These results confirm our findings and previous data by others where E2 protects neurons from oxidative stress-induced cell death (Behl et al., 1997; Sawada et al., 1998).

Apoptotic cell death is thought to represent an active process associated with new gene expression and protein synthesis (Martin, 1993). We demonstrated that RNA and protein synthesis are required for PBL to die after exposure to DA/Fe^{2+}/H_2O_2-induced oxidative stress (Fig. 4). This indicates that protein synthesis inhibitors protect from DA/iron toxicity primarily by blocking directly the synthesis of one or more proteins required for cell death. Surprisingly, CHX and AMD cannot reduce apoptosis completely caused by DA/Fe^{2+} treatment but reduced almost to control values

H_2O_2 treatment (Fig. 4). This observation could be explained by the possibility that other complementary reactions could also take place in DA/Fe^{2+} condition such as Fenton reaction [(H_2O_2) + Fe^{2+} =(·OH)] (Winterbourn, 1995), covalent cross-linking of DA to proteins, formation of protein aggregates and lipid peroxidation contributing to the cell disruption (Velez-Pardo et al., 1997). Taking together these observations, the present data indicate that DA/Fe^{2+}/H_2O_2 produce a predominant mechanism of cell death dependent on de novo protein synthesis. These findings are in agreement with the view that lymphocytes under oxidative stress insults induce activation of specific gene transcription and, subsequently, apoptosis (Staal et al., 1990; Datta et al., 1992). This idea is further supported by two related observations. First, the ability of DA to trigger cellular apoptosis through an oxidation-linked JNK activation pathway by related intermediate oxidation products such as H_2O_2 (Luo et al., 1998). Second, the ability of H_2O_2 to activate directly the oncogenic p21-ras protein which in turn activates a cascade of further phosphorylation reactions and gene expression proteins (Lander et al., 1995). All these data suggest a model wherein DA oxidation catalyzed by iron generates H_2O_2 that in turn triggers a common apoptotic death program through an oxidative stress mechanism dependent on the expression of de novo proteins in both neuronal and nonneuronal cells (Jimenez Del Rio and Velez-Pardo, 2000). These assumptions may link the oxidative stress hypothesis as a general mediator of cell death in PD.

The protection of E2 against oxidative stress induced by DA/Fe^{2+} appears to be independent of gene transcription. Compatible with this finding, we found that the ER antagonists TAM and RAL were not able to prevent the E2 effect (Table 1). These important observations are in support for a receptor-independent protective mechanism. Interestingly, TAM and RAL act synergistically with DA/Fe^{2+} to induce PBL apoptosis (Table 1). These results are in agreement with recent data showing that TAM acts as an oxidant stress agent able to facilitate apoptosis in cancer cells not expressing ERs (Ferlini et al., 1999).

At present, internucleosomal cleavage of the chromatin with the formation of DNA fragments of 180–200 has often been regarded to evidence one of the main biochemical characteristic of apoptosis in many cells, but not all (Wyllie, 1980; Oberhammer et al., 1993). However, internucleosomal DNA laddering was not detected when PBL was exposed to H_2O_2, DA alone or DA in combination with iron (data not shown). These data are in agreement with previous observations that suggested that chromatin condensation and fragmentation may be independent of the DNA degradation and that the cleavage of DNA may be restricted to large fragments (Velez-Pardo et al., 1997, 1998). Moreover, these results comply with the observation that H_2O_2 induce cell death in PBL by apoptosis in the absence of internucleosomal DNA cleavage and that a zinc-inhibitable endonucleolytic activity is accounted for that effect (Marini and Musiani, 1998). Taking together these data may suggest that DNA fragmentation (Fig. 2B) is the consequence of a specific apoptotic pathway by the DA/Fe^{2+} system similar to neuronal (Velez-Pardo et al., 1997, 1998) and nonneuronal cells (this work).

The demonstration that DA and iron-induced cell death by an oxidative H_2O_2-mediated mechanism can be blocked by E2 may offer potential means to protect cells against oxidative stress damage in PD. Accordingly, estrogens have provided strong evidence of exerting pleitropic nonreproductive actions on the brain (Woolley, 1999; Dubal et al., 1999) and other organ/tissue (Huang and Zheng, 1999; Epstein, 1999). The E2 may utilize diverse signaling pathways to produce biological effects including (1) nuclear ER-linked modulation of gene transcription; (2) ER-dependent but nontranscriptional mechanisms; (3) non-ER-linked gene transcription; (4) cell membrane-associated activity independent of mRNA transcription and protein synthesis (reviewed by Mendelson and Karas, 1999). In the present study, we have demonstrated that E2 exerts a direct antioxidant activity by a nongenomic mechanism in agreement with previous findings (Deluzen, 1997; Sawada et al., 1998; Behl and Lezoualc'h, 1998; Behl et al., 1997).

In summary, we have demonstrated that DA and iron-induced apoptosis in PBL evidenced by morphological changes such as chromatin condensation and fragmentation. The cytotoxic effect of DA/Fe^{2+} results mainly from the generation of H_2O_2, which in turn triggers a cascade of molecular events requiring RNA and protein synthesis. We have also showed that E2 protects PBL from DA/Fe^{2+}-promoted apoptosis by a nongenomic mechanism. Therefore, we conclude that the cytoprotection antioxidant activity of E2 apply to the approach of DA/Fe^{2+}-induced oxidative stress in PBL. Moreover, the findings presented in this study further support and validate our previous hypothesis that DA/Fe^{2+} and H_2O_2 could be a general mediator of cell death through a common molecular oxidative stress mechanism in neuronal and nonneuronal cells. Our data may not only contribute to the understanding of the machinery of cell death but also provide insight into potential treatments for rescuing and prolonging the survival of neurons by E2 in patients with PD.

Acknowledgments

We gratefully acknowledge Dr. A Cadavid and B. Peña from the Reproduction Program, University of Antioquia for technical assistance and providing peripheral blood lymphocyte cells. This research was supported by "Comite para el Desarrollo de la Investigacion, CODI-code #9805," UdeA.

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