

Regulation of the apoptosis–necrosis switch

Pierluigi Nicotera^{*1} and Gerry Melino^{1,2}

¹Medical Research Council, Toxicology Unit, Leicester, UK; ²Fondazione S. Lucia, 00179 Roma; and Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, Italy

Execution of the apoptotic program involves a relatively limited number of pathways. According to a general view, these would converge to activate the caspase family of proteases. However, there is increasing evidence that apoptotic-like features can also be found when caspases are inhibited. Moreover, under pathological conditions, apoptosis and nonapoptotic death paradigms are often intertwined, which suggest that, *in vivo*, cells may use diverging execution pathways. Molecular switches between apoptosis and necrosis include adenosine triphosphate-dependent steps in the activation of caspases or steps sensitive to reactive oxygen/nitrogen species. In turn, caspase activation can cause necrosis by promoting ion overload.

Oncogene (2004) 23, 2757–2765. doi:10.1038/sj.onc.1207559

Keywords: apoptosis; necrosis; ATP; NO; nitric oxide; neurons

Introduction

Various genetically encoded programs involved in signaling or in the initiation of cell death may decide the fate of individual cells or organs during development. The term 'programmed cell death' has therefore been used primarily to describe the coordinated series of events leading to controlled cell demise in developing organisms (Schwartz and Osborne, 1993; Schwartz *et al.*, 1993). Owing to this early definition, the term 'physiological cell death' has been linked to the notion of 'programmed cell death.' Nevertheless, cells execute a biochemical program of cell death also under pathological conditions, and the consequences for the individual cell may be practically indistinguishable from those observed during developmental demise. In many circumstances, developmental cell death as well as death under pathological conditions have the morphological characteristics described by Lockshin and Williams (1965) and by Kerr *et al.* (1972). The concept of a death program is, however, not necessarily linked to a morphological appearance. For example, in non-vertebrate systems, programmed cell death does

not always display an apoptotic-like morphology (Schwartz *et al.*, 1993). This is the reason why the early pioneering work in *Caenorhabditis elegans* did not describe developmental cell death in *C. elegans* as apoptotic (Yuan and Horvitz, 1990). Nevertheless, the discovery that both developmental and nondevelopmental death can share similar execution systems and controlling proteins blurred the boundary between developmental cell death and cell demise in adult tissues. In particular, the characterization of the main executioners of apoptosis, the caspases, has linked the physiological type of death encountered in development with that observed in pathological situations throughout the whole life span. This has focused attention on the molecular mechanisms of apoptosis with less attention to distinct cell death paradigms. For example, a second type of regulated, active cell death is autophagic cell death, which is characterized by the appearance of cytoplasmic autophagic vacuoles originating from lysosomes, followed by mitochondrial dilatation and enlargement of the ER and Golgi (Schweichel and Merker, 1973). Autophagic death is frequent in neuronal development and in neurodegenerative diseases (Petersen *et al.*, 2001; Yuan *et al.*, 2003). In 1990, Clarke (1990) also described a third model of cell death with vacuolar degeneration, swelling of intracellular organelles, without lysosomal activation. This model of cell death, also found in the developmental neuronal system, is similar to necrosis. Finally, although neglected by most, cell lysis/necrosis can also be regulated and may involve in some cases caspase activation (Schwab *et al.*, 2002).

All these types of cell death are present and strictly regulated *in vivo*. This is evident, for example, in *C. elegans* (Xu *et al.*, 2001; Suzuki *et al.*, 2003). Here, specific aspartyl and calpain proteases are required for neurodegeneration (Syntichaki *et al.*, 2002). This poses several relevant questions: (i) What are the factors that determine the choice between different types of death? (ii) Do different programs only represent death subroutines, which target preferentially subsets of cells or event intracellular targets? (iii) When do they intervene together to eliminate damaged cells *in vivo*?

Here, we shall outline some molecular mechanisms that are known to switch cells' fate from apoptosis to necrosis or *vice versa*, and discuss their relevance in pathological settings.

*Correspondence: P Nicotera, Medical Research Council Toxicology Unit, Hodgkin Building, Lancaster Road, Leicester University, PO Box 138, Leicester LE1 9HN, UK; E-mail: pn10@le.ac.uk

Caspase-dependent cell death

The finding that one class of death-related genes, those expressing caspases (cysteine aspartases) (Nicholson, 1999), are involved in cell death in genetically distant organisms has directed attention on the central role of this protease family as the main effector of apoptotic cell demise. Consequently, there is a tendency to identify apoptosis conceptually with its main execution system. Caspases are constitutively expressed in mammals, similar to *ced-3* in *C. elegans* (Shaham and Horvitz, 1996). To date, at least 13 human caspases have been identified. They are expressed as inactive proenzymes and proteolytically activated to form active tetramers. Substrates include cytoskeletal proteins, nuclear structural proteins and enzymes, and some controllers of caspase activation (i.e. Bcl-2) (Nicholson, 1999). However, some caspases may eventually have death-unrelated, and perhaps vital functions and their activation may not always underlie cell death. Caspase multiplicity likely reflects their diverse roles under pathophysiological conditions. For example, caspases degrade amyloid precursor protein, presenilins (PS1, PS2), tau, huntingtin, atrophin-1, ataxin-3 and the androgen receptor (Ellerby *et al.*, 1997; Gervais *et al.*, 1999). However, cleavage of relevant substrates does not necessarily imply that caspase activation is the major mechanism leading to cell death in pathological settings. It is indeed unlikely that a single execution system, even as diversified as the one involving caspases, is alone responsible for death execution. Should this be the case, viruses and transformed cells could have easily escaped or shut down a program converging on a single execution pathway. These considerations may help to understand why different sets of caspases are recruited in different paradigms of mammalian cell death, and also why deletion of single caspases has only localized and partial effects on cell death (Kuida *et al.*, 1996, 1998). Moreover, several forms of demise seem to be caspase independent (Sarin *et al.*, 1997) or even accelerated by caspase inhibitors (Vercammen *et al.*, 1998a,b). Indeed, other protease families have been implicated in apoptosis (Adjei *et al.*, 1996; Grimm *et al.*, 1996), while caspases can be activated without causing cell death (Jaattela *et al.*, 1998). In some cases, caspase inhibition does not alter the extent of death, but rather the shape of demise (Hirsch *et al.*, 1997; Leist *et al.*, 1997).

