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MPP+

Mechanism for Its Toxicity in Cerebellar Granule Cells

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Abstract

Cerebellar granule cells constitute the largest homogeneous neuronal population of the mammalian brain. However, they are not often used in studies that involve MPP+-neurotoxicity. Currently, it is known that the toxicity of MPP+ in cerebellar granule cells as well as in other models, including dopaminergic cells, results from activation of the apoptotic machinery after an initial oxidative burst with mitochondrial damage and energetic failure. Therefore, cerebellar granule cells serve as a good model to investigate the MPP+ effects and to study in vitro the molecular mechanism implicated in the genesis of Parkinson's disease.

Index Entries: Cerebellar granule cells (CGC); 1-methyl-4-phenylpyridinium (MPP+); apoptosis; Parkinson's disease.

Cerebellar Granule Cells as a Model to Study Neuronal Cell Death

Cerebellar granule cells (CGCs) comprise the largest homogeneous neuronal population in the mammalian brain (90% of the total cell content). Primary cultures of CGCs were established almost 30 yr ago *(1–3)*. Since then, they have become the most popular in vitro model to study almost every aspect of functional and pathological neurobiology in a rather homogeneous population of neurons: between 90 and 95% of cells present in the culture are CGCs.

This article reviews the most relevant results concerning 1-methyl-4-phenylpyridinium (MPP+) toxicity in CGCs. Although these cells do not comprise the most widely used model to study the degeneration associated with MPP+-neurotoxicity and Parkinson's disease (PD), CGCs are quite sensitive to the toxic effects of MPP+ in vitro *(4–8)* and in vivo *(9)*. On the other hand, the intracellular concentration

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of MPP^+ that is achieved in CGCs following exposure to MPP^+ is comparable to the calculated intracellular concentration in dopaminergic neurons. Therefore, CGCs represent a good model to study the mechanisms of MPP^+ toxicity, which could be extrapolated to the dopaminergic system.

Morphology of Cell Death

Necrosis

The word necrosis describes the death of a cell or of a group of cells as a result of injury, disease, or pathological state. Therefore, necrosis is associated with one or more exogenous factors that lead to nonphysiological cell death. The morphological changes that are characteristic of necrosis *(10)* include clumping of the chromatin without marked changes in its distribution. Densities in the matrix of abnormally swollen mitochondria and local membrane disruption are sometimes evident *(11).* At later stages, there is a more-or-less pronounced disintegration of cell organelles and membranes, although the cell somehow maintains an overall identity.

Programmed Cell Death

According to its morphological appearance, programmed cell death can be subdivided into two major classes (12)—namely, apoptotic (type I) and autophagic (type II) cell death.

Apoptosis

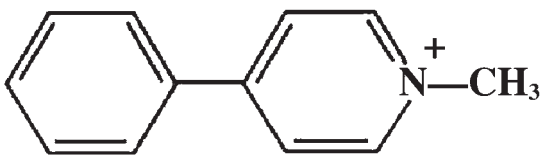
Apoptosis originally was defined as a mode of cell death with a series of characteristic events following this sequence: nuclear and cytoplasmic condensation, cell fragmentation, and phagocytosis *(13).* Changes in the nucleus represent the first unequivocal evidence of apoptosis. Chromatin condensation and segregation into sharply delineated masses typically are observed at the onset of apoptosis. Cytoplasm condensation also occurs in parallel with nuclear changes and the cell membrane becomes convoluted, with a star-like appearance. After the cell and its nucleus assume a more irregular shape, nuclear budding produces discrete fragments. The cell is fragmented into membrane-bound apoptotic bodies, which are very characteristic of this type of cell death. The nuclear alterations are often associated with internucleosomal cleavage of DNA, which is recognized as DNA laddering on conventional agarose gel electrophoresis. It is clear that specific cellular pathways are activated in apoptosis, enforcing the concept that in this type of cell death, cells are responsible of their own demise ("cell suicide"). Besides being relevant in several physiological functions (differentiation, maturation, *etc.*), apoptosis is involved in cell injury that is produced by a spectrum of physical and chemical agents *(14–16).* Apoptosis is also involved in several neurodegenerative diseases, including Alzheimer's Disease *(17)* or PD *(18).*

