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REVIEW

Monoamine and Iron-Related Toxicity: From "Serotonin-Binding Proteins" to Lipid Peroxidation and Apoptosis in PC12 Cells

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ABSTRACT. 1. Monoamines do not form coordination bonds with a preformed iron–serotonin-binding protein (SBP) complex, as initially believed. Instead, metals oxidize the monoamines either directly (manganese, copper) or by oxygen free radical formation (iron), the oxidation products bind covalently to SBP and the conjugates are able to undergo redox cycling. These interactions are denoted as a "molecular oxidative mechanism."

2. Dopamine in combination with iron induces lipid peroxidation and apoptosis in PC12 cells by a stress oxidative-Ca^{2+} independent mechanism.

3. Dopamine–iron cytotoxicity may have relevance to an understanding of the mechanism by which dopaminergic neurons are eroded in some neurodegenerative disorders. GEN PHARMAC 31;1:19–24, 1998. **© 1998 Elsevier Science Inc.**

KEY WORDS. Monoamine, iron, SBP, PC12, lipid peroxidation, apoptosis

SEROTININ-BINDING PROTEINS AS ALLEGED STORAGE PROTEINS FOR SEROTONIN

Secretory cells have developed mechanisms to reduce the free concentration of monoamines within their storage vesicles and thereby also the osmotic pressure. Whereas the association of catecholamines with ATP and divalent cations is well documented (Winkler *et al.*, 1981; Yoo *et al.*, 1990), it was proposed by Tamir and Huang (1974) that serotonin should tightly bind to specific "serotonin-binding proteins" (SBPs). SBPs from different species were reported to comprise two components with molecular masses close to 45 and 56 kDa (Barasch *et al.*, 1987; Jimenez Del Rio *et al.*, 1992; Liu *et al.*, 1985; Tamir *et al.*, 1989). They were initially thought to be localized in synaptic vesicles (Gershon and Tamir, 1981; Jonakait *et al.*, 1979; Tamir and Gershon, 1979), but subsequent studies suggested that the 56-kDa form was freely present in the cytosol, where it could transport or protect the amine or both (Adlersberg *et al.*, 1987).

Interestingly, SBP from bovine frontal cortex was also found to bind dopamine as well as other compounds that contain a catechol moiety (Jimenez Del Rio *et al.*, 1992). It was therefore proposed that SBP might also play a "housekeeping" (i.e., storage, transport, protection) function for catecholamines. This hypothesis was initially supported by the detection of SBP in soluble extracts from bovine retina and adrenal medulla, tissues that contain high levels of catecholamines and very limited amounts of serotonin (Jimenez Del Rio *et al.*, 1993a; Pinxteren *et al.*, 1993).

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MOLECULAR MECHANISM OF MONOAMINE–SBP INTERACTION

The molecular mechanism of the serotonin–SBP interaction is essential to an understanding of the physiological role of these proteins. Because the binding of serotonin to SBP is strongly enhanced by Fe^{2+} but not by Fe^{3+} ions (Tamir and Rapport, 1978), the serotonin–SBP association was initially explained by a coordinative mechanism in which Fe^{2+} first binds to SBP and then forms coordination bonds with as many as four serotonin molecules (Tamir and Liu, 1982). However, this initial model could not be reconciled with observations such as (1) the ability of reducing agents and agents that deplete superoxide radicals (superoxide dismutase) to inhibit the Fe^{2+}-stimulated binding, (2) the ability of oxidizing agents and superoxide radicals to stimulate the binding in the absence of Fe^{2+} and (3) the irreversible nature of the monoamine–SBP association. These observations rather imply that the binding requires an oxidative step. Because the oxidation products of catecholamines have been known for some time to bind to proteins in a covalent fashion (Maguire *et al.*, 1974; Misra and Fridovich, 1972), we were led to formulate an alternative model to describe the [^{3}H]dopamine– and [^{3}H]serotonin–SBP interactions (Jimenez Del Rio *et al.*, 1993b). In this model, Fe^{2+} does not participate in the binding process by itself but rather initiates the oxidation of serotonin and dopamine into electron-deficient species (quinoneimine, *o*-quinones) that are capable of forming covalent bonds with external nucleophiles such as the sulphydryl groups of SBP. This model comprises a three-step mechanism: (1) reaction of Fe^{2+} with dissolved molecular oxygen to produce superoxide radicals, (2) oxidation of the monoamines by the radicals into quinone derivatives and (3) covalent binding of the oxidation products to cysteine residues of proteins.

