Mitochondrial mechanisms involved in nitric oxide (NO)-induced apoptosis in bovine chromaffin cells in primary culture.

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Nitric oxide (NO) is a signalling molecule that plays important roles in physiological processes including relaxation of smooth muscle, neurotransmission and host defence mechanisms against tumour cells and bacteria. Endogenous NO is synthesised from L-arginine by three isoforms of NO synthase (NOS), two of which are constitutively expressed, predominantly in neurones (nNOS) and endothelial tissue (eNOS), respectively. Generally, constitutive NOSs release small amounts of NO and are acutely regulated by calcium/calmodulin and phosphorylation. A third isoform (iNOS) is induced during inflammation and other oxidative stress events such hypoxia, producing large amounts of NO for up to long periods. NO exerts its physiological effects through the activation of guanylate cyclase and cGMP formation or through posttranslational modifications of proteins (*S*-nitrosylation and nitration). However, the induction of a high output system for NO in response to cytokines or a massive production of NO following accumulation of excitatory neurotransmitter glutamate can result in cell death. Neurones, pancreatic □-cells and macrophages seem to be particularly sensitive to NO toxicity. While in some systems, NO can react with some radicals and effectively cause cell death by necrosis, in others the progressive intra- or extra-cellular generation of NO causes apoptosis.

In bovine chromaffin cells, the presence of a constitutively expressed nNOS has been demonstrated by both biochemical and immunocytochemical methods[1-3]. In addition, the presence of NOS closely associated with ChAT-positive fibres innervating rat chromaffin cells has been reported[4,5]. In these cells, the L-arginine/NO/cGMP pathway has an important inhibitory role in both basal and ACh-stimulated catecholamine (CA) secretion[1,6,8]. However, the exposure of these cells to high concentrations of NO donors, peroxynitrite or cytokines for a long time cause their death by a mixed necrotic and apoptotic mechanism, depending on NO concentration and time of exposure[7].

The cell death phenomenon, besides being an important feature in the development of the nervous system, seems to be a cause for many neurodegenerative diseases such as Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and

brain ischemia, where a gradual loss of specific sets of neurones results in disorders of movement and CNS function[9]. Regarding this subject, the study of the effects of NO on chromaffin cells, which share a common embryologic origin with neurones, is very interesting in order to examine whether these cells are good models for the study of the molecular mechanisms of catecholaminergic neuronal death underlying some neurodegenerative diseases.

Our results indicate that treatment of adrenal chromaffin cells with either NO donors or cytokines, which induce NO formation by both nNOS and iNOS activation, leads to a high output of NO and a dose-dependent apoptotic death. This apoptotic death was prevented by NO scavengers like CPTIO or haemoglobin in the case of NO donors, and by NOS inhibitors like L-NMMA and L-thiocitrulline in the case of cytokines, thus indicating that the effects are due to NO production (Vicente *et al.*, 1999). The NO-induced apoptosis in chromaffin cells takes place with an increase in hypodiploid cell number, activation of caspase-3 enzyme and DNA fragmentation, accompanied by arresting of cell cycle in the G_0G_1 phase and a decreased number of chromaffin cells in the G_2M and S phases[10]. Furthermore, the treatment of these cells with peroxynitrite mediates both necrosis and apoptosis depending on the dose and the time of stimulation. Therefore, we wondered whether the intensity of the initial insult could be related to the pathway leading to chromaffin cell death[10].

The molecular mechanisms of apoptosis involve several pathways and activation of caspases, a family of cysteine proteases, represents a common event for several pro-apoptotic stimuli. Regarding on the characterisation of the events upstream from caspase activation, mitochondrial damage has been reported to trigger this process. Consistent with this hypothesis, anti-apoptotic proteins such as Bcl-2 are located in the mitochondria, suggesting a role for this organelle in the induction of apoptotic death. Moreover, the release of mitochondrial proapoptotic factors, such as cytochrome c, is blocked by Bcl-2.