Autophagy

Autophagy is a third type of cell death in which the cytoplasm is actively destroyed by lysosomal enzymes far before nuclear changes become visible. The most characteristic feature is the appearance of large autophagic vacuoles of lysosomal origin in the cytoplasm *(19).* Although many of the characteristic changes of apoptosis eventually become evident in these cells, changes are notably delayed, and substantial cellular degradation is evident before the typical nuclear alterations of apoptosis occur. Finally, when about three-quarters of the cytoplasm has been destroyed, it begins to condense, and chromatin coalescence and margination become apparent *(20).*

MPP^+: A Tool to Study the Cell Death in Neurodegenerative Disorders

In 1983, Langston et al. *(21)* reported that a group of people who were addicted to a new synthetic fentanyl derivative developed an irre-



MPP+

Fig. 1. Chemical structure of MPP+.

versible disease, with symptoms very similar to those of PD. Analysis of the drug showed a content of 3% of a bipiridinium derivative, the MPTP. Postmortem investigations clearly confirmed the lesion of *substancia nigra (22).* Additionally, in the monkey, MPP+ (*see* chemical structures in Fig. 1) induces a massive loss of dopamine (DA) in the *striatum (23,24)* and a depletion of dopaminergic markers in the nigrostriatal tract *(25).* Interestingly, in monkeys, the topology of the nigral lesion after MPP+ treatment is similar to that observed in human PD.

With these facts, MPTP/MPP+-induced neurotoxicity is one of the experimental models that is most commonly used to study the pathogenesis of PD *(5,6,26–29).* However, many aspects for its toxicity remain unclear, especially the early events that occur during the first hours after MPP+ exposition. One controversial aspect is whether MPP+ induces structural changes that are related to cell death via apoptosis (I or II) or necrosis. Investigations aimed at clarifying this aspect were carried out both after in vivo administration of MPP+ to animals and by treating neuronal cultures with MPP+ *(30).* Methods allowing a distinction between apoptosis and necrosis were then applied. However, the results gave no univocal explanation, although it is generally accepted that low doses of MPP+ lead to "classical" apoptosis, whereas high doses of MPP+, which cause acute toxicity, lead to necrotic degeneration. There are currently no studies describing autophagical cell death induced by MPP+. Despite the properties of CGCs that make them suitable for neurodegeneration studies and the fact that primary cultures of CGCs have been shown to be susceptible to MPP+ toxicity in the 0.01- to 1-m*M* range *(6,7,31,32),* these cells are not extensively used for the study of MPP+ neurotoxicity. Briefly, this article shows the most important results obtained with this model.

MPP+ Incorporation Into CGC

To be active, MPTP must be converted into MPP+, which is the true toxic agent, by the monoamino oxidase B in the inner mitochondrial membrane *(33).* Because MPP+ is a polar molecule, it cannot freely enter into cells but depends on the plasma membrane carriers to gain access. Three transporters have been implicated in the entrance of MPP+ into CGCs: dopamine transporter (DAT), organic cation transporter (OCT), and cationic amino acid transporter (CAT).

It is known that MPP+ shows a high affinity for DAT in several cells *(34–36).* The possible incorporation of MPP+ inside CGCs mediated by DAT has been studied by blockage of the neurotoxic effects of MPP+ by the DAT antagonist mazindol *(4)* or, more recently, by GBR-12909 *(7).* Despite this fact, a lack of expression of DAT has been described in CGC *(37),* and it has been hypothesized that GBR-12909 is neuroprotective by a mechanism not involving DAT. The data suggests that GBR-12909 protects CGCs against MPP+ toxicity as a result of binding to the Σ receptor.

The cationic charge of MPP+ pushed us to investigate the possibility that an OCT was implicated in MPP+ uptake. CGCs express OCT3 but do not express OCT1 or OCT2 *(37).* Experiments using methy1-[^{3}H] MPP+ and selective inhibitors of OCT3 as 17β-estradiol, corticosterone, or decynium-22 show a strong inhibition of MPP+ accumulation in CGCs. This fact demonstrated that MPP+ is transported into CGCs through OCT3 with high affinity.