The degree of caspase activation may also be of importance. Caspase activation varies quantitatively from system to system, and it is unclear whether a threshold has to be reached to turn caspase activation into a lethal reaction. For example, a moderate degree of caspase activation does not always cause cell demise (Jaattela *et al.*, 1998). Finally, an apoptotic-like death can also occur in a caspase-independent manner (see, for review, Borner and Monney, 1999). Part of the problem may be due to the fact that caspase inhibitors can prevent the appearance of certain, but not all, features of apoptosis in certain model systems (McCarthy *et al.*, 1997). In this context, caution should be exercised,

because of possible nonspecific effects of broad-range caspase inhibitors. Although generally considered specific for the caspase family, these inhibitory peptides may in fact block only some, but not all, caspases. It is also clear that caspase inhibitors can also inhibit unrelated proteases, whose activity may be necessary for survival. While the existence of alternative pathways may be conceivable, to date, the evidence for effective alternative execution systems is limited. The crosstalk between caspases and other proteases is also worth considering in this context. In particular, calpains, and also other proteases can synergize or in contrast hinder caspases in the execution of cell death. Notably, recent work suggests that caspases can also trigger secondary necrosis when they cleave and inactivate plasma membrane Ca^{2+} transport systems. This causes secondary Ca^{2+} overload and plasma membrane lysis (Schwab *et al.*, 2002).

Energy requirement for the shape of cell death

While the occurrence of a caspase-independent apoptosis is strongly debated, it is instead widely accepted that, in some cases, caspase inhibitors only delay cell demise. Cells die eventually with morphologically different characteristics (Hirsch *et al.*, 1997; Leist *et al.*, 1997). Evidence that cells triggered to undergo apoptosis are instead forced to die by necrosis when energy levels are rapidly compromised has been recently provided (Leist *et al.*, 1997), see Figure 1.

In vivo, under pathological conditions, apoptosis and necrosis may often coexist (Leist *et al.*, 1995). Moreover,

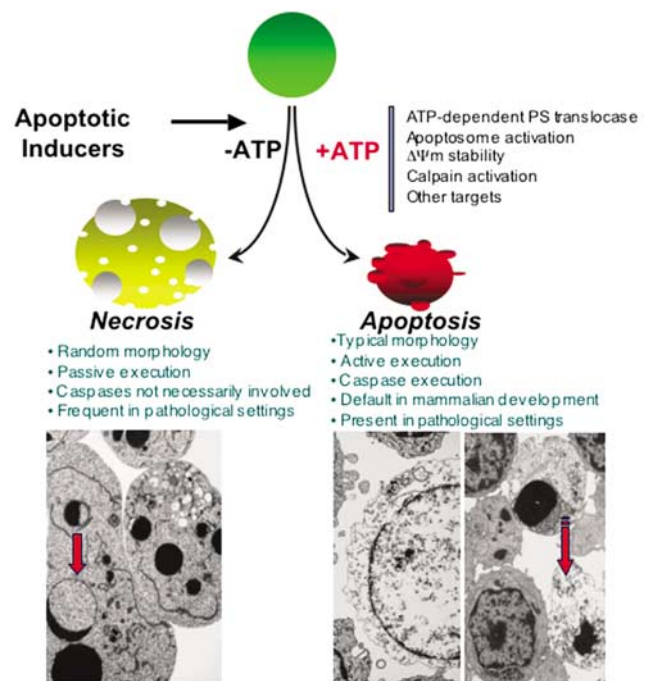


Figure 1 Apoptosis–necrosis switch by ATP. Switch of the morphology of cell death, shown by electron microscopy, and its regulation by the intracellular ATP concentration levels

intracellular energy levels are rapidly dissipated in necrosis, but not in apoptosis (Ankarcrona *et al.*, 1994).

By manipulating the intracellular adenosine triphosphate (ATP) level, it has been possible to determine that when intracellular ATP is low, typical apoptotic stimuli cause necrosis (Leist *et al.*, 1997). ATP can be either depleted or repleted to defined levels and for defined periods of time. This allows identification of a defined period of time after the exposure of cells to apoptogenic stimuli during which energy-dependent steps are required to complete the apoptotic program. If ATP concentrations are markedly reduced during this period, activation of downstream caspases and all typical apoptotic features are blocked. Stimulated cells die nonetheless. However, death has a necrotic appearance. These findings provide direct evidence that the complete apoptotic program involves energy-requiring steps, one of which is at the level of the formation of the protein complex between Apaf-1, cytochrome *c* (cyt *c*) and procaspases (Liu and Stämmer, 1999), outlined in Figure 2. Lack of ATP at this step would prevent the resulting downstream degradative processes including caspase-3 activation.

Nitric oxide (NO) as an inhibitor or activator of apoptosis and molecular switch between apoptosis and necrosis

NO is a second messenger molecule with the unique property, being a gas, of freely moving from one cell to the other (Moncada *et al.*, 1991; Bredt and Snyder, 1994). In fact, NO is an extremely versatile messenger in biological systems, and has been implicated in a number of different physiopathological roles, such as smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, cell differentiation and tissue morphogenesis (Stämmer and Piantadosi, 1996; Stämmer *et al.*, 1997; Brune *et al.*, 1999; Nicotera *et al.*, 1999a; Gu *et al.*, 2002) and cytotoxicity (Brune *et al.*, 1999). Cell

death elicited by endogenously derived NO plays a central role in pathologic phenomena such as septic shock, nonspecific ‘host’ defense against tumors and intracellular pathogens, acute and chronic neurodegenerative diseases, pancreatic β -cell destruction in diabetes mellitus and transplant rejection (Choi, 1988; Moncada *et al.*, 1991; Bredt and Snyder, 1994; Kaneto *et al.*, 1995; Bonfoco *et al.*, 1996; Nicotera *et al.*, 1997, 1999b; Leist and Nicotera, 1998). Figure 3 shows a schematic pattern of the action of NO, from its synthesis, chemical interactions, intracellular biochemical effectors, leading to its biological actions.

NO is a hydrophobic molecule and a highly diffusible free radical, generated from the oxidation of L-arginine to L-citrulline by a family of constitutive or cytokine-inducible nicotinamide adenine dinucleotide phosphate-dependent isoenzymes, the NO synthases (NOS) (part 2 in Figure 3). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are usually constitutively expressed in selective tissues, and are transiently activated by an increase in intracellular concentration of free Ca^{2+} . On the contrary, the cytokine-inducible NOS (iNOS) is transcriptionally regulated, resulting in NO production at basal Ca^{2+} levels for up to several days (Nicotera *et al.*, 1999a). Following its production, NO is preserved in its molecular structure by a chemically heterogeneous group of spontaneously decomposing NO donors (part 3 in Figure 3). This deposit stabilization finely tunes the biological activity of NO after its liberation.

