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Monoamine neurotoxins-induced apoptosis in lymphocytes by a common oxidative stress mechanism: involvement of hydrogen peroxide (H_2O_2), caspase-3, and nuclear factor kappa-B (NF-κB), p53, c-Jun transcription factors

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Abstract

The destruction of dopaminergic and serotonergic nerve cells by selective 6-hydroxydopamine (6-OHDA), 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT), respectively, is a commonly used tool to investigate the mapping of neuronal pathways, elucidation of function and to mimic human neurodegenerative disease such as Parkinson's and Alzheimer's diseases. Despite intense investigations, a complete picture of the precise molecular cascade leading to cell death in a single cellular model is still lacking. In this study, we provide evidence that 6-OHDA, 5,6- and 5,7-DHT toxins-induced apoptosis in peripheral blood lymphocytes cells in a concentration-dependent fashion by a common oxidative mechanism involving: (1) the oxidation of toxins into quinones and production of the by-product hydrogen peroxide, reflected by desipramine—a monoamine uptake blocker—and antioxidants inhibition, (2) activation and/or translocation of nuclear factor-κB, p53 and c-Jun transcription factors, showed by immunocytochemical diaminobenzidine-positive stained nuclei, (3) caspase-3 activation, reflected by caspase Ac-DEVD-CHO inhibition, (4) mRNA and protein synthesis *de novo* according to cycloheximide and actinomycin D cell death inhibition. These results are consistent with the notion that uptake and intracellular autoxidation of those toxins precede the apoptotic process and that once H_2O_2 is generated, it is able to trigger a specific cell death signalisation. Thus, taken together these results, we present an ordered cascade of the major molecular events leading peripheral blood lymphocytes to apoptosis. These results may contribute to explain the importance of H_2O_2 as a second messenger of death signal in some degenerative diseases linked to oxidative stress stimuli.

Keywords: Apoptosis; Caspase-3; 5,6-Dihydroxytryptamine; 5,7-Dihydroxytryptamine; 6-Hydroxydopamine; Transcription factor

1. Introduction

The dopaminergic (6-OHDA) and serotonergic (5,6-DHT and 5,7-DHT) related toxins have been extensively used *in vivo* as a tool to generate animal models of Parkinson's (PD) and Alzheimer' disease (AD) by selective degeneration of the nigrostriatal and cortical/hippocampal neurons [1]. Although no universal view regarding the exact neurotoxin mechanism(s) underlying monoamine-related toxins has emerged, the oxidative stress theory has been proposed over the years. According to this theory, during spontaneous or enzymatically catalysed oxidation of 6-OHDA, 5,6- and 5,7-DHT toxins, they may undergo covalent attachment to proteins [2,3] forming conjugates wherein the monoamine should constantly cycle between its oxidised and reduced form [4], thereby depleting the cells' oxygen. Indeed, the formation of covalent bonds between oxidation products (*o*-quinones) and proteins and its redox cycling activity have been proposed as a possible mechanism to explain their neurodegenerative effects [4–7]. Alternatively, it has been suggested that monoamine-related toxins are rapidly and non-enzymatically oxidised by molecular oxygen to generate highly reactive oxygen species (ROS) such as free superoxide radicals ($O_2^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$),

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Abbreviations: AA, ascorbic acid; Ac-DEVD-CHO, Ac-Ala–Ala–Val–Ala–Leu–Leu–Pro–Ala–Val–Leu–Leu–Ala–Leu–Leu–Ala–Pro–Asp–Glu–Val–Asp-CHO; AO, acridine orange; AMD, actinomycin D; CHX, cycloheximide; DES, desipramine; E2, 17β-estradiol; EB, ethidium bromide; 5,6-DHT, 5,6-dihydroxytryptamine; 5,7-DHT, 5,7-dihydroxytryptamine; 6-OHDA, 6-hydroxydopamine; H_2O_2, hydrogen peroxide; JNKs/SAPKs, c-Jun N-terminal kinases/stress activated protein kinases; NAC, *N*-acetyl-cysteine; NF-κB, nuclear factor-kappa B; PBLs, peripheral blood lymphocytes.

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and hydrogen peroxide (H_2O_2) [8–10], which in turn may damage DNA, proteins and lipids [11]. These reactive oxygen species may act also as a second intermediate in intracellular signalling [12] or inhibited the mitochondria respiratory chain complexes (I and IV) leading to cell death [9,13,14]. Accordingly, it has been demonstrated that the treatment with 6-OHDA induces apoptosis (a mode of cell death) *in vivo* [15] as well as *in vitro* [16].

Apoptosis is a complex process in which catabolic enzymes degrade essential macromolecules leading to a characteristic biochemical and morphological death phenotype including compaction of chromatin against the nuclear membrane, membrane blebbing, cell shrinkage with preservation of organelles, nuclear and cytoplasmic budding to form membrane-bound fragments (apoptotic bodies) and cleavage of nuclear DNA at nucleosomes, producing a characteristic 180-bp multiple banding pattern on electrophoresis [17]. Remarkably, the central component of the apoptosis machinery is a proteolytic system involving a family of 13 cysteine-dependent aspartate-direct proteases called caspases [18]. Among them, caspase-3 has been involved as a key executor protease in neuronal death in developmental models and dopaminergic neurons in PD [19,20]. Interestingly, it has been shown that caspase-3 can be activated by H_2O_2 exposure as a final effector in apoptotic death *in vitro* [21,22]. While it has been demonstrated that caspases mediate 6-OHDA-induced apoptosis [23], it is yet to be determine whether caspase-3 plays a direct role in mediating 6-OHDA and dihydroxytryptamine neurotoxicity.

Proper performance of the apoptotic programme requires the coordinated activation and execution of multiple sub programmes (reviewed by [24]). Recently, we have postulated a hypothetical cell death model ([25] and references within) in which oxidation of dopamine (DA) generated quinone products and H_2O_2. This last compound may trigger the activation of a well-organised cascade of molecular events of the cell death machinery. In this model, H_2O_2 turns on the c-Jun N-terminal kinases (JNKs)/stress activated protein kinases (SAPKs) pathway through p21-ras protein activation. JNK/SAPK activate subsequently the nuclear factor-κB (p50, p65, I-κB) complex. Then, NF-κB (p50/p65) activates the transcriptional factor p53 and, in turn, it activates the pro-apoptotic Bax protein leading the neuron to cell death by apoptosis. Additionally, Bax has been shown to induce cytochrome *c* release from mitochondria and further activation of caspase-3 by the apoptosome cytochrome *c*-dependent complex (cyt C + dATP + Apaf1/caspase-9) [26]. Alternatively, JNK/SAPK activate the c-Jun transcription factor (activator protein-1, AP-1) leading to cell death [36]. Despite this knowledge, there remains conflicting evidence regarding the importance of MAPKs, NF-κB, p53 and c-Jun as mediators of cell death from one study to another where different cell types are used. Therefore, a complete picture of the precise molecular cascade leading to cell death linked to dopaminergic- and serotonergic-H_2O_2 generating neurotoxins in one cell model is still lacking.