However, block of OCT3 does not completely inhibit MPP^+ accumulation or cell death *(37)*. As indicated previously, MPP^+ is a permanently charged cation *(38)*. Therefore, the CAT family is a putative transporter implicated in neurotoxin uptake. These transporters show a relatively low specificity and could use L-Arg, L-Lys, or L-His as substrate. Gonzalez-Polo et al. *(7)* studied the role of the CAT family in the transport of MPP^+ and measured the effect of several cationic amino acids in the neurotoxicity mediated by MPP^+. Their results showed an increase of cell survival and a blockage of DNA laddering, suggesting that CAT is implicated in the MPP^+ uptake into the cells.

Molecular Machinery Implicated in MPP^+ Toxicity in CGCs

Oxidative Stress: The Beginning

Several works have suggested involvement of reactive oxygen species (ROS) in MPP^+-induced neurotoxicity in various cell types *(39–41)*, including CGCs *(7,42,43)*. Generation of ROS has been detected using the oxidation-sensitive probes H_2 2′-7-dicthorodihydrofluorescein acetate (DCFDA) (moderately selective for peroxide) and dihydroetidine (DHE) (moderately selective for superoxide anion). Classically, ROS production is attributed to the inhibition of mitochondrial complex I. This blockage induces ROS peaks measured at 6 or 24 h after addition of MPP^+ *(43,44)*. However, ROS generation (detected by the oxidation-sensitive fluorescent probe DHE) is one of the earliest cellular responses to MPP^+ toxicity, and it is observed 30 min after MPP^+ exposure *(42)*. This early ROS production may be a result of MPP^+ interaction with xanthine oxidase (XO) *(42)*, which might lead to the formation of superoxide radicals. The reversion of cell death mediated by allopurinol (an XO inhibitor) strongly supports this fact. Participation of superoxide anions produced by XO during the neurotoxic process in CGCs were recently published *(45–47)*. This participation is apparently mediated by changes in cytosolic calcium concentrations, particularly after glutamate excitotoxic pulse *(48)*.

Implication of early and late MPP^+ ROS production is also demonstrated by the protection with several antioxidants *(49,50)*, especially vitamin E *(42)*. Concomitant with the ROS production, a decrease of glutathione levels was observed *(42)*. These data are complementary with the increase of resistance to MPP^+ toxicity that is observed in CGCs after overexpression of glutathione peroxidase (GPx1) *(43)*. These findings suggest that superoxide can play a pivotal role in MPP^+ toxicity in CGCs. Superoxide facilitates hydroxy1 radical production in the Fenton reaction by interaction with iron. Deferoxamine mesylate or N,N′-bis(2-hydroxybenzyl ethylenediamine-N) N′-diacetic acid (HBED) (two potent iron chelators) protect against loss of viability in CGCs that are exposed to MPP^+ *(43,44)*, indicating iron's essential role for MPP^+ toxicity in CGCs, as it is well-known for other cellular systems *(51,52)*. However, deferoxamine mesylate (DFx) does not directly affect DHE or DFCH responses, indicating that it does not directly affect ROS production *(44)*.

Superoxide can also react with nitric oxide (NO) to produce peroxynitrite, another potent oxidant *(53)*. The protective effect of 5,10,15,20-tetrakis (4-sulphonatephenyl) porphyrinate iron (III) chloride (FeTPPS) (a ferric porphyrin complex that catalytically isomerises peroxynitrite to nitrite [*54*]) provide the role of peroxitrite production in MPP^+ mediated toxicity in CGCs *(44)*. Indirect determination of NO by nitrite measurement shows a moderate increase in CGCs that are exposed to MPP^+. This increase is abolished using NO synthase (NOS) inhibitors such as 2-ethyl-2-thiopseudourea (ETPU) or 7-nitroindazole (7-NI). *S*-methylisothiourea (SMIT) also partially protects against cell death that is induced by MPP^+ exposition *(44)*. The participation of NO in MPP^+ toxicity has also been reported in several other models *(55–57)*. The more effective ETPU (1 m*M*) and 7-NI (10 μ*M*)

allow the activation of the NOS isoform involved in NO production: neuronal nitric oxide synthesis (nNOS) is likely involved (iNOS is located mainly in glia [*58*]), and ablation of endothelial nitric oxide synthesis (eNOS) has no bearing on MPP+-induced neurotoxicity *(59).* Additionally, time-dependent nNOS, but not inducible nitric oxide (iNOS), expression was observed after 24 h of exposition to MPP+ *(44).*