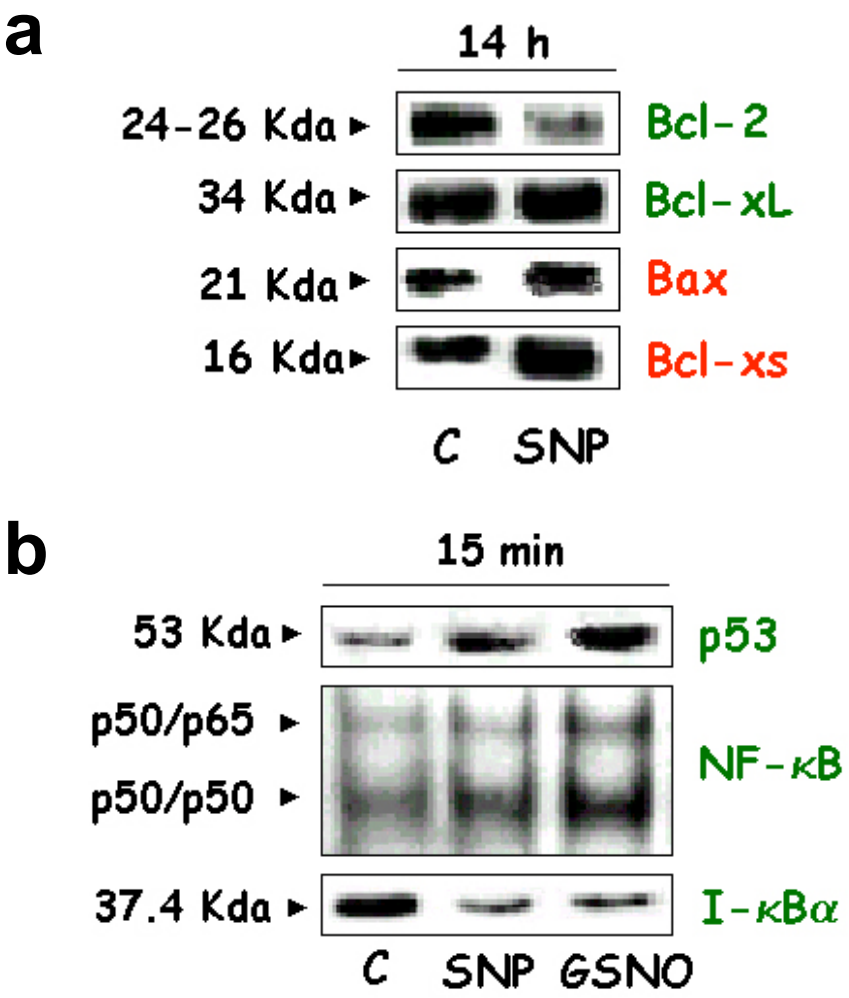

Figure 1: Effects of NO on expression of different protein involved in apoptosis. A) Western blots showing the effect of 1 mM SNP on expression of anti-apoptotic (Bcl-2 and Bcl-XL) and pro-apoptotic (Bax and Bcl-Xs) proteins of Bcl-2 family.at 14 h of incubation. B) Effect of 1 mM SNP on p53 expression and on NF-□B binding to iNOS promoter (EMSA) and I□B□ degradation at 15 min incubation..

The aim of this work was to assess the suspected involvement of mitochondrial mediators in the apoptotic death induced by NO in chromaffin cells, emphasizing the time course of these events. Us-

ing bovine chromaffin cells in primary culture and different NO donors (SNP, SNAP and GSNO) at apoptotic concentrations (100-1000 μM), we have demonstrated that NO induces a time-dependent decrease in trans-mitochondrial membrane potential ($\Delta\Psi_m$), measured by decrease in fluorescence of TMRM, being this effect detected after 4 hours of incubation with the NO donors. This effect preceded both NO donor-induced activation of caspase-3, which could be reversed by the inhibitor CPP32 at 50 nM, and appearance of hypodiploid cells, measured by flow cytometry. These events occurred after 8 hours of treatment and were maximal after 24 hours. The disruption of $\Delta\Psi_m$ is followed by cytochrome c release from the mitochondria to the cytosol, being this effect maximal after 14-16 h incubation with NO donors, and accompanied by a decrease in the mitochondrial content of the protein. Thus, both events occurred upstream from the caspase 3 activation and subsequent apoptosis in chromaffin cells.

The involvement of the Bcl-2 protein family in the NO-induced apoptosis was demonstrated by evaluating the effect of NO donors on the expression of different antiapoptotic (Bcl-2 and Bcl-x_L) and proapoptotic (Bax and Bcl-x_s) members of this family. Results (Figure 1a) show that NO donors mediate the inhibition of Bcl-2 expression, which was minimal after 14-16 hours incubation (25% of control value at 1 mM SNP). This effect was preceded by a time-dependent increase in the expression of this protein, thus indicating the activation of a survival pathway as an attempt to protect chromaffin cells against NO-induced apoptosis. The most significant effect was the dose-dependent induction of the proapoptotic protein Bcl-x_s expression, which was maximal between 14-16 hours of incubation with the NO donors (2,5 fold higher than the control value, at 1 mM SNP). On the other hand, the changes in the expression of the proapoptotic Bax and antiapoptotic Bcl-x_L were smaller (20-30% over the control). Main changes in the expression of Bax and Bcl-x_s preceded the decrease in Bcl-2, which indicates that both effects may be regulated by different cell signalling mechanisms.