In the present study, we have used peripheral blood lymphocytes (PBLs) to investigate (1) the mechanism of cell death induced by 6-OHDA, 5,6- and 5,7-DHT neurotoxins, and (2) whether these toxins share a common oxidative stress mechanism through H_2O_2 production, activation of NF-κB, p53 and c-Jun/AP-1 transcription factors and caspase-3 protease. The reason to select PBL as a model is based on the fact that lymphocytes have been demonstrated to express several dopaminergic [27–30] as well as serotonergic neuroreceptors [31]. Most importantly, dopamine in combination with iron (Fe^{2+}) has been shown to induce apoptosis by H_2O_2 in PBL [32]. Thus, lymphocytes represent a remarkable example of non-neuronal model that can provide insight into the biological processes of response to oxidative stress stimulus by displaying different cell death pathways [33].

2. Materials and methods

2.1. Materials

If not otherwise specified, substances were purchased from Sigma and were of analytical grade or better. Dihydrorhodamine (DHR) was purchased from Molecular Probes (Eugene). Caspase-3 inhibitor (Ac-Ala–Ala–Val–Ala–Leu–Leu–Pro–Ala–Val–Leu–Leu–Ala–Leu–Leu–Ala–Pro–Asp–Glu–Val–Asp-CHO) was from Alexis Biochemicals (Ac-DEVD-CHO, Cat. no. 260-046-M001).

2.2. Isolation of lymphocytes

PBL cells from venous blood of healthy adult male (range age 30–40-year-old) were obtained by Ficoll–Hypaque gradient centrifugation. Isolated PBLs were washed three times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH = 7.4) and finally suspended in RPMI 1640 (Gibco) plus 10% foetal calf serum (FCS, Gibco). The PBL in suspension were cultured in RPMI 1640 supplemented with 10% foetal calf, 2 mM L-glutamine, 100 unit/mL penicillin and 100 mg/mL streptomycin. The PBLs were plated in 24-wells (1×10^6 cells/mL per well).

2.3. Experiments with peripheral blood lymphocytes

2.3.1. Assessment of apoptotic index

PBLs were pre-incubated for 30 min at 37° in culture medium containing 1 mM pargyline and then incubated with 50, 150, 250 μM 6-OHDA, 5,6- and 5,7-DHT, respectively, in the absence or presence of different products of interest for 24 hr. PBLs were then used for parallel microscopic examinations. 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). Based on the differential uptake of the fluorescent DNA binding dyes acridine orange and ethidium bromide, normal PBL cells (NL, bright green chromatin) can be discriminated from early apoptotic cells (EA, 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) [84]. Quantification of apoptosis was done by counting a minimum of 250 total cells as follows: percent 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 was recorded blind by the authors.

2.4. Assessment of DNA fragmentation

PBLs were incubated as described above. The DNA was obtained according to manufactures' protocol (Wizard Genomic DNA purification kit, Promega, Cat. no. A1120). After standard procedures described elsewhere [32], DNA was rehydrated with 100 μL of rehydration solution and supplemented with glycerol—0.05% bromophenol blue. Electrophoresis was performed for 5 hr 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 transiluminator.

2.5. Determination of ROS

Detection of H_2O_2 in cellular systems can be obtained by the use of the sensitive uncharged and non-fluorescent DHR according to [34]. PBL (1×10^6 cells/mL) were incubated with 150 μM 6-OHDA, 5,6- and 5,7-DHT and 25 μM menadione (a redox cycling toxin used as a positive control) for 6 and 24 hr, respectively. To evaluate H_2O_2 generation, aliquots of 50 μL untreated and treated PBL was incubated in the presence of 1 mM DHR (20 mM stock solution in DMSO) for 15 min and at both intervals of time (6 and 24 hr). DHR is oxidised to the cationic green fluorescent dye rhodamine 123 which accumulated intracellularly owing to the electrically negative cytoplasmic and mitochondrial membrane potential. The quantification of fluorescent cells under a fluorescence microscope (Nikon) was performed by counting a minimum of 250 total cells as follows: percent fluorescent 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 monoamine toxicity and fluorescent cells were counted blind by the authors. Experiments were performed in three separate and independent settings.

2.6. Inhibition of caspase-3 activity

Inhibition of caspase-3 activity was performed using the cell permeable and irreversible inhibitor Ac-DEVD-CHO [35]. The compound Ac-DEVD-CHO (20 μM) was added to PBL (1×10^6 cells/mL) at the time of incubation with monoamine-related toxins and incubated for 24 hr. Assessment of apoptotic indexes was determined as previously described. Experiments were performed in three separate and independent settings.

2.7. Immunocytochemical detection of NF-κB, p53 and c-Jun transcription factor proteins

Immunocytochemical staining was performed according to suppliers' protocol (Santa Cruz Biotechnology, goat ABC staining System, Cat. no. sc-2023) using the primary goat poly-clonal antibodies NF-κB p65 (C-20)-G (Santa Cruz Biotechnology Cat. no. sc-372-G), p53 (FL-393) (Santa Cruz Biotechnology Cat. no. sc-6243-G), and c-Jun/AP-1(N)-G (Santa Cruz Biotechnology Cat. no. sc-45-G). After toxins treatment, cells were plated on poly-L-lysine coated cover slip and fixed in 4% methanol in 0.1 M phosphate buffer, pH 7.4 for 25 min and then washed with phosphate-buffered saline (PBS). Slides were exposed to 1% hydrogen peroxide in PBS for 10 min. After several washes, cells were permeabilised with Triton X-100 solution in PBS for 5 min. Cells were incubated with primary antibodies for 1 hr at 37° and subsequently incubated with biotinylated antibody at 37° for 1 hr. Finally, the specimens were stained with the ABC enzyme kit. After staining, they were cover-slipped with cover glasses. Positive diaminobenzidine (DAB) stained nuclei (dark-brown colour) were counted from five random fields per treatment at 40× magnification. Quantification of DAB-positive nuclei was done by counting a minimum of 250 total cells as follows: percent DAB (+) nuclei = 100 × (total number of dark-brown colour nuclei/total number of nuclei counted). PBL cells were counted blind by the authors from one to two experimental settings from images imported to a TV screen using a high resolution CCD colour video camera (Toshiba Model No. IK-627AT).