The inhibition of mitochondria also leads to a decrease in cellular adenosine triphosphate (ATP) levels *(60).* In CGCs, exposure to low concentrations of MPP+ produced a time- and dose-dependent decrease in ATP levels *(61,62),* with a depletion of cytosolic NAD^+ levels and activation of lactate dehydrogenase activity *(62).* These depletions (observed only after 6 h and with a maximum at 24 h after MPP+ exposition) can be protected by the addition of glucose but not of pyruvate *(62).* These results indicate that MPP+, similarly to other cell models *(63–65),* causes impairment of cellular energy metabolism, with a major dependence on glycolisis as a source of energy.

The Middle Act: Cytochrome c, Bax, and Colleagues

Numerous studies have suggested that MPP+ can induce apoptosis in vitro in other cell models, such as GH3 pituitary cells, PC12, SH-SY-5Y, or primary mesencephalic dopaminergic cells *(5,6,26–29),* as well as in cerebellar granule cells *(5–7,31,42).* MPP+ toxicity in CGCs causes translocation of cytochrome c from mitochondria to the cytosol *(6,31,42).* The classical time-course for cytochrome c release of the apoptosis cascade begins 3 h after MPP+ exposition *(6,31),* with a marked time dependence *(6).* However, recently *(42),* an early release (30 min after MPP+ exposition) of cytochrome c was described. This early apoptotic event is strongly associated with an early free-radical production, because it is demonstrated by the inhibition of cytochrome c release by antioxidants such as vitamin E *(42).* These free radicals are probably the result of the interaction between MPP+ and XO, because allopurinol (a potent and specific XO inhibitor) blocks release of cytochome c *(42).* Interestingly, release of cytochrome c is not accompanied by a change in the mitochondrial transmembrane potential ($\Delta\Psi_m$). However, this parameter diminishes later (3 h after MPP+ exposition) *(31,32).*

The release of cytochrome c is associated with translocation to the mitochondria of certain Bcl-2-family proteins (e.g., Bad, Bax, or Bid) *(66)* in other cellular MPP+ models *(67).* Bax may play a central role in neuronal mitochondria-dependent apoptosis *(68).* Bax can translocate from the cytosol to mitochondria following a death signal. In CGCs, this signal is early production of ROS. In CGCs that are exposed to MPP+, an early (15–30 min after MPP+ addition) increase in mitochondrial Bax levels is observed that parallels free-radical production and cytochrome c release *(42).* Two observations support the dependence on the early free-radical burst: (a) Bax translocation is blocked by vitamin E and (b) allopurinol blocks Bax translocation. These data agree with recent reports showing the involvement of free radicals and gluthatione depletion (observed early in CGCs that are exposed to MPP+) in Bax activation *(69).* Bax could form a channel in mitochondria, allowing cytochrome c release without mitochondrial damage *(70).* No observations of early changes in mitochondrial membrane potential in MPP+ mediated toxicity in CGCs support this hypothesis.

Bad is another proapoptotic Bcl-2-related protein. In absence of apoptotic stimuli, Bad is phosphorylated and sequestered in the cytosol by binding to 14-3-3 protein *(71).* During apoptosis, Bad is dephosphorylated and promotes cell death by binding to bcl-X_L *(71).* Bad is preferably, but not exclusively, phosphorylated by Akt *(72)* and dephosphorylated by PP1 and PP2 phosphatases *(66).* In CGCs treated with MPP+, Bad is strongly dephosphorylated simultaneously (15–30 min after addition of MPP+ to Bax translocation to the mitochondria