It is evident that SBP does not participate in the housekeeping of monoamines, because such functions cannot be reconciled with (1) the binding of oxidation products, (2) the covalent nature of this binding and (3) the fact that this binding requires only the presence of exposed nucleophiles such as sulfydryl groups. This suggests that any protein could form a potential target for monoamine-derived oxidation products, provided that it possesses a sufficiently exposed sulfydryl group. The preferential labeling of two components with respective molecular masses of 45 and 58 kDa in extracts from brain and other tissues (Gershon and Tamir, 1984; Gershon *et al.*, 1983; Jimenez Del Rio *et al.*, 1992) could therefore merely be a result of their relative preponderance compared with other proteins. In this respect, Small and Wurtman (1984, 1985) had already noticed that the molecular mass of actin is similar to that of the 45-kDa form of SBP, and the possibility was addressed that the two proteins may be identical. Further support for this suggestion arose from the observation that [^{3}H]serotonin and [^{3}H]dopamine bind with the same characteristics to rabbit skeletal muscle actin and to SBP from bovine brain (Velez Pardo *et al.*, 1995a). In the same vein, the higher molecular mass SBP is suspected to be tubulin.

MONOAMINE/IRON-ASSOCIATED TOXICITY: STUDIES ON CELL EXTRACTS

There is ample evidence that catecholamine- and serotonin-derived oxidation products form covalent bonds with several intracellular proteins (Ito *et al.*, 1988; Kato *et al.*, 1986). The most thoroughly examined protein, catechol *O*-methyltransferase, can be irreversibly inactivated by several of such oxidation products (Borchardt, 1975). G proteins, which play a key role in the regulation of signal transduction pathways, were recently also shown to be inactivated by oxidation products of serotonin (Fishman *et al.*, 1991). These findings led us to the notion that the covalent monoamine–SBP binding could represent an *in vitro* tool for investigating neurotoxicity. This assumption is further strengthened by the following series of findings.

Besides Fe^{2+}, cations such as Mn^{2+} and Cu^{2+} also were observed to increase the binding of dopamine to SBP (Velez-Pardo *et al.*, 1995b). These metal ions are well known to oxidize catecholamines, and it is particularly striking that diseases that are characterized by neurological symptoms and can be attributed to the destruction of dopaminergic neurons are also associated with increased levels of iron (idiopathic Parkinsonism), manganese (chronic manganese intoxication) and copper (Wilson disease) (Gorell *et al.*, 1997; Oder *et al.*, 1994; Segura-Aguilar and Lind, 1989; Sengstock *et al.*, 1992; Sloot *et al.*, 1994).

Catecholamine and serotonin-related neurotoxins such as adrenochrome, 6-hydroxydopamine, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine and 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline are potent inhibitors of the binding of monoamines to SBP (Jimenez Del Rio *et al.*, 1994). The ability of SBP to constitute a target for those neurotoxins further illustrates the potential cytotoxic implications of covalent monoamine–SBP binding.

Molecular mechanisms such as free oxygen radical formation, alkylation of cell constituents, aggregation of proteins and redox cycling (in which the monoamine should constantly cycle between its oxidized and its reduced form, thereby depleting the cell's oxygen as well as certain reducing agents) have been proposed to explain the oxidation-related toxic effects of serotonin-related neurotoxins (Cohen and Heikkila, 1974; Floyd and Wiseman, 1979; Goyal and Garg, 1996; Ito *et al.*, 1988; Saner and Thoenen, 1971; Sinhababu and Borchardt, 1988). It is likely that some of these mechanisms could act in concert to produce neurodegeneration. In this respect, it has been demonstrated that "quinoproteins" (i.e., proteins that possess covalently attached quinone cofactors) are capable of producing "redox cycling" (McIntire, 1994), and it was recently found that SBP-associated dopamine is also able to catalyze this process (Velez-Pardo *et al.*, 1996). It is thus conceivable that, after covalent binding of their oxidation products to proteins, dopamine, serotonin or related neurotoxins could still be capable of depleting the cell from essential oxidant and reductant components.