NO is highly reactive (see part 3 in Figure 3), and its half-life is extremely short in biological systems, being estimated below microseconds. The chemistry of NO involves inter-related redox forms ($\text{NO}^\bullet$, NO^+ , NO^-), with different chemical reactivity toward distinct target groups, thus explaining the pleiotropic effects of NO in biological systems (see Figure 3) (Stämmer *et al.*, 1992a, b). $\text{NO}^\bullet$ reacts with molecular oxygen, superoxide anion and transition metal cations, leading to the formation of reactive nitrogen intermediates that

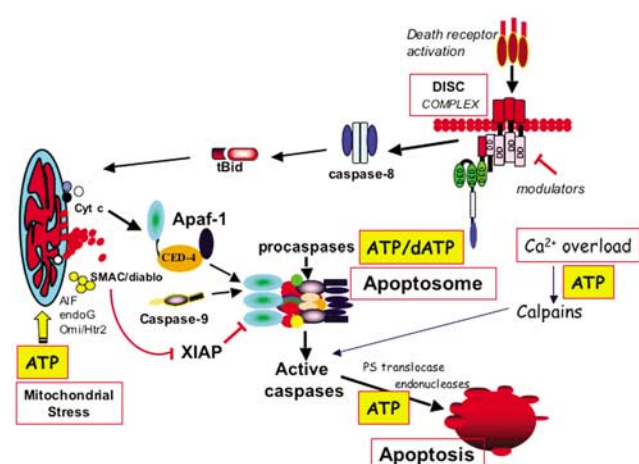


Figure 2 ATP-dependent steps in apoptosis. Potential regulation of the cell death machinery by ATP

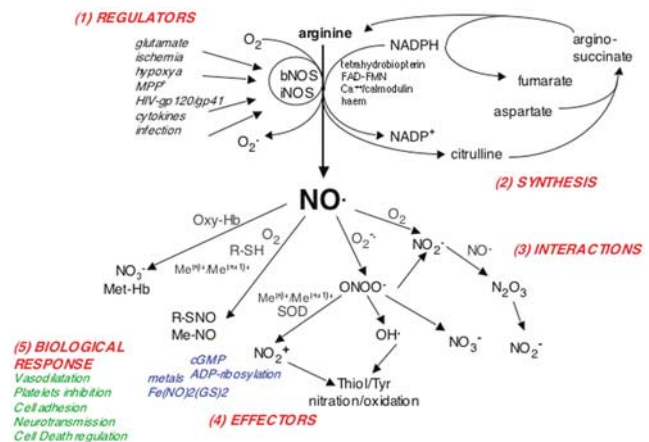


Figure 3 Generation, storage, reactivity and activity of nitric oxide. Regulation (1), synthesis (2), molecular interactions (3), activity on effectors (4) and the biological effects (5) of NO are indicated in a simplified scheme. See text for details

directly or indirectly support additional nitrosative reactions (Figure 3). In particular, $\text{NO}^\bullet$ binds to hemoproteins and iron–sulfur centers. The nitrosonium cation (NO^+) character found in nitroso compounds in biological systems is involved in nitrosation reactions with nucleophilic groups such as thiols, amines, carboxyls, hydroxyls, and aromatic rings (Figure 3). The nitroxyl anion (NO^-) rapidly undergoes dimerization and dehydration, thus generating dinitrogen oxide, in addition to reacting with thiols leading to sulfhydryl oxidation and with metals (Figure 3).

NO -mediated intracellular signaling pathways can be mediated by cyclic guanylic acid (cGMP) (Figure 3); cGMP formation results from NO binding to the heme group of soluble guanylyl cyclase, thus leading to enzyme activation. Alternatively, cGMP-independent mechanisms include inhibition of iron–sulfur centers, DNA damage, poly(adenosine 5'-diphosphate-ribosylation) activation with cellular energy deprivation and protein modification due to S-nitrosylation of thiol groups, thiol oxidation to sulfenic and sulfinic acid and tyrosine nitration (Stamler, 1994; Becker *et al.*, 1998; Stamler and Hausladen, 1998; Foster *et al.*, 2003). cGMP-independent NO -induced responses account for the antimicrobial, cytostatic and cytotoxic ability of NO . S-nitrosylation of cysteine residues is a common protein modification achieved by NO in the presence of oxygen and is usually associated with the loss of protein function (Hess *et al.*, 2001; Matsumoto *et al.*, 2003).

S-nitrosylation may regulate cellular signal transduction by influencing signaling proteins such as p21ras (Lander *et al.*, 1995) and c-Jun N-terminal kinase 2 (So *et al.*, 1998). It affects the cellular gene transcription machinery by inhibiting the DNA-binding activity of nuclear factor κB (NF- κB) (Matthews *et al.*, 1996; Marshall and Stamler, 2001) and activator protein-1 (AP-1) (Tabuchi *et al.*, 1994) or by activation of OxyR (Hausladen *et al.*, 1996). NO nitrosylates thiol groups of cytosolic enzymes such as transglutaminase (Melino *et al.*, 1997; Bernassola *et al.*, 1999; Rossi *et al.*, 2000), AP-1 (Bernassola *et al.*, 2001; Melino *et al.*, 2000a), caspases (Dimmeler *et al.*, 1997; Melino *et al.*, 1997), glyceraldehyde-3-phosphate dehydrogenase (Mohr *et al.*, 1996) hemoglobin (Jia *et al.*, 1996; Gow and Stamler, 1998) and skeletal muscle (Stamler and Meissner, 2001; Eu *et al.*, 2003), and it can modulate the biological activity of extracellular molecules such as coagulation factor XIII (Catani *et al.*, 1998).

In addition to regulating directly the 'apoptotic' machinery, see Figure 4, NO finely regulates the NMDA receptors, and therefore excitotoxicity in neuronal cells (Lipton *et al.*, 1993), including neuroblastoma cells exposed to the gp120 HIV protein (Corasaniti *et al.*, 1992, 1994, 1995; Bagetta *et al.*, 1993; Clementi *et al.*, 1996; Maccarrone *et al.*, 2000), or even tamoxifen (Maccarrone *et al.*, 1998) or fenretinide (Lovat *et al.*, 2003). Via S-nitrosylation or nitration of proteins, NO can be a bifunctional modulator of cell death capable of either triggering or inhibiting cell demise. In addition, NO can regulate the apoptosis–necrosis switch.