(42). Bad can bind Bcl-X_L, which could contribute to the inhibition of the death-repressor activity of Bcl-X_L, facilitating the Bax-induced cytochrome c release. Preincubation of CGCs with okadaic acid, a broad-range protein phosphatase inhibitor, partially inhibits both early Bad dephosphorylation and cytochrome c release *(42).* This result indicates that phosphatases are activated during the 30 min after exposition of MPP^+ and that this activation is involved in cytochrome c release. Because Bad dephosphorylation is also inhibited by vitamin E and allopurinol *(42),* one could hypothesize that phosphatase activities are activated by the ROS that is produced in the first minutes after MPP^+ exposition. Bid is the third proapoptotic-bcl2 member implicated in cytochrome c release in MPP^+-induced neurotoxicity in CGCs. The ability of Bid to induce cytochrome c release has been suggested to be mediated by Bax, because Bid can facilitate the insertion of Bax into the mitochondrial membrane to form functional oligomers *(73,74).* The active form of Bid is the truncated tBid, which is formed by cleavage by caspase 8 after Fas engagement. Indeed, caspase 8 activity (measured using Ac-IETD as a substrate) increases in a time-dependent manner in CGCs that are exposed to MPP^+ starting from 30 min (with a maximum at 60 min) after the beginning of the experiment (González-Polo et al., unpublished data). Interestingly, in CGCs, the tBid form increases in parallel with Bax translocation to mitochondria and Bad dephosphorylation and precedes the release of cytochrome c (González-Polo et al., unpublished data). These data strongly indicate the participation of these three proapoptotic BCP-member proteins in MPP^+-mediated cytochrome c release (and induction of apoptosis) in CGCs.

5.3. The End: Caspase Activation

In the apoptotic cascade, release of cytochrome c from cytosol precedes the activation of several of the executors of the apoptotic program: caspases—especially caspase-3. Caspase-3 is activated for proteolytic cleavage, which is detected by fluorescence assays using Ac-DEVD as a substrate or by Western blot detection of 17 kDa. Caspase-3 implication in CGC death is demonstrated by the fact that both pancaspase inhibitor Z-VAD-fmk and the specific caspase-3 inhibitor DEVD significantly attenuates MPP^+-induced cell death in CGCs *(6,42).* Indeed, some works *(6,42)* describe the increase of caspase-3 activity in CGCs treated with MPP^+. In two cases, cytochrome c preceded caspase-3 activity but had a different schedule. González-Polo et al. *(42)* detected that an early (60 min) caspase-3 activation preceded, as described previously, by a release of cytochrome c (30 min) is associated with an early production of ROS (5–10 min). Therefore, the apoptotic cascade appears to begin with an early production of ROS because all events are blocked by antioxidants such as vitamin E, suggesting that the early events implicated in MPP^+-induced apoptosis in CGCs are related to oxitative stress—probably as a result of activation of the XO system *(42).* Apoptotic cell death can be detected from 6 h after MPP^+ exposition by MTT assay *(75)* and analysis of typical DNA fragmentation *(5–7,42,43).* Similarly to the other previous events, CGC death and DNA fragmentation can be prevented by antioxidants like vitamin E *(42),* indicating that oxidative stress plays a crucial role in MPP^+-mediated apoptotic cell death in CGCs.

Conclusions

This survey of recent literature has confirmed the extreme usefulness of the cerebellar granule neurons as a model to study the cellular pathways and the molecular signals related to the balance of survival/death of neural cells. Although CGCs have not been extensively studied as a prominent target for MPP^+ neurotoxicity, the data illustrated in this article indicate that CGCs are quite sensitive to the toxic effects of MPP^+ in vitro. The MPP^+ mode of