MONOAMINE/IRON-ASSOCIATED TOXICITY: STUDIES ON INTACT CELLS

Several animal models as well as neuronal and nonneuronal cell lines have been used to investigate the toxic effect of iron and catecholamines (Ben-Shachar and Youdim, 1991; Glinka *et al.*, 1996; Michel and Hefti, 1990; Mytilineou *et al.*, 1993; Sengstock *et al.*, 1992). PC12 cells, a clonal catecholaminergic cell line derived from rat pheochromocytoma that responds to nerve growth factor by undergoing differentiation into a sympathetic-like neuronal phenotype (Greene and Tischer, 1976), have been particularly fruitful for the study of catecholamine toxicity. A particularly striking observation was that dopamine-related compounds such as L-DOPA and the neurotoxin 6-hydroxydopamine are able to induce apoptosis, a type of cell death exhibiting distinct morphological and biochemical features such as cell shrinkage, chromatin condensation, breakup of the nucleus and plasma membrane blebbing followed by fragmentation of the cell into discrete apoptotic bodies (Walkinshaw and Waters, 1994, 1995; Zilkhafalb *et al.*, 1997).

The combined action of catechols and iron on intact cells was first investigated by Tanaka *et al.* (1991). Using a dorsal root ganglion neuronal culture, they demonstrated that both dopa and dopamine cause cell death in the presence of iron. Recently, we also investigated the combined action of dopamine and iron on PC12 cells. To avoid the possibility that the cytotoxic actions of dopamine and iron could result from an alternative pathway involving the turnover of dopamine by monoamine oxidase B enzyme, the monoamine oxidase B inhibitor pargyline was included in the incubation media (Velez-Pardo *et al.*, 1997). Because iron under the form of $FeSO_4$ interferes with microscopic evaluations and staining of PC12 cells, it was replaced by ferrocene. This form of iron had already been used for iron loading in living systems, and it has the advantage of being taken up by cells in a relatively short time (Ward *et al.*, 1991). Under these conditions, iron and dopamine were shown to act synergistically to induce apoptosis in undifferentiated PC12 cells. This event was associated with a variety of structural changes such as the covalent linking of dopamine to proteins, the formation of protein aggregates and lipid peroxidation.

In PC12 cells, lipid peroxidation could also be produced by dopamine and ferrocene alone. Experiments with ascorbic acid revealed that it was able to inhibit the effect of dopamine but not the effect of ferrocene (Velez-Pardo *et al.*, 1997). This latter finding is compatible with the reported use of ascorbic acid–iron combinations to generate free radicals (Ramassamy *et al.*, 1994), and it suggests that ascorbic acid does not prevent lipid peroxidation by free oxygen radicals. The ability of ascorbic acid to block the effect of dopamine fits with its well-known ability to reduce quinones and semiquinones back to catechols (MacDonald and Sirvio, 1993; Pardo *et al.*, 1993). It is therefore likely that the dopamine-mediated lipid peroxidation is provoked by its oxidation products rather than by free oxygen radicals (Velez-Pardo *et al.*, 1997). A quite similar conclusion was reached by Tanaka *et al.* (1991), who found that superoxide and hydroxyl radicals are not essential for the catecholamine–iron-mediated lipid peroxidation in cultured dorsal