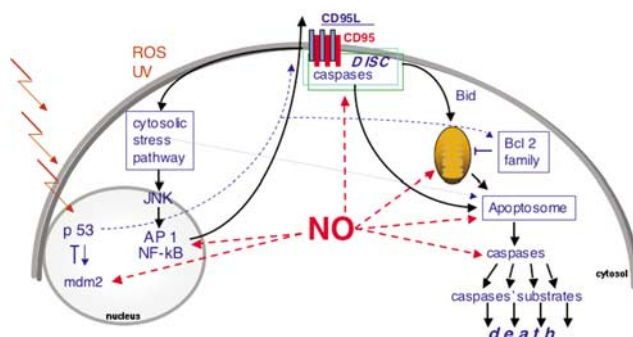


Figure 4 Interaction of NO with the apoptotic molecular machinery. NO has been shown to interact and regulate different steps in the apoptotic pathway: death receptor signaling, mitochondrial sensitivity, transcription factors and caspase activation. This regulation is able to alter the sensitivity of the cell to die

Mechanisms of NO inhibition of cell death

S-nitrosylation

NO can inhibit apoptosis. Figure 4 shows the ability of NO donors to inhibit receptor-mediated cell death. This was first demonstrated in neuronal cells during excitotoxicity (Lipton *et al.*, 1993). NO can also inhibit cell death in endothelial cells (Dimmeler *et al.*, 1997) and in lymphoid cells (Mannick *et al.*, 1994; Melino *et al.*, 1997). S-nitrosylation of several critical factors may be important for cell survival. S-nitrosylation of the *N*-methyl-D-aspartate receptor reduces neuronal demise due to excitotoxicity (Lipton *et al.*, 1993; Kim *et al.*, 1999; Lipton, 1999; Bossy-Wetzel and Lipton, 2003; Manabe and Lipton, 2003). The apoptosis execution pathway can also be interrupted by NO at different steps. Figure 4 shows several protein targets of the apoptotic machinery, which can be inhibited by NO through direct S-nitrosylation of catalytic cysteine residues. NO may interfere with the downstream apoptotic machinery acting either at the level of the executioner caspases or upstream of these events to inhibit initiator caspases such as caspase-8 and transcriptional factors such as AP-1 and NF- κB . The action of NO could therefore differ in distinct cells, depending on the specific pathway activated.

For example, exogenous NO can inhibit CD95 signaling in Jurkat T or endothelial cells via S-nitrosylation of caspase-3-like proteases (Melino *et al.*, 1997). Other studies have shown NO -mediated inactivation of caspases in apoptotic models such as CD95-ligated human leukocytes (Mannick *et al.*, 1997; Mannick *et al.*, 1999) and tumor necrosis factor α (TNF α)-, proatherosclerotic factor-, and reactive oxygen species (ROS)-stimulated rat hepatocytes and endothelial cell lines (Kim *et al.*, 1997a; Dimmeler and Zeiher, 1999; Li and Billiar, 1999). The effects of NO can directly inhibit caspase activity by S-nitrosylation of the active-site cysteine residue (Li *et al.*, 1997; Melino *et al.*, 1997). NO is also able to suppress a mechanism for the initiation or amplification of apoptotic signaling. Kim *et al.* (1998) have reported that both Bcl-2 cleavage and cyt *c* release are inhibited in TNF α - and actinomycin-

cin D-treated MCF-7 adenocarcinoma cells exposed to NO donors. Finally, NO can deplete mitochondrial ATP generation and thereby switch apoptosis into necrosis (see below). Interestingly, caspase zymogens are endogenously S-nitrosylated in nonstimulated human lymphocyte cell lines, and CD95 ligation can activate caspase-3 also by stimulating the denitrosylation of its catalytic site cysteine (Liu and Stamler, 1999; Mannick *et al.*, 1999).

NO as a scavenger of radical species

NO can be protective against cellular damage resulting from cerebral and myocardial ischemia:reperfusion. NO exerts this protective action through the reaction with alkoxyl and peroxy radicals, thus inhibiting lipid peroxidation (Rubbo *et al.*, 1994); it also suppresses the superoxide:hydrogen peroxide-mediated cytotoxic effect by acting as a scavenger of ROS (Wink *et al.*, 1993; Brune *et al.*, 1997; Enikolopov *et al.*, 1999).

Induction of protective proteins

Exogenous NO or preinduction of iNOS has been shown to stimulate the expression of the inducible heat-shock protein 70 (Hsp70) and subsequently prevent hepatocytes from TNF α - and actinomycin D-induced apoptosis (Kim *et al.*, 1997a). Even though it has been recently suggested that Hsp70 acts downstream of caspase activation (Jaattela *et al.*, 1998), the mechanisms through which it suppresses apoptosis remain largely unknown. NO can also inhibit cell death by inducing the expression of other protective proteins such as cyclooxygenase-2 (Brune *et al.*, 1999), hemoxygenase-1 (Kim *et al.*, 1995) and metallothionein (Schwarz *et al.*, 1995). Recent studies have demonstrated that protection from ultraviolet-A-induced apoptosis is strongly correlated with NO-elicited upregulation of Bcl-2 expression (Suschek *et al.*, 1999).

cGMP-dependent mechanisms

In hepatocytes, NO prevents increases in caspase activity by a cGMP-dependent mechanism that does not involve S-nitrosylation (Kim *et al.*, 1997b; Li and Billiar, 1999). Furthermore, eNOS activation, which exerts a protective effect on the susceptibility of activated T-lymphocytes to CD95-triggered apoptosis, acts via cGMP-dependent pathways (Sciorati *et al.*, 1997). The effect of cGMP on apoptosis seems to be mediated through cGMP-dependent kinase (Kim *et al.*, 1997b).

Inhibition of ANT

NO can directly regulate the activity of the ANT mitochondrial protein, crucial for the activation of mitochondrial depolarization in certain apoptosis models (Vieira *et al.*, 2001). Therefore, NO can directly regulate the threshold of release of mitochondrial proteins such as cyt *c*, AIF, Omi/HtrA2 and Diablo/Smac, thus finely tuning apoptosis.

Induction of cell death by NO

While the reactions described above can inhibit the execution of cell death, NO can also cause cell death. NO is implicated in both apoptotic and necrotic cell death depending on the NO chemistry and the cellular biological redox state. Also, exposure to different levels of NO can produce different outcomes: high NO levels in neurons trigger necrosis, while longer exposure to lower NO concentrations can trigger apoptosis (Bonfoco *et al.*, 1995), see Figure 4a. The reactivity of NO, Figure 1, is the key to understanding when and how NO causes death or survival, Figure 4b, but its intrinsic complexity makes it difficult to pinpoint a specific chemical species (NO $^{\bullet}$, NO $^+$, NO $^-$), with a direct biochemical reaction (S-nitrosylation, ROS reactions, Fe–heme binding, mitochondrial $\Delta\Phi$ and ATP regulation) and the biological effect (survival, apoptosis, necrosis).