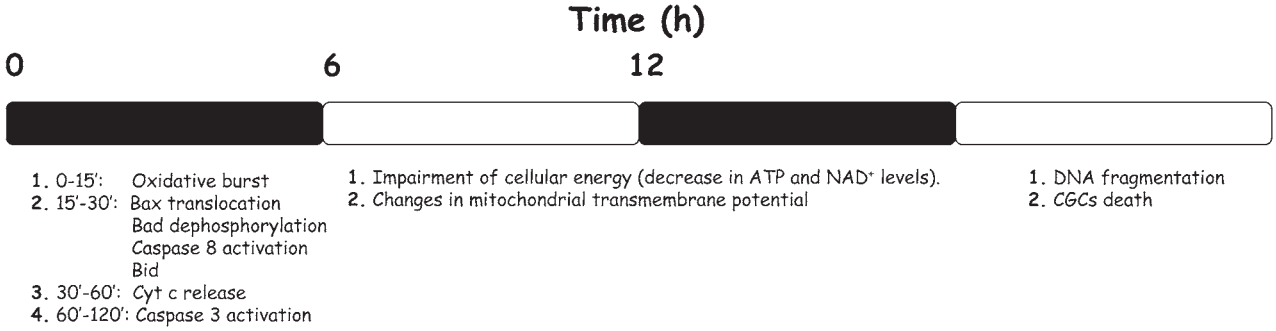


Fig. 2. Summary of the proposed schedule for the CGCs death induced by MPP+. Only the most representative events have been included.

action in CGCs is closely related to its effect in dopaminergic cells, including mitochondrial dysfunction, oxidative stress, energetic failure, and activation of the molecular pathways involved in apoptosis (*see* Figs. 2 and 3). The initial oxidative burst and the subsequent recruitment of the mitochondrion-dependent apoptotic factors seem to play a pivotal role, with caspase-3 as the main executioner of cell death. These data support the use of CGCs as a valid model to study MPP+ neurotoxicity and its application to the understanding the genesis of PD, like the participation of presynaptic proteins (as synuclein) or autophagy.

Typical Preparation of Cerebellar Granule Cells

This protocol is optimized for the preparation of CGCs from rat cerebellum but can essentially be used to obtain CGCs from others animals.

Primary cultures of CGCs are obtained from 7- to -8 d-old Wistar rats of either sex. The animals are housed in a temperature-controlled room maintained at 12 h light–dark cycles. The standard laboratory animal food and tap water were freely available for the mothers *ad libitum.* The experimental protocol resumed here is in accordance with the National Institutes of Health guidelines and is designed to minimize pain or discomfort of the animals. Cerebella dissected that are free of meninges are chopped into small pieces and digested with trypsin (2.5 mg/mL, 10 min at 37°C) in a Krebs-Ringer buffer solution, pH 7.4, containing 3 mg/mL of bovine serum albumin. After addition of soybean trypsin inhibitor (0.5 mg/mL) and DNAse (0.1 mg/mL), the tissue is disrupted by 10 passages through a fine-tip plastic transfer pipet. The resulting cell suspension is filtered through a 100-µm nylon cloth; centrifuged and resuspended in DMEM that is supplemented with 10% fetal calf serum, 25 m*M* of KCP, 2 m*M* of glutamine, penicillin (50 U/mL), and streptomycin (50 µg/mL). Then cells are seeded in poly-L-lysine-pretreated plates at a density of 5×10^5 cells/mL and grown in a humidified atmosphere of 5% CO_2 at 37°C. Cytosine arabinoside (10 µ*M*) is added 24 h after plating to arrest the growth of no neuronal cells (mostly astrocytes and microglia).

After extraction of the CGCs, the experiments are normally carried out after 7 d in culture in a DMEM medium (without fetal calf serum) supplemented with 25 m*M* of KCP, 2 m*M* of glutamine, 50 U/mL of penicillin, 50 µg/mL of streptomycin, 0.1 mg/mL of sodium pyruvate, 20 n*M* of progesterone, and 5 µg/mL of insulin.