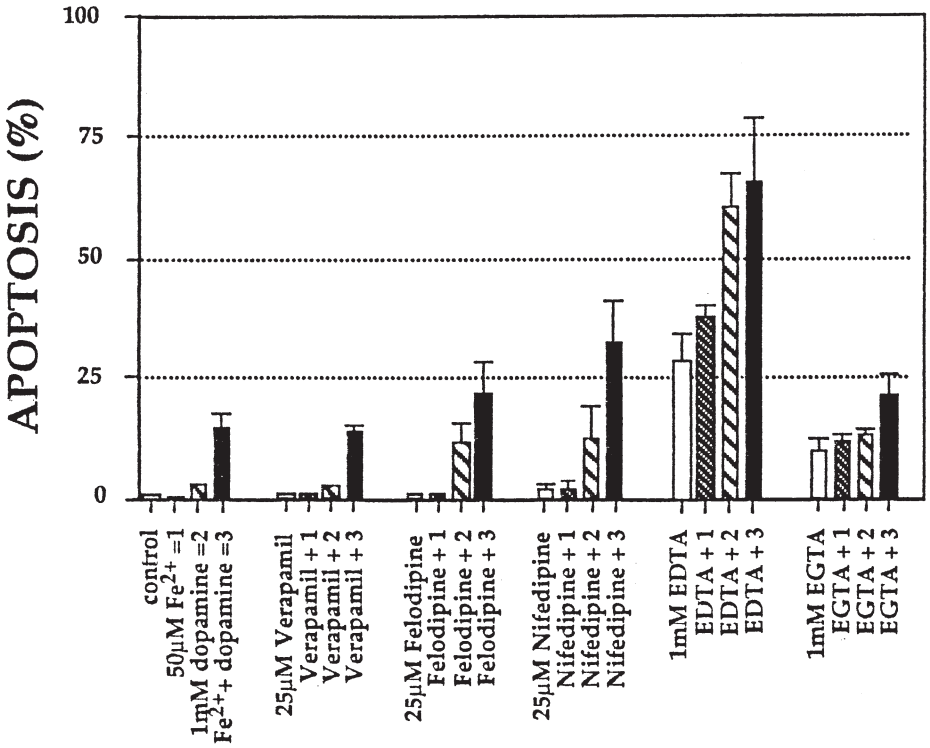

FIGURE 1. Effect of calcium channel blockers and chelators in PC12 cells. PC12 cells were incubated for 24-hr at 37°C in culture medium containing 1 mM dopamine in the presence of 50 μM ferrocene or 25 μM of Ca^{2+} blockers or both (verapamil, felodipine and nifedipine) and 1 mM Chelators (EDTA, EGTA). Cells were then used for microscopic examination. Apoptotic index was evaluated in percentage as described earlier (Velez Pardo *et al.*, 1997). Values are means±SD of six experiments.

root ganglia. After 24-hr treatment with a combination of dopamine and ferrocene, PC12 cells underwent morphological changes that are characteristic of apoptosis; that is, cell shrinkage, chromatin condensation, breakup of the nucleus, plasma membrane blebbing and cell fragmentation (Velez-Pardo *et al.*, 1997). This effect was markedly reduced by ascorbic acid, and oxidation products of dopamine may therefore also constitute potential triggers for apoptosis in PC12 cells.

Taken together, the experiments with ascorbic acid suggest that the oxidation products of dopamine may elicit both lipid peroxidation and apoptosis in PC12 cells. The link between both phenomena could be causal, as suggested by Tanaka et al. (1991) because apoptosis of PC12 cells may be induced by serum deprivation (Batistatou and Greene, 1991; Kerr *et al.*, 1972; Martin *et al.*, 1994) in the absence of detectable lipid peroxidation.

MONOAMINE/IRON-ASSOCIATED APOPTOSIS: IMPLICATION OF CALCIUM?