3. Results

3.1. Dopaminergic (6-hydroxydopamine) and serotonergic (5,6- and 5,7-dihroxytryptamine) toxins induce apoptosis in lymphocytes in a concentration-dependent fashion

Previous studies from our laboratory have shown that DA in presence of Fe^{2+} induce apoptosis in PBL evidencing

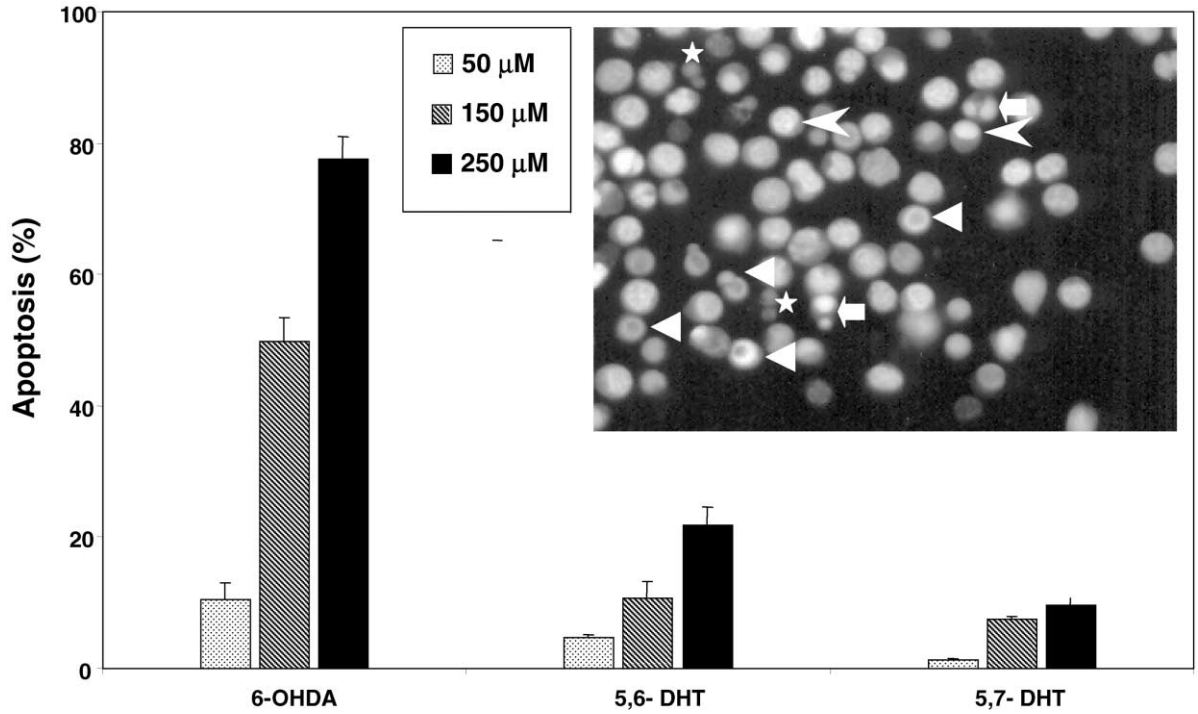

Fig. 1. Dose-dependence of dopaminergic- and serotonergic-related toxin induce apoptosis in PBLs. PBL cells were incubated with increasing concentrations of 6-OHDA, 5,6- and 5,7-DHT for 24 hr. The evaluation of apoptosis was performed as described in Section 2. The amount of apoptosis is expressed as a mean of percentage ± SD from three independent experiments. Inset: morphological changes of PBL treated with 150 μM 6-OHDA and stained with 1 μL AO/EB (0.1 mg/mL) after 24 hr. Notice that early apoptotic cells show highly condensed green chromatin (arrow) or nuclear fragmentation (arrowheads for U- and/or ring-like forms), and apoptotic bodies (thick arrow) and late apoptotic cells show highly condensed and/or fragmented red chromatin (asterisk). Notice that some cells show normal bright green chromatin. Similar morphological characteristics were observed when PBLs were treated with 250 μM 5,6/5,7-DHT for the same period of incubation time. Picture represent one out of three independent observations. Magnification 400×.

the morphological hallmarks of apoptosis such as chromatin condensation/fragmentation and formation of apoptotic bodies [32]. Therefore, the cytotoxic effect of 6-OHDA, 5,6- and 5,7-DHT was evaluated 24 hr after the treatment of PBL cells with concentrations from 50 to 250 μM. As shown in Fig. 1 (inset), toxins induced the typical morphology of apoptosis such as chromatin condensation (arrows) and fragmentation (U- and ring-like forms (arrowheads)) and apoptotic bodies (thick arrow). Although there was a dose-dependent increase in numbers of apoptotic cells by the three toxins as assessed by AO/EB staining, they displayed a marked difference in effectiveness at the same concentration. For instance, 50, 150 and 250 μM 6-OHDA induce 10 ± 3, 50 ± 4, 78 ± 4% AO/EB index, respectively, whereas 5,6-DHT (at the same concentration range) induces 5 ± 1, 11 ± 3 and 22 ± 3% AO/EB index, respectively, and much less is induced by 5,7-DHT (1 ± 0.5, 8 ± 1 and 10 ± 1 AO/EB index, respectively) (Fig. 1). Consequently, we used either 150 or 250 μM toxin concentration for further experiments.

3.2. Antioxidants and desipramine, a monoamine uptake blocker, protect lymphocytes from 6-OHDA, 5,6- and 5,7-DHT-induced apoptosis

Because autoxidation of monoamine toxins into *o*-quinones and ROS has been implicated as a first critical step in their cytotoxic mechanism, we evaluate the potential ability of ascorbic acid (AA), *N*-acetyl-cysteine (NAC) [37] and 17β-estradiol (E2) [38] as antioxidants. As shown in Table 1, when AA, NAC or E2 were incubated in combination with 250 μM toxins, they completely suppressed the noxious effect of neurotoxins to control or untreated PBL values. To determine whether 6-OHDA, 5,6- and 5,7-DHT were initiating apoptosis via a specific intracellular mechanism of action, the ability of desipramine (DES) to inhibit PBL cell death was examined. Effectively, DES blocked 6-OHDA, 5,6- and 5,7-DHT-induced cell death to control values (Table 1).