NO-induced apoptosis

NO induces apoptosis in a variety of cell types, including macrophages (Brune *et al.*, 1999), neurons (Bonfoco *et al.*, 1995), pancreatic β cells (Ankarcrona *et al.*, 1994; Kaneto *et al.*, 1995), thymocytes (Fehsel *et al.*, 1995), chondrocytes (Blanco *et al.*, 1995) and hepatocytes (Leist *et al.*, 1995). The signaling pathways that lead to apoptosis still remain poorly understood. NO-induced apoptosis is often accompanied by accumulation of the tumor suppressor gene p53, changes in the expression of pro- and antiapoptotic Bcl-2 family members, caspase-3-like protease activation and cyt *c* translocation (Brune *et al.*, 1999). This is mediated by a chemical interaction with mdm2, which fails to degrade and inactivate p53 (Wang *et al.*, 2002, 2003; Schneiderhan *et al.*, 2003). In the excitotoxic death of cultured neurons, NO-triggered apoptosis requires a Ca $^{2+}$ signal triggered by the activation of the NMDAR channels (Bonfoco *et al.*, 1996). Depletion of ATP in PC12 cells, caused via the NO-mediated inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, is one of the mechanisms responsible for NO neurotoxicity (Yasuda *et al.*, 1998). However, the NO-mediated modification glyceraldehyde-3-phosphate dehydrogenase modification cannot necessarily be linked to energy depletion, at least in monocytic macrophage cell lines (Messmer and Brune, 1996).

Mechanisms of NO-mediated necrosis

NO induces necrosis in several cell systems via multiple target interactions, with thiol groups, heme, iron–sulfur and tyrosine residues containing proteins and DNA, and have been implicated in NO-mediated necrosis (Figure 5a and b). Some of these effects may be direct, whereas others may arise from the reaction of NO with superoxide anion and, at high concentrations, with oxygen to form peroxynitrite (ONOO $^-$) and nitrosative agents, respectively. As stated above, NO high concentrations or short time inhibit essential cellular processes, such as DNA synthesis, mitochondrial respiration and metabolic

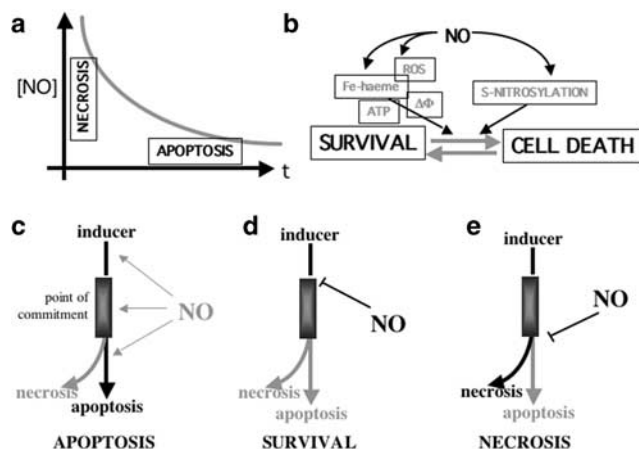


Figure 5 Apoptosis-necrosis switch by NO. The intensity of the stimulus and the time of exposure affect the type of cell death induced (a), via interaction with several molecular targets (b and also Figure 4), which can also result in the complete inhibition of death (b). Depending on the site and on the type of interaction, the action of NO can indeed result in the induction of apoptosis (c), in the inhibition of cell death (d), as well as in the induction of necrosis (e), when the insult is not compatible with life and the cell death machinery is inhibited. The intracellular redox status and the redox defenses of the cell finely regulate the biological effect of NO

reactions (Figure 4 and 5). Thus, NO reacts with ribonucleotide reductase, targeting both thiol groups and the tyrosyl-free radical required for catalysis, inhibiting deoxyribonucleotide synthesis (Roy *et al.*, 1995). NO can disrupt mitochondrial function by reversibly inactivating cytochrome *c* oxidase, thus stimulating superoxide anion production by the respiratory chain (Cleeter *et al.*, 1994). The resulting superoxide anion, through the formation of ONOO⁻, irreversibly inhibits complexes I and III of the mitochondrial electron transport chain (Bolanos *et al.*, 1995). It also inactivates crucial metabolic enzymes such as aconitase, by forming iron-nitrosyl complexes with its Fe-S centers. Even though inhibition of aconitase does not necessarily induce ATP depletion depending on the cell model, it may lead to a reduction in glucose and amino-acid metabolism and subsequent energy loss (Welsh *et al.*, 1991).

NO therefore seems to kill via ONOO⁻-mediated pathways. ONOO⁻ causes lipid peroxidation (Radi *et al.*, 1991), chemical cleavage of DNA (Salgo *et al.*, 1995) and nitration of both free and protein-bound tyrosine with subsequent alterations of protein phosphorylation or perturbation of protein tertiary structure. ONOO⁻ seems to contribute to cell death through the activation of the nuclear enzyme poly(adenosine 5'-diphosphoribose) synthetase, which in turn results in ATP depletion (Zhang *et al.*, 1994). It is not clear as to whether this is the primary mechanism of NO-induced necrosis.

NO shifts the apoptotic response elicited by different stimuli into necrotic cell death

The combined exposure of Jurkat T cells to an agonist anti-CD95 antibody and increasing concentrations of

SNAP prevent apoptosis (Melino *et al.*, 1997). Inhibition of apoptosis and the shift to necrosis in Jurkat (Leist *et al.*, 1999) and neuronal cells (Volbracht *et al.*, 2001) can occur by NO-mediated ATP depletion or by directly preventing caspases activation (Melino *et al.*, 1997). It is evident from these results that NO can control the switch from apoptosis into necrosis.

In neuronal apoptosis, NO can have unique features. While it can trigger glutamate release and excitotoxic cell death, NO can also prevent caspases activation and switch cell death from apoptosis into necrosis. This may be relevant in neurodegeneration when a persistent proinflammatory condition may cause sustained generation of NO. Neurons exposed to a microtubule-disassembling agent, colchicine die by apoptosis (Volbracht *et al.*, 1999). However, if ATP is depleted by NO, cell death occurs by necrosis. ATP depletion prevents in this system both the activation of caspases and the exposure of phagocytosis-recognition molecules.