It has been suggested for some time that a sustained increase in Ca^{2+} occurs in the early stages of apoptosis (Bellomo *et al.*, 1992). In this context, the calcium ionophores A23187 and ionomycin are both potent inducers of apoptosis (Aznu *et al.*, 1996; Cohen and Duke, 1984). Extracellular removal of Ca^{2+} or pretreatment with calcium chelators also have been shown to prevent either DNA cleavage or morphological apoptotic changes (McConkey *et al.*, 1989; Ojcius *et al.*, 1991; Perotti *et al.*, 1990; Zheng *et al.*, 1991). Although little is known about the precise signaling cascade by which calcium promotes cell death, calcium is thought to play an important regulatory function in apoptosis and even to act as a signaling molecule in this process (Golstein *et al.*, 1991; Lee *et al.*, 1993; McConkey and Orrenius, 1994; Trump and Berezesky, 1992). However, it should be noted that an increase in intracellular calcium may not be a universal initiator signal of this process, and there is even growing evidence against a universal requirement for Ca^{2+}. Indeed, not all apoptosis models exhibit an increase in intracellular Ca^{2+} (Kluck *et al.*, 1994; Kruman *et al.*, 1992). For instance, in promyelocytic leukemia (HL-60) cells, it has been shown that elevation in cytosolic free Ca^{2+} is not required to trigger apoptosis (Lennon *et al.*, 1992). Moreover, the calcium ionophore A23187 has even been shown to block apoptosis in certain systems (Rodriguez-Tarduchy *et al.*, 1990), and a decrease in intracellular Ca^{2+} was reported to induce apoptosis by interleukin 3 (IL-3) withdrawal from the IL-3-dependent hemopoietic 32D cell line (Baffy *et al.*, 1993).

To get a better understanding of the molecular mechanism of the dopamine–iron-mediated apoptotic process in undifferentiated PC12 cells (Velez-Pardo *et al.*, 1997), we examined the role of Ca^{2+} in this process. This was performed by adding Ca^{2+} chelators (EDTA, EGTA) and three Ca^{2+} channel blockers (nifedipine, felodipine and verapamil) to the assay medium. In this respect, undifferentiated PC12 cells were shown to possess the L-type Ca^{2+} channels (Toll, 1982).

For viability studies, treated PC12 cells were mixed with 1 μl acridine orange–ethidium bromide (0.1 mg/ml), and 10 μl of the suspension was placed onto a slide and examined under fluorescence on a confocal microscope (Leitz Wetzlar, Germany). On the basis of the differential uptake of the fluorescent DNA binding dyes acridine orange and ethidium bromide, normal cells (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 (less than 1%, bright orange chromatin). Quantification of apoptosis was recorded by counting a minimun of 200 total cells as follows: % apoptotic cells={[Total number of apoptotic cells (EA+LA)]/total number of cells counted}×100 (McGahon *et al.*, 1995).

After 24-hr incubation, 50 μM ferrocene caused apoptosis of 1% of the cells and 1 mM dopamine alone 3±1% (Fig. 1). Combined addition of dopamine and ferrocene induced apoptosis to 14±4% of the PC12 cells. Addition of either 25 μM felodipine or nefidipine to this mixture provoked a further increase in the percentage of apoptotic cells: 22±7 and 32±9% of the cells, respectively. In contrast, verapamil did not increase dopamine–iron-mediated apoptosis (i.e.,

14±3%). As control, no significant apoptosis was observed when the Ca^{2+} channel blockers were added to PC12 cells alone.

Ca^{2+} chelators also increased the percentage of apoptosis. As shown in Figure 1, EDTA induced apoptosis alone (29±10%) and in the presence of ferrocene (38±3%), dopamine (60±13%) or dopamine plus ferrocene (65±24%). EGTA was less potent; it produced only 21±5% of apoptosis in combination with dopamine and ferrocene.

The increased apoptosis by calcium chelators and by L-type Ca^{2+} channel blockers suggests that high concentrations of calcium do not constitute a major factor in triggering the apoptotic process in PC12 cells. In agreement with the apoptosis models that do not exhibit an increase in intracellular Ca^{2+} (Baffy *et al.*, 1993; Kluck *et al.*, 1994; Kruman *et al.*, 1992; Lennon *et al.*, 1992; Rodriguez-Tarduchy *et al.*, 1990), the present findings further challenge the universality of models in which a sustained increase in intracellular Ca^{2+} should be required for apoptosis (McConkey and Orrenius, 1994; Perotti *et al.*, 1990). In fact, these results suggest that depletion of Ca^{2+} may contribute or even lead to apoptosis in PC12 cells.