Table 1
Antioxidants and monoamine uptake blocker protect PBL cells from dopaminergic- and serotonergic-related toxins-induced apoptosis[a]

Treatment	Apoptosis (%)			
	Untreated	6-OHDA	5,6-DHT	5,7-DHT
Control	<1 ± 0.5	75 ± 4	22 ± 2	10 ± 2
DES (100 nM)	1 ± 0	1.5 ± 0.3	3 ± 0.5	1 ± 0
AA (1 mM)	0.8 ± 0.4	1.3 ± 0.2	1.7 ± 0.3	1.5 ± 0.2
NAC (1 mM)	1.3 ± 0.3	9 ± 2	2.5 ± 0.5	2 ± 0.4
E2 (4 μM)	1 ± 0	3 ± 1	1 ± 0	1 ± 0.2

[a] PBL cells were incubated for 24 hr with 6-OHDA, 5,6- and 5,7-DHT (250 μM) only or with NAC, AA and E2, as antioxidants and DES, as specific monoamine uptake blocker. The evaluation of apoptosis was performed as described in Section 2. Quantification of apoptosis is expressed as a mean of percentage ± SD from three independent experiments.

3.3. H_2O_2-generated by 6-OHDA, 5,6- and 5,7-DHT toxins is a condition to induce cell death in lymphocytes

It is well known that 6-OHDA is a H_2O_2 generating compound [39] and H_2O_2 has been implicated in mediating apoptosis in several *in vitro* cell models. Therefore, as an indication of monoamine oxidation-induced oxidative stress and cell death, we measured the generation of H_2O_2 by toxins (reflected as fluorescent cells) and menadione (used as control) in parallel with apoptotic index determination. We observed that 150 μM 6-OHDA, 5,6-DHT and 25 μM menadione produced the highest percentage of H_2O_2-fluorescent cells evident as early as 6 hr with sustained production of H_2O_2-values at 24 hr when compared with untreated PBL at both intervals of time (Table 2), whereas 5,7-DHT produced the low percentage of H_2O_2-fluorescent cells. In contrast, a significant percentage of apoptotic cells was observed only after 24 hr of 6-OHDA, 5,6- and 5,7-DHT and menadione treatments (Table 2).

Table 2
Dopaminergic- and serotonergic-related toxins generate H_2O_2 and concomitantly provoked apoptosis in PBL[a]

Phenomenon treatment	6 hr		24 hr	
	H_2O_2 (%)	Apoptosis (%)	H_2O_2 (%)	Apoptosis (%)
Untreated	0 ± 0	<1 ± 0	0 ± 0	1 ± 0
6-OHDA (150 μM)	42 ± 5	1 ± 0	48 ± 3	47 ± 4
5,6-DHT (150 μM)	14 ± 3	1 ± 0	13 ± 1	10 ± 2
5,7-DHT (150 μM)	2 ± 1	1 ± 0.5	2 ± 1	8 ± 2
Menadione (25 μM)	16 ± 2	1 ± 1	13 ± 2	58 ± 4

[a] The cells were exposed to toxins (150 μM) for the time indicated and left with fresh medium for 24 hr. After 6 and 24 hr of incubation, intracellular peroxide production and apoptosis was determined using DHR fluorescence and AO/EB staining respectively. Values are the means ± SD of three separate experiments. Menadione was used as a positive control.

3.4. Caspase-3 is involved in dopaminergic and serotonergic toxins-induced apoptosis in lymphocytes

Recent data suggest that caspase-3 is a major executor protease in apoptosis process in response to stress stimuli [21,22]. Therefore, we sought to examine whether caspase-3 activation was crucial to induce apoptosis by dopamine/serotonin-related toxins. Since zinc ion (Zn^{2+}) has been demonstrated to specifically inhibit caspase-3 [40,41], we initially determined the effect of increasing concentration of zinc sulphate ($ZnSO_4$) alone on PBL to determine optimal concentration at which they do not promote cell death by themselves. As shown in Fig. 2, 100 or 250 μM zinc was innocuous for PBL (1 and 2.5 ± 0.5% AO/EB index, respectively) but 500 μM provoked 17 ± 2% AO/EB apoptotic death (data not shown) compared with untreated cells (1% AO/EB index). Thus, PBLs were challenged with either 100 or 250 μM zinc in combination with 150 μM 6-OHDA, 250 μM 5,6- and 5,7-DHT. As indicated in Fig. 2, only 250 μM zinc reduced significantly the toxin effect of 6-OHDA and 5,6-DHT (2.6- and 3.7-folds, respectively) whilst at both (100 and 250 μM) concentrations of zinc completely abridged the effect of 250 μM 5,7-DHT (10-folds). To ascertain that caspase-3 activation occurs during the apoptotic process in PBL cells, we examined the effect of the cell-permeable and highly selective inhibitor Ac-DEVD-CHO of caspase-3 [35]. As