Conclusions

It is perhaps not surprising that initially simple death programs, developed early during phylogeny, undergo complex modifications in mammalian cells. Large gene families have evolved to provide a more intricate control of cell death in higher organisms, in part, perhaps to accommodate the need of individual organ differentiation. A consequence of the increased complexity may be that an increasing number of feedback loops gives rise to multiple possibilities of initiation, control and execution. The main implication of this standpoint is the exclusion of a single, predominant commitment step. It seems likely that accumulation of damage incompatible with cell survival would require disruption of several vital cell functions. Once such a threshold is trespassed, multiple positive feedback loops would ensure the progression of

	SIGNALING			TOXICITY	
	SNO	SSR	SOH	SO ₂ H	SO ₃ H
	Reversible			Irreversible	
<u>1. NITROSANT</u>					
NO	+	-	-	-	-
R-SNO	-	+	-	-	-
N ₂ O ₃	-	-	(-)	-	-
DNIC	-	-	-	+	-
<u>2. NITROSANT / OXIDANT</u>					
ONO ⁻	-	-	-	+	+
<u>3. OXIDANT</u>					
O ₂	-	-	-	-	+
O ₂ ⁻	-	-	(-)	+	-
H ₂ O ₂	-	-	-	+	-
OH ⁻	-	-	-	+	-

Figure 6 Signaling versus toxic effects of NO. NO can react with different chemical groups resulting in reversible or irreversible reactions. These can be simplified as a progressive scale of modification. Stamler (Stamler, 1994; Stamler and Hausladen, 1998; Stamler *et al.*, 2001, 1992a, b, 1997; Stamler and Piantadosi, 1996;) proposed that while reversible reactions are related to signaling, therefore to the life of the cell, irreversible reactions result in toxic effects. These chemical mechanisms are responsible for the biological effects outlined in Figure 5

the death program to the end, and the safe disposal of the injured cell. This also implies that the morphological appearance of cell death (apoptosis or necrosis) is not linked to a single commitment point, but rather is a result of a more or less complete execution of subroutines deciding on the shape of dying cells.

Physiological mediators such as NO have emerged as potent modulators of death pathways (Melino *et al.*, 1997, 2000b; Brune *et al.*, 1999; Li and Billiar, 1999; Nicotera *et al.*, 1999a) and may act as effective switches between cell death paradigms *in vivo*. The ability of NO to diffuse intracellularly rapidly and from cell to cell would represent an efficient mechanism to prevent the activation of apoptotic effector molecules such as caspases, resulting from cell injury or exposure to other activators (CD95L, TNF α , ceramides, retinoids, chemotherapeutic drugs). Alternatively, the ability of NO to prevent apoptosis may decide the degree of inflammation in pathological states. Finally, NO can induce cell death depending on the biological milieu, its

redox state, concentration, exposure time and the combination with oxygen, superoxide and other molecules. One way to look at the pathophysiologically relevant switches between apoptosis and necrosis has been suggested by Stamler (Stamler *et al.*, 1992a, b, 1997, 2001; Stamler, 1994; Stamler and Piantadosi, 1996; Stamler and Hausladen, 1998), who conceptually separate reversible NO reactions, involved in signaling, from nonreversible NO reactions, involved in toxicity (Figure 6). Regardless of the mediator or tissue condition that may decide the switch between different cell death modes, it is apparent that targeting individual death routines may not be sufficient to ameliorate disease states effectively.

Acknowledgements

The work was supported by the Medical Research Council, and by grants from Telethon (E1224), AIRC, EU (QLG1-1999-00739 and QLK-CT-2002-01956), MIUR, MinSan to GM.

References

- Adjei PN, Kaufmann SH, Leung W-Y, Mao F and Gores GJ. (1996). *J. Clin. Invest.*, **98**, 2588–2596.
- Ankarcrona M, Dypbukt JM, Brune B and Nicotera P. (1994). *Exp. Cell Res.*, **213**, 172–177.
- Bagetta G, Corasaniti MT, Melino G, Paoletti AM, Finazzi-Agro A and Nistico G. (1993). *Biochem. Biophys. Res. Commun.*, **197**, 1132–1139.
- Becker K, Savvides SN, Keese M, Schirmer RH and Karplus PA. (1998). *Nat. Struct. Biol.*, **5**, 267–271.
- Bernassola F, Catani MV, Corazzari M, Rossi A and Melino G. (2001). *J. Cell. Biochem.*, **82**, 123–133.
- Bernassola F, Rossi A and Melino G. (1999). *Ann. NY Acad. Sci.*, **887**, 83–91.
- Blanco FJ, Ochs RL, Schwarz H and Lotz M. (1995). *Am. J. Pathol.*, **146**, 75–85.
- Bolanos JP, Heales SJ, Land JM and Clark JB. (1995). *J. Neurochem.*, **64**, 1965–1972.
- Bonfoco E, Krainc D, Ankarcrona M, Nicotera P and Lipton SA. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 7162–7166.
- Bonfoco E, Leist M, Zhivotovsky B, Orrenius S, Lipton SA and Nicotera P. (1996). *J. Neurochem.*, **67**, 2484–2493.
- Borner C and Monney L. (1999). *Cell Death Differ.*, **6**, 497–507.
- Bossy-Wetzel E and Lipton SA. (2003). *Cell Death Differ.*, **10**, 757–760.
- Bredt DS and Snyder SH. (1994). *Annu. Rev. Biochem.*, **63**, 175–195.
- Brune B, Gotz C, Messmer UK, Sandau K, Hirvonen MR and Lapetina EG. (1997). *J. Biol. Chem.*, **272**, 7253–7258.
- Brune B, von Knethen A and Sandau KB. (1999). *Cell Death Differ.*, **6**, 969–975.
- Catani MV, Bernassola F, Rossi A and Melino G. (1998). *Biochem. Biophys. Res. Commun.*, **249**, 275–278.
- Choi DW. (1988). *Neuron*, **1**, 623–634.
- Clarke PG. (1990). *Anat. Embryol. (Berl.)*, **181**, 195–213.
- Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S and Schapira AH. (1994). *FEBS Lett.*, **345**, 50–54.
- Clementi E, Racchetti G, Melino G and Meldolesi J. (1996). *Life Sci.*, **59**, 389–397.
- Corasaniti MT, Melino G, Navarra M, Garaci E, Finazzi-Agro A and Nistico G. (1995). *Neurodegeneration*, **4**, 315–321.
- Corasaniti MT, Melino G, Tartaglia RL, Finazzi-Agro A and Nistico G. (1994). *Neuropharmacology*, **33**, 1071–1077.
- Corasaniti MT, Tartaglia RL, Melino G, Nistico G and Finazzi-Agro A. (1992). *Neurosci. Lett.*, **147**, 221–223.
- Dimmeler S, Haendeler J, Nehls M and Zeiher AM. (1997). *J. Exp. Med.*, **185**, 601–607.
- Dimmeler S and Zeiher AM. (1999). *Cell Death Differ.*, **6**, 964–968.
- Ellerby HM, Martin SJ, Ellerby LM, Naiem SS, Rabizadeh S, Salvesen GS, Casiano CA, Cashman NR, Green DR and Bredesen DE. (1997). *J. Neurosci.*, **17**, 6165–6178.
- Enikolopov G, Banerji J and Kuzin B. (1999). *Cell Death Differ.*, **6**, 956–963.
- Eu JP, Hare JM, Hess DT, Skaf M, Sun J, Cardenas-Navina I, Sun QA, Dewhirst M, Meissner G and Stamler JS. (2003). *Proc. Natl. Acad. Sci. USA*, **100**, 15229–15234.
- Fehsel K, Kroncke KD, Meyer KL, Huber H, Wahn V and Kolb-Bachofen V. (1995). *J. Immunol.*, **155**, 2858–2865.
- Foster MW, McMahon TJ and Stamler JS. (2003). *Trends Mol. Med.*, **9**, 160–168.
- Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg LH, Ruffolo SC, Thornberry NA, Xanthoudakis S, Zamboni RJ, Roy S and Nicholson DW. (1999). *Cell*, **97**, 395–406.
- Gow AJ and Stamler JS. (1998). *Nature*, **391**, 169–173.
- Grimm LM, Goldberg AL, Poirier GG, Schwartz LM and Osborne BA. (1996). *EMBO J.*, **15**, 3835–3844.
- Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC and Lipton SA. (2002). *Science*, **297**, 1186–1190.
- Hausladen A, Privalle CT, Keng T, DeAngelo J and Stamler JS. (1996). *Cell*, **86**, 719–729.
- Hess DT, Matsumoto A, Nudelman R and Stamler JS. (2001). *Nat. Cell Biol.*, **3**, E46–9.
- Hirsch T, Marchetti P, Susin SA, Dallaporta B, Zamzami N, Marzo I, Geuskens M and Kroemer G. (1997). *Oncogene*, **15**, 1573–1581.
- Jaattela M, Wissing D, Kokholm K, Kallunki T and Egeblad M. (1998). *EMBO J.*, **17**, 6124–6134.