CLINICAL CONTEXT

The preceding findings suggest that serotonin, dopamine and their related oxidative products could have toxic effects under certain unbalancing conditions—for example, elevated concentrations of iron or other metals such as copper and manganese. In this respect, it is remarkable that Parkinsonism is associated with selective increases in iron in the substantia nigra and reduced ferritin buffering (Dexter *et al.*, 1989; Reiderer *et al.*, 1989). Dopamine is the primary neurotransmitter in the nigrostriatal system, and a loss of dopaminergic nerve cells in this brain area is the major neuropathological feature of Parkinson disease. Although the cause (or causes) of the degeneration of dopaminergic neurons is still unknown, the oxidant stress hypothesis is regarded by some investigators (Ebadi *et al.*, 1996; Fahn and Cohen, 1992; Jenner 1992; Jenner *et al.*, 1996) to be the best currently available. Several indications for oxidant stress in Parkinson disease have indeed been found: depletion of reducing substances such as glutathione (Sofic *et al.*, 1992), a high concentration of iron (Dexter *et al.*, 1991; Riederer *et al.*, 1989; Sengstock *et al.*, 1992; Sofic *et al.*, 1991), oxidation of proteins and membrane lipids (Dexter *et al.*, 1989) and deficiencies in mitochondrial complex I (Mitzuno *et al.*, 1990). It is thus conceivable that iron-mediated oxidation of dopamine could contribute to the progression of this disease. In the same vein, Volicer and Crino (1990) suggested that certain oxidation products of serotonin are also implicated in Ahlzeimer disease.

The monoamine–SBP binding has already been proposed to constitute an *in vitro* test for investigating certain forms of drug toxicity and, in particular, for investigating potential factors that may contribute to the degeneration of dopaminergic neurons in Parkinson disease. Cultured PC12 cells are now proposed to represent a more refined model for investigating different cellular aspects of iron and dopamine-related oxidative stress.

SUMMARY

Past *in vitro* studies have shown that Fe^{2+} increases the binding of monoamines to "serotonin-binding proteins" from calf brain extracts and to skeletal muscle actin by an oxidative mechanism, in which ferrous ions are able to generate oxygen free radicals from molecular oxygen. These radicals oxidize catecholamines and serotonin into products that are able to form covalent bonds with the external sulfhydryl groups of SBPs (Jimenez Del Rio *et al.*, 1993; Velez-Pardo *et al.*, 1995a). In subsequent studies, we also found that the monoamine–SBP conjugates were able to catalyze redox cycling processes (Velez-Pardo *et al.*, 1996). These results support the view that the iron-mediated covalent binding of monoamines to proteins represents an *in vitro* model for cytotoxicity.

Recently, dopamine was demonstrated to induce apoptosis in undifferentiated PC12 cells in the presence of iron (Velez-Pardo *et al.*, 1997). This process was evidenced by a series of compartmentalized cytotoxic events at the level of nucleus (chromatin condensation and fragmentation), cytoplasm (high-molecular-weight protein aggregates, vacuolization) and membranes (lipid peroxidation). These findings are in agreement with current views about iron and catecholamine-mediated neurotoxicity; that is, (1) iron greatly accelerates the autoxidation of monoamines such as dopamine (Langston *et al.*, 1987) and (2) the autoxidation of dopamine produces reactive quinones and oxyradicals (Brunmark and Cadenas, 1989; Graham, 1978), whose accumulation induces oxidative stress in tissues (Ben-Shachar *et al.*, 1995; Halliwell, 1992; Halliwell and Aruoma, 1991; Orrenius *et al.*, 1992). The cytotoxic actions of dopamine and iron could also result from an alternative pathway involving the turnover of dopamine by the monoamine oxidase B enzyme but, because the present experiments were carried out in the presence of pargyline, our data suggest that iron also is capable of increasing the cytotoxicity of dopamine by increasing its rate of oxidation by a molecular oxidative mechanism (Jimenez Del Rio *et al.*, 1993b; Velez-Pardo et al, 1997). Moreover, taken together all these findings support the hypothesis that oxidative stress could be a general mediator of cell death in neurodegenerative diseases (Hensley *et al.*, 1996; Owen *et al.*, 1996; Schubert *et al.*, 1995).

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