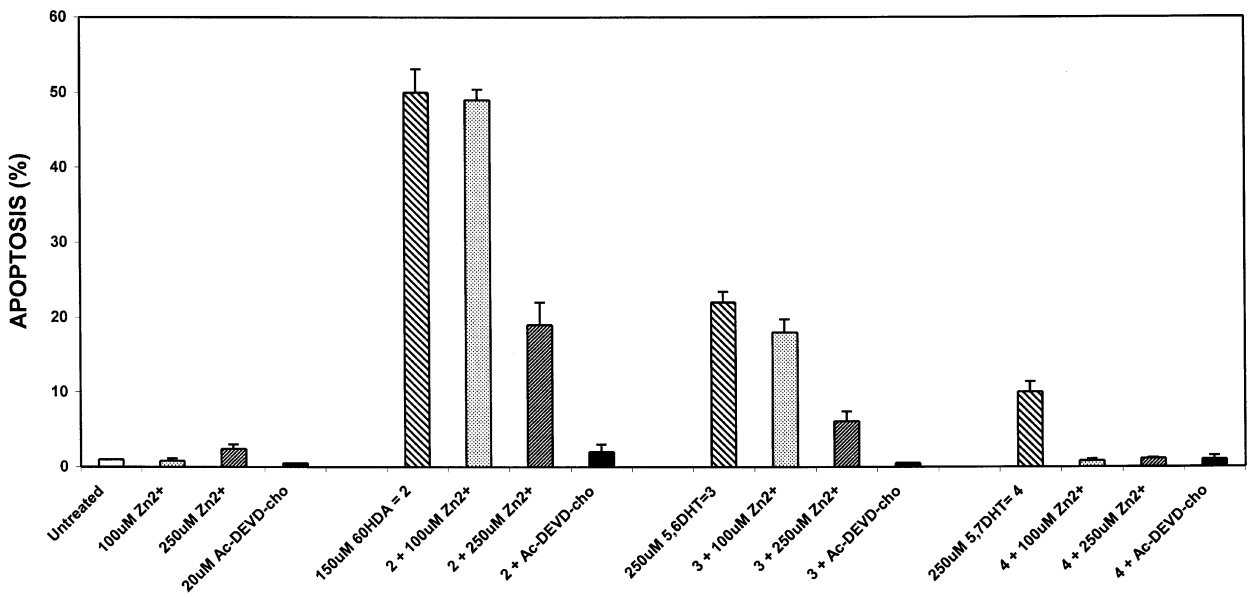

Fig. 2. Effects of zinc and caspase-3 inhibitor on monoamine toxins-induced apoptosis in PBLs. The cells were pre-treated with indicated concentrations of $ZnSO_4$ or Ac-DEVD-CHO, caspase-3 inhibitor, alone or in combination with 6-OHDA, 5,6- and 5,7-DHT (150 μM) for 24 hr. Values (percent apoptosis) are the mean of three separate experiments with error bars representing standard deviations.

Table 3
Percentage of transcription factors translocation into the nucleus induced by dopaminergic- and serotonergic-related toxins alone or in the presence of AMD[a]

Treatments	Apoptotic index (%)
Untreated cells (control)	1 ± 0
6-OHDA (150 μM)	51 ± 4
6-OHDA + CHX (5 pM)	10 ± 4
6-OHDA + AMD (5 pM)	8 ± 2
5,6-DHT (250 μM)	20 ± 3
5,6-DHT + CHX	2 ± 1
5,6-DHT + AMD	3 ± 2
5,7-DHT (250 μM)	10 ± 1
5,7-DHT + CHX	1 ± 0
5,7-DHT + AMD	1 ± 1

[a] The data shown represent the means ± SD of two independent experiments.

evidenced, 20 μM Ac-DEVD-CHO markedly reduced PBL cell death by 150 μM 6-OHDA, 250 μM 5,6- and 5,7-DHT treatments similar to control values (Fig. 2).

3.5. 6-OHDA, 5,6- and 5,7-DHT toxins-induced apoptosis are dependent on de novo proteins expression

Recently, it has been shown that inhibition of protein or RNA synthesis rescue undifferentiated PC12 cells and PBL from apoptosis induced by either 6-OHDA [16] or DA/Fe^{2+} [32]. In order to further characterise the molecular mechanism(s) involved in monoamine-related toxins provoked death, we investigate whether new gene expression is involved during the apoptotic process. PBL cells treated with 150 μM 6-OHDA, 250 μM 5,6- and 5,7-DHT in the presence of either 5 pM cycloheximide (CHX) (an inhibitor of protein synthesis) or actinomycin D (AMD) (an inhibitor of RNA synthesis) for 24 hr, resulted in a significant reduction in the percentage of apoptosis when compared with the PBL treated with toxins alone (Table 3). Remarkably, both CHX and AMD treatment reduced effectively the apoptotic index for 5,6/5,7-DHT (10-fold) but moderately reduced apoptosis induced by 6-OHDA (5–6-folds, respectively).

3.6. 6-OHDA, 5,6- and 5,7-DHT toxins-induced apoptosis are associated with activation of NF-κB, p53 and c-Jun transcription factors

Based on our above findings and previous studies that showed the ability of 6-OHDA to induce apoptosis in PC12 cells by activation of p53 and Bax [43], we decide to investigate the effect of those toxins alone or in the presence of AMD on the activation of transcription factors such as NF-κB, p53 and c-Jun in PBL and to evaluate whether new mRNA is under regulatory control by those transcription factors. Thus, as a first approach, we performed immunocytochemical staining to visualise the cellular (cytoplasmic or nuclear) localisation of those transcription factors in PBL under neurotoxin exposure. As depicted in Fig. 3, 24 hr following 150 μM 6-OHDA

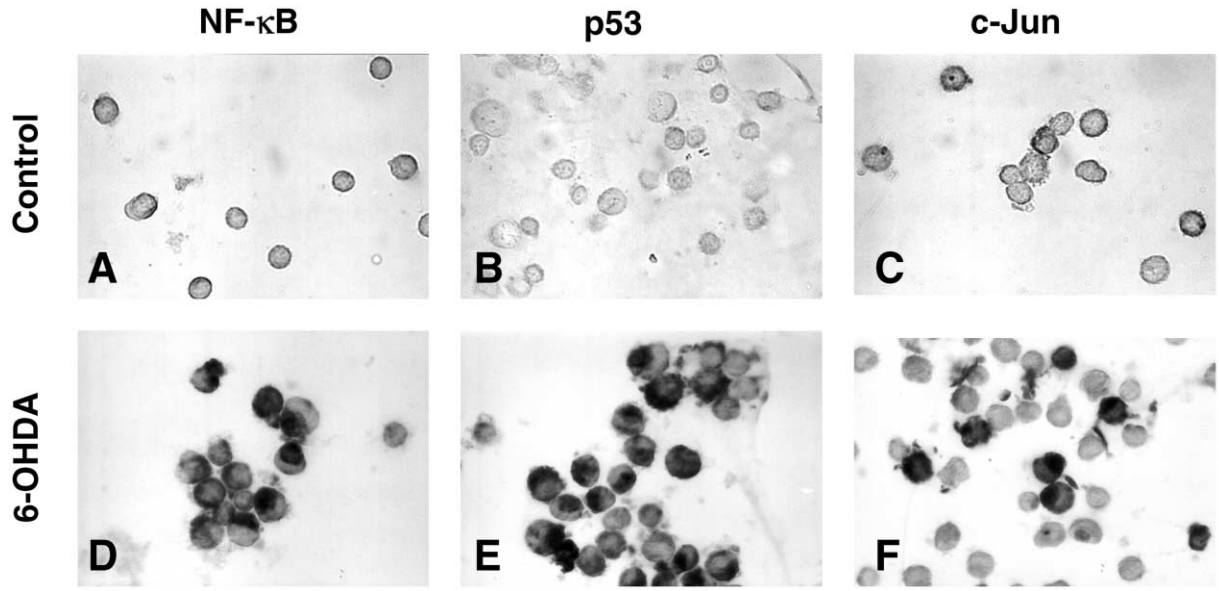

Fig. 3. Dopaminergic- and serotonergic-related toxins-induced activation of NF-κB, p53 and c-Jun transcription factors. PBL cells were left untreated (A–C) or exposed to 6-OHDA (150 μM) alone (D–F) for 24 hr. After this time of incubation, cells were stained with anti-NF-κB-p65 (A and D), anti-p53 (B and E) and anti-c-Jun (C and F) antibodies according to procedure described in Section 2. Notice that NF-κB, p53 and c-Jun-positive nuclei (dark brown colour) reflect their nuclear translocation and appear to correlate with the apoptotic nuclear morphology, i.e. condensed/fragmented nuclei when compared with untreated cells (A–C). Similar observations were obtained for PBL cells treated with 250 μM 5,6- and 5,7-DHT toxins (photomicrographs not shown). Magnifications of the photomicrographs 400× (A–F).