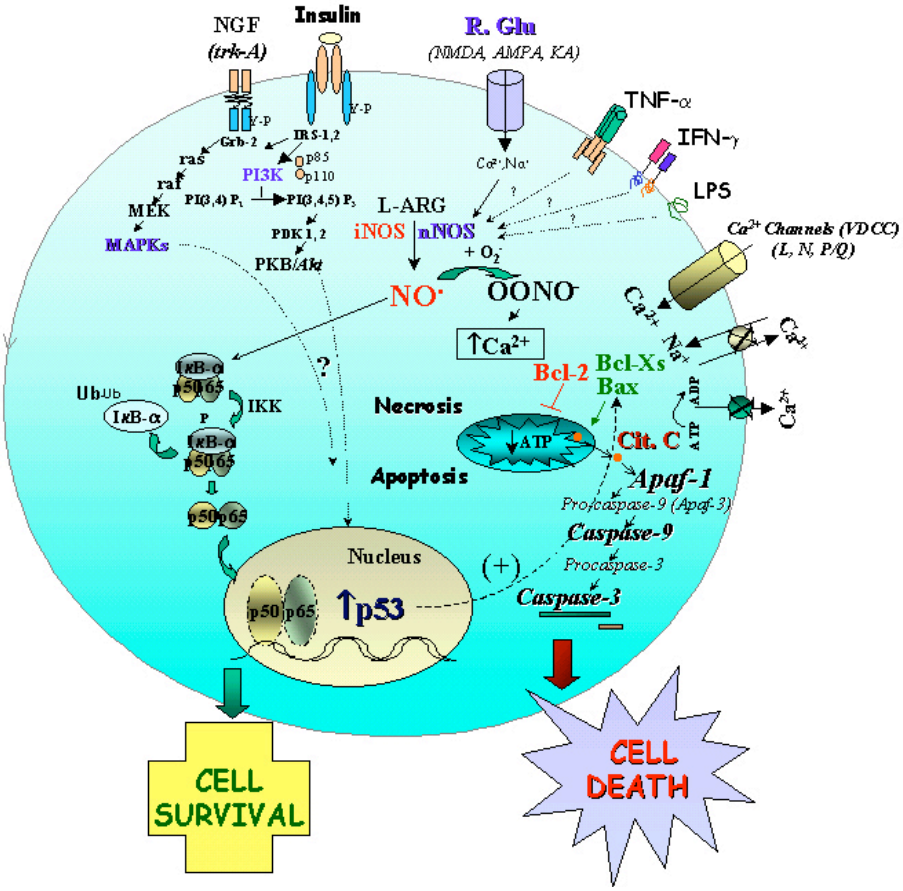

Figure 2: Summary of the proposed mechanism for the apoptotic events induced by NO in primary cultures of bovine chromaffin cells. NO, which could be generated in the cells by induction of nNOS and/or iNOS by cytokines or activation of glutamate receptors (Vicente 2000), mediates a decrease in $\Delta\Psi_m$, which is followed by a decrease in the Bcl-2 expression, an increase in cytochrome C (Apaf 2) release into the cytosol, the activation of caspase 9 and 3 and apoptotic cell death. It is likely that the induction of p53 expression is one of the earliest events after the initial insult, which could be responsible for the regulation of the expression of Bcl-2 family proteins. The expression of p53 could be, in turn, regulated by NF-κB. The growth factors NGF and IGF-1 are able to block these events, thus preventing both caspase activation and cell death.

One of the early events associated with NO-dependent apoptosis is a rapid rise and accumulation of the tumour suppressor protein p53, which reflects the cell stress elicited by NO. In chromaffin cells, an induction of the expression of p53 occurred between 15-30 minutes of incubation with different NO donors, and the effect was time-dependent and maximal between 1-24 hours, depending on the NO donor (Figure 1B). The NO-dependent increase of p53 has been consistently observed and it has been suggested that this accumulation leads to the expression of several proteins, which ultimately participates in apoptosis, like Bax. Following p53 upregulation, Bax levels could increase and heterodimerizate with other members of the Bcl-2 family, thus triggering the apoptosis. Indeed, as in chromaffin cells, in other cell types it has been observed that the overexpression of Bcl-2 inhibits p53-dependent apoptosis, which suggests a role for p53 as an initial step in the NO-dependent apoptotic process.

Finally, the activation of NF-κB to the nucleus, measured by the binding of NF-κB to nuclear proteins and I-Bα degradation, occurred after 15-30 minutes of incubation with NO donors and cytokines (Figure 1b), and seems to be another early mechanism involved in NO-induced apoptosis. However, NF-κB is a survival factor in these cells, since 10 μM SN50 (an inhibitor of NF-κB translocation) enhanced the NO-induced apoptosis in chromaffin cells. Therefore, in spite of its activation, NF-κB is not able to rescue cells from NO-induced apoptosis.

These results taken together strongly support the role of mitochondrial mediators in NO-induced apoptosis in chromaffin cells and point out these cells as good models for investigating the molecular mechanisms involved in neurodegenerative diseases with catecholaminergic neuronal death and the mechanisms of neuroprotection against apoptotic death underlying these important diseases.

Figure 2 summarises the molecular mechanism proposed for the involvement of mitochondrial mediators in NO-induced apoptosis in chromaffin cells.

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