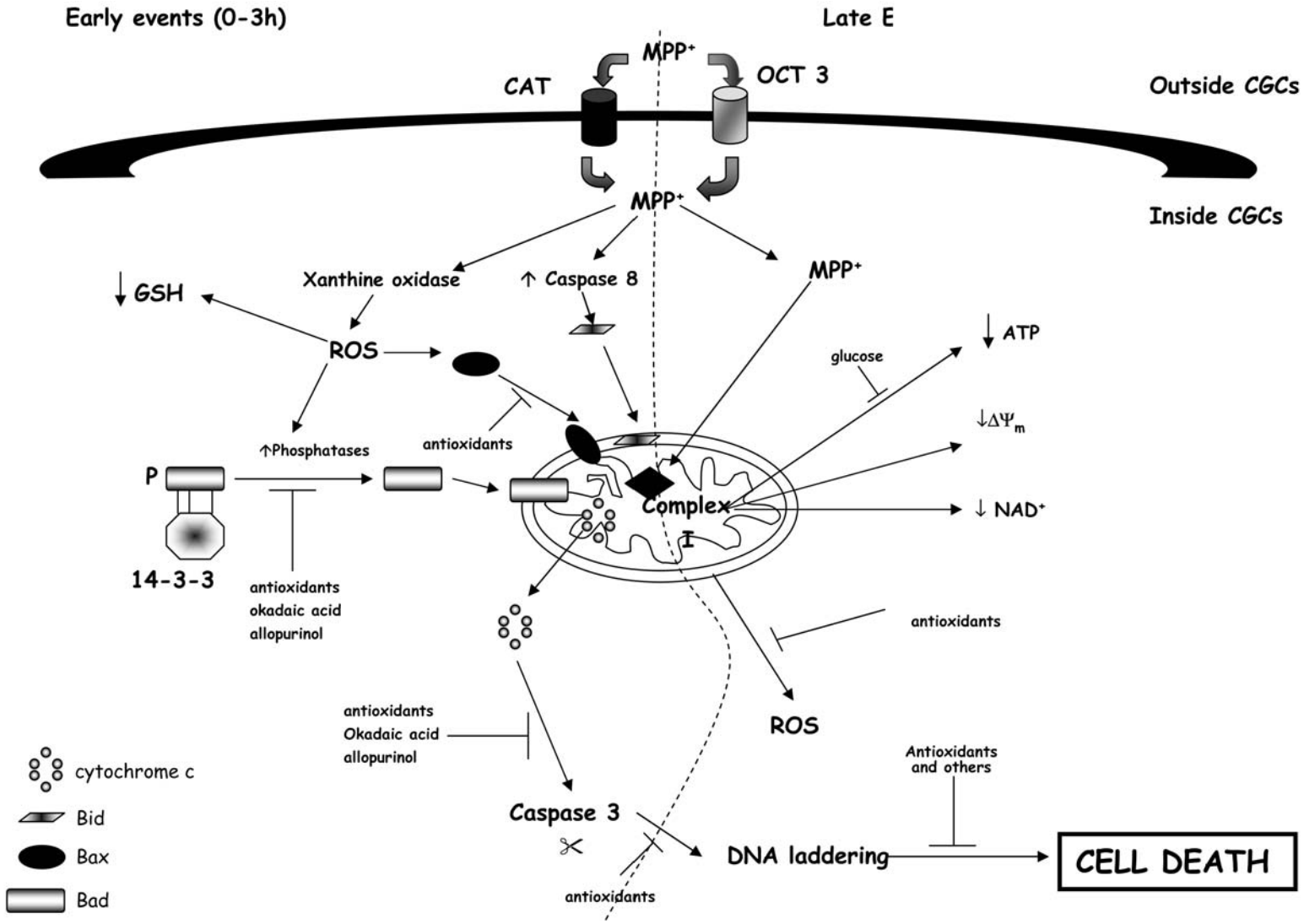


Fig. 3. Summary of the proposed mechanism for the early and late events implicated in MPP^+ toxicity in primary cultures of CGCs. MPP^+ production of ROS could be mediated by xanthine oxidase. The oxidative stress would contribute to Bad dephosphorylation (by protein phosphatase activation) and Bax translocation to the mitochondria. Additionally, caspase-8 is activated and Bid is transformed into the active form tBid. These three apoptotic BCl2-protein members would mediate cytochrome c release (without loss of $\Delta\Psi_m$ in early phase) and caspase-3 activation. In the late phase, mitochondrial complex I inhibition occurs with energetic machinery failure, a new ROS burst and alteration of the mitochondrial membrane potential. Finally, the cells die.

Acknowledgments

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