Table 4
Effects of CHX and AMD on 6-OHDA, 5,6- and 5,7-DHT-induced apoptosis[a]

Phenomenon treatment	DAB-positive stained nuclei (%)					
	6-OHDA	6-OHDA + AMD	5,6-DHT	5,6-DHT + AMD	5,7-DHT	5,7-DHT + AMD
Apoptosis	49 ± 4	8 ± 2	22 ± 3	3 ± 2	10 ± 1	1 ± 1
NF-κB	42 ± 2	30 ± 4	12 ± 1	21 ± 3	3 ± 1	4 ± 1
p53	38 ± 3	2 ± 1	22 ± 2	2 ± 1	6 ± 1	1 ± 1
c-Jun	15 ± 2	6 ± 2	5 ± 1	1 ± 0	2 ± 1	1 ± 0

[a] The data shown represent the means ± SD of three independent experiments.

treatment, DAB-positive stained nuclei NF-κB (Fig. 3D), p53 (Fig. 3E) and c-Jun (Fig. 3F) accumulated in the nuclear compartment in some PBL cells when compared with untreated cells (Fig. 3A–C). In contrast, few DAB-positive p53 and c-Jun stained nuclei were observed under AMD treatment whereas NF-κB was slightly affected. Similar observations were made for 5,6- and 5,7-DHT treatments (photomicrographs not shown). Following quantification of NF-κB, p53 and c-Jun DAB-positive nuclei under monoamine-related toxin treatments, it showed significantly higher percentage values of NF-κB and p53 translocation than c-Jun for all treatments and, except for 6-OHDA treatment, NF-κB showed lower translocation values than p53 (Table 4). In contrast, PBL cells under exposure to AMD provoked a dramatic decrease in p53 (e.g. 6-OHDA, Table 4) for all treatments but NF-κB and c-Jun were moderately affected (Table 4). Interestingly, the apoptotic nuclear morphology (i.e. chromatin condensation Fig. 1, inset) coincided with DAB-positive nuclear translocation of those transcription factors (Fig. 3D–F).

3.7. Analysis of DNA fragmentation by agarose gel electrophoresis

Previous studies by Marini *et al.* [42] have shown that H_2O_2 induced in human lymphocytes an apoptotic response that was characterised by chromatin fragmentation and the appearance of 50 kb DNA fragments in the absence of internucleosomal DNA. Consequently, we assessed whether dopaminergic/serotonergic-related toxins may be associated with DNA laddering. Accordingly, though the bulk of DNA extracted from control, 6-OHDA/5,6- and 5,7-DHT treatments was largely preserved, none of the DNA samples exhibited visible ladder pattern of DNA fragmentation (three independent experiments, data not shown).

4. Discussion

The destruction of dopaminergic and serotonergic nerve cells by selective 6-OHDA, 5,6- and 5,7-DHT, respectively, is a widely used tool to investigate the mapping of neuronal pathways, elucidation of function and to mimic human neurodegenerative disease such as PD and AD [1,44]. Although increasing evidence demonstrates that 6-OHDA induce apoptosis in dopaminergic cells in rat substantia nigra brain [15], its exact action mechanism remains to be determined. Additionally, very little attention has been paid to the molecular cytotoxic effect of 5,6- and 5,7-DHT. Therefore, it is of our immediate interest to investigate whether the dopaminergic and serotonergic neurotoxins share a common molecular mechanism of toxicity. The mechanism of cell death induced by 6-OHDA, 5,6- and 5,7-DHT has been investigated here using PBL cells as a model system. These cells have been demonstrated to be susceptible to DA alone [45] or in combination with iron [32] toxicity and to display different apoptotic pathways [33].

In this study, we provide evidence that 6-OHDA, 5,6- and 5,7-DHT-derivated toxins provoke apoptosis in PBL cells by a common oxidative mechanism involving the oxidation of toxins into quinones and production of the by-product H_2O_2, which in turn triggers a specific cell death signalisation through caspase-3, NF-κB, p53 and c-Jun transcription factors activation. Specifically, treatment with 6-OHDA, 5,6- and 5,7-DHT induce apoptosis in a concentration-dependent fashion (Fig. 1) characterised by the presence of apoptotic bodies and highly condensed and fragmented chromatin (Fig. 1, inset). These findings are in agreement with previous apoptotic morphology identified on PBL treated with DA/Fe^{2+} [32]. However, we found a dramatic disparity in the percentages of apoptosis induced by dopaminergic and serotonergic toxins (Fig. 1). This discrepancy may reflect chemical intrinsic differences on their autoxidation rates into *o*-quinone products and generation of H_2O_2 as previously demonstrated by our group [3,4,32]. This last notion was corroborated by two experimental observations: firstly, significant reduction in the percentage of apoptosis was observed when 250 μM 6-OHDA, 5,6- and 5,7-DHT toxins were incubated in the presence of reductant agent AA, reactive oxygen scavengers NAC and E2 (Table 1). This data comply with the notion that AA has the ability to recycle 6-OHDA, 5,6- and 5,7-DHT from its quinoid product, *o*-quinone, and the ability of NAC and E2 to effectively remove the oxidative by-product H_2O_2 [32,37]. Moreover, the ability of DES to reduce cell death suggests that neurotoxins-induced apoptosis in PBL via a specific intracellular mechanism of

action in accordance with previous data [16,46]. Secondly, we also have demonstrated that autoxidation of these toxins went along with a significant production of H_2O_2 and concomitantly provoke apoptosis in a time-dependent fashion (Table 2). These results suggest that both phenomena occur dependently from each other and that toxin oxidation into H_2O_2 play an important role in the apoptotic process. Indeed, the fast oxidation of 6-OHDA and 5,6-DHT or slow oxidation of 5,7-DHT produce a significant increased in the percentage of H_2O_2-fluorescent PBL after 6 hr, in contrast to the percentage of apoptosis that was similar to untreated cells. However, by 24 hr, a dramatic increased in cell death is observed with sustained production of H_2O_2. Thus, apoptotic death induced by these toxins requires more than 6 hr. This notion is further supported by the observation that the well-known redox cycling and H_2O_2-generator-menadione (25 μM, used as a positive control) provokes cell death after 6 hr of exposure to PBL cells (Table 2).