- Jia L, Bonaventura C, Bonaventura J and Stamler JS. (1996). *Nature*, **380**, 221–226.
- Kaneto H, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi H, Yamasaki Y, Kamada T and Taniguchi N. (1995). *Diabetes*, **44**, 733–738.
- Kerr JF, Wyllie AH and Currie AR. (1972). *Br. J. Cancer*, **26**, 239–257.
- Kim YM, Bergonia HA, Muller C, Pitt BR, Watkins WD and Lancaster Jr JR. (1995). *J. Biol. Chem.*, **270**, 5710–5713.
- Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnette DR, Stamler JS and Lipton SA. (1999). *Neuron*, **24**, 461–469.
- Kim YM, de Vera ME, Watkins SC and Billiar TR. (1997a). *J. Biol. Chem.*, **272**, 1402–1411.
- Kim YM, Kim TH, Seol DW, Talanian RV and Billiar TR. (1998). *J. Biol. Chem.*, **273**, 31437–31441.
- Kim YM, Talanian RV and Billiar TR. (1997b). *J. Biol. Chem.*, **272**, 31138–31148.
- Kuida K, Haydar TF, Kuan C-Y, Gu Y, Taya C, Karasuyama H, Su MS-S, Rakic P and Flavell RA. (1998). *Cell*, **94**, 325–337.
- Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P and Flavell RA. (1996). *Nature*, **384**, 368–372.
- Lander HM, Ogiste JS, Pearce SF, Levi R and Novogrodsky A. (1995). *J. Biol. Chem.*, **270**, 7017–7020.
- Leist M, Gantner F, Jilg S and Wendel A. (1995). *J. Immunol.*, **154**, 1307–1316.
- Leist M and Nicotera P. (1998). *Exp. Cell Res.*, **239**, 183–201.
- Leist M, Single B, Castoldi AF, Kuhnle S and Nicotera P. (1997). *J. Exp. Med.*, **185**, 1481–1486.
- Leist M, Single B, Naumann H, Fava E, Simon B, Kuhnle S and Nicotera P. (1999). *Exp. Cell Res.*, **249**, 396–403.
- Li J and Billiar TR. (1999). *Cell Death Differ.*, **6**, 952–955.
- Li J, Billiar TR, Talanian RV and Kim YM. (1997). *Biochem. Biophys. Res. Commun.*, **240**, 419–424.
- Lipton SA. (1999). *Cell Death Differ.*, **6**, 943–951.
- Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ and Stamler JS. (1993). *Nature*, **364**, 626–632.
- Liu L and Stamler JS. (1999). *Cell Death Differ.*, **6**, 937–942.
- Lockshin RA and Williams CM. (1965). *J. Insect Physiol.*, **11**, 123–133.
- Lovat PE, Ranalli M, Corazzari M, Raffaghello L, Pearson AD, Ponzoni M, Piacentini M, Melino G and Redfern CP. (2003). *J. Cell. Biochem.*, **89**, 698–708.
- Maccarrone M, Fantini C, Ranalli M, Melino G and Agro AF. (1998). *FEBS Lett.*, **434**, 421–424.
- Maccarrone M, Lorenzon T, Bari M, Melino G and Finazzi-Agro A. (2000). *J. Biol. Chem.*, **275**, 31938–31945.
- Manabe S and Lipton SA. (2003). *Invest. Ophthalmol. Vis. Sci.*, **44**, 385–392.
- Mannick JB, Asano K, Izumi K, Kieff E and Stamler JS. (1994). *Cell*, **79**, 1137–1146.
- Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ and Stamler JS. (1999). *Science*, **284**, 651–654.
- Mannick JB, Miao XQ and Stamler JS. (1997). *J. Biol. Chem.*, **272**, 24125–24128.
- Marshall HE and Stamler JS. (2001). *Biochemistry*, **40**, 1688–1693.
- Matsumoto A, Comatas KE, Liu L and Stamler JS. (2003). *Science*, **301**, 657–661.
- Matthews JR, Botting CH, Panico M, Morris HR and Hay RT. (1996). *Nucleic Acids Res.*, **24**, 2236–2242.
- McCarthy NJ, Whyte MK, Gilbert CS and Evan GI. (1997). *J. Cell Biol.*, **136**, 215–227.
- Melino G, Bernassola F, Catani MV, Rossi A, Corazzari M, Sabatini S, Vilbois F and Green DR. (2000a). *Cancer Res.*, **60**, 2377–2383.
- Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistico G and Finazzi-Agro A. (1997). *Nature*, **388**, 432–433.
- Melino G, Catani MV, Corazzari M, Guerrieri P and Bernassola F. (2000b). *Cell. Mol. Life Sci.*, **57**, 612–622.
- Messmer UK and Brune B. (1996). *Eur. J. Pharmacol.*, **302**, 171–182.
- Mohr S, Stamler JS and Brune B. (1996). *J. Biol. Chem.*, **271**, 4209–4214.
- Moncada S, Palmer RM and Higgs EA. (1991). *Pharmacol. Rev.*, **43**, 109–142.
- Nicholson DW. (1999). *Cell Death Differ.*, **6**, 1028–1042.
- Nicotera P, Ankarcrona M, Bonfoco E, Orrenius S and Lipton SA. (1997). *Adv. Neurol.*, **72**, 95–101.
- Nicotera P, Bernassola F and Melino G. (1999a). *Cell Death Differ.*, **6**, 931–933.
- Nicotera P, Leist M and Manzo L. (1999b). *Trends Pharmacol. Sci.*, **20**, 46–51.
- Petersen A, Larsen KE, Behr GG, Romero N, Przedborski S, Brundin P and Sulzer D. (2001). *Hum. Mol. Genet.*, **10**, 1243–1254.
- Radi R, Beckman JS, Bush KM and Freeman BA. (1991). *Arch. Biochem. Biophys.*, **288**, 481–487.
- Rossi A, Catani MV, Candi E, Bernassola F, Puddu P and Melino G. (2000). *J. Invest. Dermatol.*, **115**, 731–739.
- Roy B, Lepoivre M, Henry Y and Fontecave M. (1995). *Biochemistry*, **34**, 5411–5418.
- Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M and Freeman BA. (1994). *J. Biol. Chem.*, **269**, 26066–26075.
- Salgo MG, Bermudez E, Squadrito GL and Pryor WA. (1995). *Arch. Biochem. Biophys.*, **322**, 500–505.
- Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CM and Henkart PA. (1997). *Immunity*, **6**, 209–215.
- Schneiderhan N, Budde A, Zhang Y and Brune B. (2003). *Oncogene*, **22**, 2857–2868.
- Schwab BL, Guerini D, Didszun C, Bano D, Ferrando-May E, Fava E, Tam J, Xu D, Xanthoudakis S, Nicholson DW, Carafoli E and Nicotera P. (2002). *Cell Death Differ.*, **9**, 818–831.
- Schwarz MA, Lazo JS, Yalowich JC, Allen WP, Whitmore M, Bergonia HA, Tzeng E, Billiar TR, Robbins PD, Lancaster Jr JR and Pitt BR. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4452–4456.
- Schwartz LM and Osborne BA. (1993). *Immunol. Today*, **14**, 582–590.
- Schwartz LM, Smith SW, Jones MEE and Osborne BA. (1993). *Proc. Natl. Acad. Sci.*, **90**, 980–984.
- Schweichel JU and Merker HJ. (1973). *Teratology*, **7**, 253–266.
- Sciorati C, Rovere P, Ferrarini M, Heltai S, Manfredi AA and Clementi E. (1997). *J. Biol. Chem.*, **272**, 23211–23215.
- Shaham S and Horvitz HR. (1996). *Genes Dev.*, **10**, 578–591.
- So HS, Park RK, Kim MS, Lee SR, Jung BH, Chung SY, Jun CD and Chung HT. (1998). *Biochem. Biophys. Res. Commun.*, **247**, 809–813.
- Stamler JS. (1994). *Cell*, **78**, 931–936.
- Stamler JS and Hausladen A. (1998). *Nat. Struct. Biol.*, **5**, 247–249.
- Stamler JS, Lamas S and Fang FC. (2001). *Cell*, **106**, 675–683.
- Stamler JS and Meissner G. (2001). *Physiol. Rev.*, **81**, 209–237.
- Stamler JS and Piantadosi CA. (1996). *J. Clin. Invest.*, **97**, 2165–2166.

- Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ and Loscalzo J. (1992a). *Proc. Natl. Acad. Sci. USA*, **89**, 444–448.
- Stamler JS, Singel DJ and Loscalzo J. (1992b). *Science*, **258**, 1898–1902.
- Stamler JS, Toone EJ, Lipton SA and Sucher NJ. (1997). *Neuron*, **18**, 691–696.
- Suschkew CV, Krischel V, Bruch-Gerharz D, Berendji D, Krutmann J, Kroncke KD and Kolb-Bachofen V. (1999). *J. Biol. Chem.*, **274**, 6130–6137.
- Suzuki H, Kerr R, Bianchi L, Frokjaer-Jensen C, Slone D, Xue J, Gerstbrein B, Driscoll M and Schafer WR. (2003). *Neuron*, **39**, 1005–1017.
- Syntichaki P, Xu K, Driscoll M and Tavernarakis N. (2002). *Nature*, **419**, 939–944.
- Tabuchi A, Sano K, Oh E, Tsuchiya T and Tsuda M. (1994). *FEBS Lett.*, **351**, 123–127.
- Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W and Vandenabeele P. (1998a). *J. Exp. Med.*, **187**, 1477–1485.
- Vercammen D, Brouckaert G, Denecker G, van de Craen M, Declercq W, Fiers W and Vandenabeele P. (1998b). *J. Exp. Med.*, **188**, 919–930.
- Vieira HL, Belzacq AS, Haouzi D, Bernassola F, Cohen I, Jacotot E, Ferri KF, El Hamel C, Bartle LM, Melino G, Brenner C, Goldmacher V and Kroemer G. (2001). *Oncogene*, **20**, 4305–4316.
- Volbracht C, Leist M, Kolb SA and Nicotera P. (2001). *Mol. Med.*, **7**, 36–48.
- Volbracht C, Leist M and Nicotera P. (1999). *Mol. Med.*, **5**, 477–489.
- Wang X, Michael D, de Murcia G and Oren M. (2002). *J. Biol. Chem.*, **277**, 15697–15702.
- Wang X, Zalacstein A and Oren M. (2003). *Cell Death Differ.*, **10**, 468–476.
- Welsh N, Eizirik DL, Bendtzen K and Sandler S. (1991). *Endocrinology*, **129**, 3167–3173.
- Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J and Mitchell JB. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 9813–9817.
- Xu K, Gerstbrein B and Driscoll M. (2001). *Sci. World J.*, **1**, 129.
- Yasuda M, Fujimori H and Panhou H. (1998). *J. Toxicol. Sci.*, **23**, 389–394.
- Yuan J and Horvitz HR. (1990). *Dev. Biol.*, **138**, 33–41.
- Yuan J, Lipinski M and Degterev A. (2003). *Neuron*, **40**, 401–413.
- Zhang J, Dawson VL, Dawson TM and Snyder SH. (1994). *Science*, **263**, 687–689.