Previous studies indicated that H_2O_2 induces apoptosis through the activation of caspase-3 [21,22] and that this last executor protease could be inhibited by Zn^{2+} [40]. In this report, we found that 250 μM zinc is moderately effective to inhibit 6-OHDA (2.6-folds) and 5,6-DHT (3.7-folds) induced apoptosis and that it suppressed completely the toxicity of 5,7-DHT (10-folds) (Fig. 2). This result may imply that the ability of zinc ions to block apoptosis through caspase-3 inhibition depends either on the rate of oxidation of toxins and subsequent generation of H_2O_2 or depends on an alternative cell death pathway that might be involved under toxins exposure. However, using a more specific cell permeable caspase-3 inhibitor, Ac-DEVD-CHO (20 μM), we observed a dramatic reduction in the percentage of apoptosis induced by monoamine-related toxins in PBL cell (Fig. 2). This observation demonstrated, for the very first time, that the cytotoxicity of dopaminergic and serotonergic neurotoxins is directly related to the activation of caspase-3 mediated by H_2O_2.

It is known that among the many intracellular substrates of caspase-3 [18] is a 45 kDa molecular chaperone called ICAD or DFF45 (for inhibitor of caspase-activated DNase or DNA fragmenting factor), whose cleavage results in release of a previously inactive, 40-kDa nuclear homing nuclease (called CAD or DFF40) that inserts double-strand breaks at internucleosomal sites in DNA [47] producing DNA fragments of 180–200 bp which are universally detected as a DNA ladder pattern by agarose gel electrophoresis. Thus, internucleosomal cleavage is compatible with the notion that this is a chromosomal DNA caspase-3-dependent degradation process. However, we and other investigators have consistently found no internucleosomal DNA fragmentation (or DNA laddering) under different oxidative stress stimuli such as 6-OHDA, 5,6- and 5,7-DHT (this work), H_2O_2 [32,42,87], DA alone or in combination with iron in PC12 cells [46], PBL cells [32] and in Jurkat T cells [48]. At present, the significance of the apparent discrepancy between the observation that caspase-3-mediated activation by H_2O_2 is associated with typical apoptotic nuclear condensation (according to AO/EB staining criteria, Fig. 1 inset) and the absence of DNA internucleosomal cleavage is unknown. Our data suggest that chromatin condensation and fragmentation may be independent of the DNA degradation [50,51] and that the cleavage of DNA may be restricted to large fragments [42,46,47,49]. Taking together our results suggest that dopamine- and serotonin-related H_2O_2 generating toxins induce a specific apoptotic pathway involving activation of caspase-3 (Fig. 2), which in turn might activate CAD, inducing a type of morphological and biochemical nuclei characteristics compatible with chromatin condensation/fragmentation in the absence of internucleosomal cleavages [51].

Past work have shown that mRNA and protein *de novo* synthesis is required for apoptosis induced by oxidant stimuli [16,32]. Here, we demonstrated that CHX and AMD could reduce significantly apoptosis induced by 6-OHDA, 5,6- and 5,7-DHT (Table 3). However, CHX and AMD cannot completely diminish apoptosis caused by 6-OHDA exposures. This observation could be explained by two possibilities. Firstly, mRNA and protein *de novo* synthesis are dependent on the intrinsic rate of neurotoxin oxidation and therefore, on the rate of H_2O_2-generation (e.g. 6-OHDA vs. 5,7-DHT). Secondly, a complementary mechanism independent of mRNA and protein synthesis could also take place in dopamine and serotonin toxins-induced apoptosis. These assumptions prompted us to evaluate mRNA and protein *de novo* synthesis related with down stream proteins expressed following stress toxin insult. Hence, we focused on the analysis of translocation of the cytoplasmic transcription factors such as NF-κB, p53 and c-Jun to nucleus, involved in apoptosis [54–56]. At present, whether activated NF-κB and c-Jun transcription factors play an antiapoptotic [58–60,83] or pro-apoptotic role [53,85,86] against stressful conditions remain a controversial issue [36,52,57]. Although the nature of those disagreements are not yet well established (e.g. dopamine effect on NF-κB [60] vs. [85,86]), our present data support consistently the notion that p53, NF-κB and c-Jun activation by oxidative stress stimuli are involved in apoptotic cell death. This assumption is supported by direct observation of p53, NF-κB and c-Jun DAB-positive nuclei (Fig. 3D–F) and this event coincides with apoptosis upset (Fig. 1, inset). Remarkably, the DAB-nuclear positive transcription factors detection (Fig. 3D–F) is associated with typical apoptotic morphology such as nuclear margination and condensation of the chromatin when compared with untreated PBL (Fig. 3A–C). These results clearly demonstrated that the molecular mechanism by which the dopaminergic- and serotonergic-related toxins mediated apoptosis in PBL involves the cytoplasmic activation and translocation to nucleus of NF-κB, p53 and nuclear activation of c-Jun through an ordered cascade of

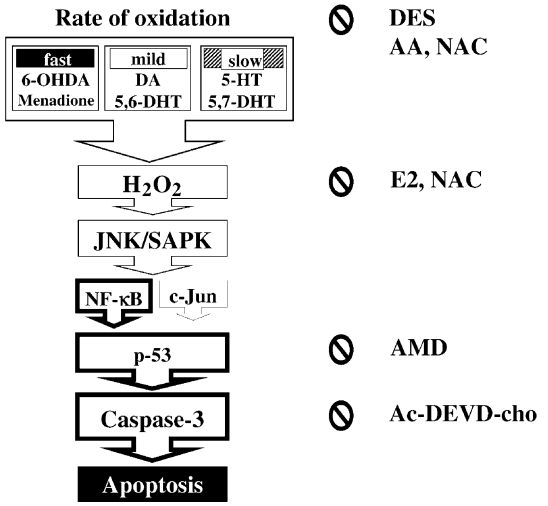

Fig. 4. Schematic representation of the major molecular sequential steps of apoptosis induced by dopaminergic- and serotonergic-related toxins in PBL. The dopaminergic (6-OHDA) and serotonergic (5,6- and 5,7-DHT) toxins upon fast, mild and slow autoxidation, respectively [3,4,32], generate H_2O_2 (this work). This compound activates JNK/SAPKs pathway (MEKK1 [77]) which activate in parallel both NF-κB and c-Jun transcription factors (this work). In turn, NF-κB is able to activate the transcriptional factor p53 (this work, and [70]). p53 subsequently activates the pro-apoptotic Bax [73] protein which induce cytochrome *c* release from mitochondria [76] to activate the apoptosome complex [26]. This complex activates caspase-3 (this work) leading the PBL cells to cell death by apoptosis (this work). The symbol (⊘) represents the inhibition (by indicated compound) of the critical steps of the molecular cascade leading to apoptosis by neurotoxins.

events mediated by H_2O_2 ([25] and Fig. 4). Moreover, our findings showed that p53 is under NF-κB regulation control in agreement with the idea that NF-κB activates p53 [70]. Therefore, these results suggest that monoamine-related H_2O_2-generating toxins induce apoptosis via a p53- and c-Jun-dependent pathways (Table 4). This notion is further supported by a related observation: when PBLs were exposed to toxin in combination with AMD, a mRNA inhibitor, there were a dramatic decreased in p53 nuclear translocation by those neurotoxins (e.g. 6-OHDA, Table 4) that coincides with low percentage of apoptotic index, whereas NF-κB and c-Jun were both moderately reduced by 6-OHDA, up-regulated by 5,6-DHT (only NF-κB) or left unchanged by 5,7-DHT (Table 4). A possible explanation for these effects of AMD on NF-κB and c-Jun might be due to the fact that these factors are constitutively expressed as a cytoplasmic p50/p65, heterodimer bound to an inhibitory subunit, I-κB [71] and c-Jun/c-Jun homodimers, respectively. Thus, under AMD exposure the dynamic cellular process of protein maintenance and degradation (protein turnover) is slightly altered to an extend to interfere with a proper intracellular levels of NF-κB and c-Jun transcription factors. In contrast, AMD blocked *de novo* mRNA of p53 which is under NF-κB up-regulation. Since it has been demonstrated that H_2O_2 activates NF-κB [67], therefore, our data suggest that high levels of H_2O_2 production by neurotoxins (Table 2) correlated with high percentage of NF-κB nuclear translocation (Table 4) and apoptotic index. In agreement with our findings, Dumont *et al.* have recently shown that H_2O_2-induced apoptosis requires the activation of NF-κB and that this detrimental effect relies on the induced expression of p53, which is up regulated in the presence of H_2O_2 [61]. This last conclusion has also been corroborated by Uberti *et al.* in oligodendroglia cells [62]. Furthermore, Vollgraf *et al.* have reported the involvement of AP-1 (c-Jun) and NF-κB activation in H_2O_2-induced apoptosis [63]. Taken together these observations suggest that irrespective of the source of H_2O_2 (e.g. 6-OHDA, menadione) and cell type [32,43,61–66,72], it mediates apoptosis by direct activation of MAPK (JNK/SAPK pathway) [66] and subsequent activation of both NF-κB (Fig. 3B and [61,67]) and c-Jun (Fig. 3D and [63]) most likely through MEKK1 phosphorylation. Indeed, MEKK1, an upstream kinase of the JNK, links c-Jun and NF-κB pathways by phosphorylation of I kappa-B complex with subsequent dissociation of NFB/IB [77] and phosphorylation of MKK4 [78,79] which phosphorylates JNK leading to the activation of c-Jun. These ideas are supported by two additional observations such as the ability of DA to induce cellular apoptosis through its oxidation linked to JNK activation pathway [68] and the ability of H_2O_2 to activate directly the oncogenic p21-ras protein [69].

Our present results indicate that activation of caspase-3 is involved in dopaminergic- and serotonergic-related toxins-induced apoptosis. Thus, how could caspase-3 be activated and related with those transcription factors? According to our data, there is a positive relation between the dramatic decrease of apoptosis and (a) caspase-3 inactivation (Fig. 2) and (b) diminution of the expression of p53 and c-Jun in the presence of AMD (Table 3). These results suggest that p53 and c-Jun may mediate the activation of caspase-3. In agreement with this view, it has been reported that p53 may induce caspase-3 activation via mitochondrial pathway [24] through direct transcription of the pro-apoptotic *bax* gene [73]. Furthermore, it has also been demonstrated that 6-OHDA-induced apoptosis in PC12 cells involving p53 and Bax activation [43]. In fact, Bax has been shown to induce cytochrome *c* (cyt C) release from mitochondria [76], involved in p53-dependent apoptosis [75] and activation of caspase-3 [26]. Alternatively, the H_2O_2-generated toxin may directly induced release of cyt C [74] and activation of caspase-3 [26]. However, under the present experimental conditions this last possibility is improbable since we found that AMD (and CHX) reduced neurotoxins-induced apoptotic process to control values (Tables 1 and 3). Thus, this implies that toxin H_2O_2-induced apoptosis is dependent on mRNA and protein synthesis *de novo* as demonstrated above. According to our present findings and accumulated data, a schematic picture is proposed to integrate the major molecular cascade signalisation events of cell death induced by dopaminergic- and serotonergic-related toxins in PBL (Fig. 4).

In summary, we provide evidence that 6-OHDA, 5,6- and 5,7-DHT-derivated toxins provoke apoptosis in PBL cells by a common oxidative mechanism involving the oxidation of toxins into quinones and production of the by-product, H_2O_2 which in turn triggers a specific cell death signalisation through NF-κB, p53, c-Jun transcription factors and caspase-3 activation pathway. We also provide evidence suggesting that the dopaminergic- and serotonergic-related toxins-induced apoptosis is mediated mainly by H_2O_2 generation, and required *de novo* mRNA and protein synthesis. The elucidation of the molecular cascade of events induced apoptosis by those toxins may provide some clues in the understanding of cell death in PD. Indeed, our findings support the notion that oxidative stress may play an important role in PD [80] in agreement with recent data showing a relationship between the nuclear localisation of NF-κB in dopaminergic neurons of PD patients and oxidative stress [81] and a positive correlation between the degree of neuronal loss in dopaminergic mesencephalic neurons and the percentage of caspase-3 positive neurons in PD patients [82]. Thus, the present data indicate that PBL cells represent a convenient model system to investigate the molecular cascade(s) involved in the dopaminergic death process and to investigate potential pharmaceutical agents that may rescue or retard cell death in neurodegenerative diseases.

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