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Toxicology 208 (2005) 213–221
TOXICOLOGY
www.elsevier.com/locate/toxicol

Apoptotic volume decrease and nitric oxide

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Available online 19 December 2004

Abstract

Apoptosis is a physiological cell death process whose well-defined characteristics distinguish it from more accidental cell death processes. The loss of cell volume, or cell shrinkage, recently termed apoptotic volume decrease (AVD), is considered a hallmark of the apoptotic process. The activation and/or repression of the AVD process has been shown to be quite complex during apoptosis, with the involvement of multiple ionic transport mechanisms acting in both a cell type and stimulus specific manner. Similarly, the role of nitric oxide (NO) during apoptosis has also been shown to be just as complex, specifically in its ability to either induce and/or prevent apoptosis. This review examines current evidence for a link between AVD and NO and how they may interact during the programmed cell death process.
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Keywords: Apoptosis; Programmed cell death; Apoptotic volume decrease (AVD); Cell shrinkage; Regulatory volume decrease (RVD); Ions

1. Introduction

Cellular homeostasis requires a delicate balance between cell proliferation and cell death. While many cells in the body constantly cycle, thus generating new cells through the process of mitosis, unwanted cells are eliminated at specific times or in response to specific stimuli through the counter process of apoptosis. Although these processes play a key role in maintaining cellular homeostasis, mitotic and apoptotic defects can lead to various disease states. Apoptosis, also known as programmed cell death, is defined by a discrete set of morphological and biochemical characteristics that include cell shrinkage, nuclear condensation, internucleosomal DNA fragmentation, and eventual apoptotic body formation. Interestingly, these cell death characteristics, while defining apoptosis, do not necessarily occur in all cell types undergoing apoptosis. However, a universal trait of apoptosis is the loss of cell volume. Additionally, cell shrinkage has been used as the key discriminator between the physiological cell death process "apoptosis" and the accidental cell death process "necrosis". In fact, the original term used to describe this distinct mode of physiological death was "shrinking necrosis" highlighting the unique property of cell volume loss in these dying cells (Kerr, 1965, 1971).

Over the past 8–10 years, tremendous advances in the field of apoptosis have been made. Many new properties of apoptotic cells have been discovered, includ-

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0300-483X/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2004.11.024

ing the activation of specific proteases known as caspases (Miura et al., 1993; Lazebnik et al., 1994; Chen and Wang, 2002), changes at the level of the mitochondria (Ravagnan et al., 2002; Roucou et al., 2001), along with the identification of an increasing number of gene products that either repress or enhance the apoptotic process (Cory and Adams, 2002; Verhagen and Vaux, 2002). Two distinct programmed cell death pathways have been described: an extrinsic pathway involving the activation of cell surface death receptors; and an intrinsic pathway involving apoptotic stimuli that impinge at the level of the mitochondria (Wajant, 2002; Sartorius et al., 2001; Gupta, 2001). Recently, it has been suggested that other intracellular organelles are the targets of pro-apoptotic signals that integrate their response through the mitochondria, thus leading to cell death (Ferri and Kroemer, 2001). This review focuses is on the characteristic change in cell volume during apoptosis and how nitric oxide may play a role in this key feature of the programmed cell death process.

2. The volume response

Most cells have the inherent ability to control cell volume upon changes in the extracellular environment (Hoffmann, 1987; Al-Habori, 1994). When a cell perceives a change in the ionic composition of the extracellular environment, a series of ionic channels, exchangers, and co-transporters is activated which regulates the intracellular ionic environment to maintain continual cellular homeostasis. As a result of osmotic changes in the extracellular environment, a concomitant increase or decrease in intracellular water occurs in most cells, which in turn corresponds to a change in the intracellular ionic environment. For example, exposure of a cell to hypotonic conditions results in cellular swelling due to an increase in intracellular water. Depending on the cell type, compensatory ionic transport mechanisms termed a regulatory volume decrease (RVD) response, are activated that may include the K^+/Cl^- co-transporter, the K^+/H^+ exchanger coupled to the Cl^-/HCO_3^- exchanger, and/or individual K^+ and Cl^- channels. In contrast, a cell exposed to hypertonic conditions shrink due to a decrease in intracellular water. Again, compensatory ionic transport mechanisms termed a regulatory volume increase (RVI) response are activated that includes the $Na^+/K^+/2Cl^-$ co-transporter and/or the Na^+/H^+ exchanger coupled to the Cl^-/HCO_3^- exchanger. In the hypertonic condition, compensation for the initial increase in intracellular Na^+ due to the RVI response is achieved by increased activity of the Na^+/K^+-ATPase that exchanges intracellular Na^+ for extracellular K^+, thus re-establishing the original cellular ionic composition. The overall goal of these responses is to return the cell to a near normal cell size. While the specific response to changes in the external environment is known for many different cell types, the mechanism that signal changes in cell size are not completely defined.

3. The apoptotic volume decrease

The loss of cell volume or cell shrinkage during apoptosis has been shown to occur in parallel with a dramatic loss of both intracellular potassium and sodium ions (Barbiero et al., 1995; Bortner et al., 1997; McCarthy and Cotter, 1997; Yu et al., 1997). Interestingly, and in contrast to the volume changes and responses described above, the loss of cell volume during apoptosis, termed apoptotic volume decrease (AVD), occurs under normotonic conditions, suggesting that AVD is triggered independent of changes in the extracellular osmotic environment. The intracellular potassium concentration is normally high compared to intracellular sodium, and many studies examining AVD have focused on intracellular potassium loss and demonstrated its importance during apoptosis. Walev et al. (1995) reported that processing of proIL-1β to its mature form by the interleukin β converting enzyme (ICE), also known as caspase-1, occurs at low intracellular potassium levels in human monocytes, suggesting that the intracellular ionic environment can affect the activation of apoptotic proteins. Hughes et al. (1997) went on to directly show both enhanced caspase activation and optimal apoptotic nuclease activity in response to the AVD-initiated decrease in intracellular potassium. Additionally, exposure of apoptotic-stimulated cells to high extracellular potassium resulted in the inhibition of apoptosis at various levels of the cell death process (Bortner et al., 1997; Thompson et al., 2001; Cain et al., 2001), suggesting that prevention of potassium loss can protect cells from cell death. Therefore, maintenance of the normal intracellular ionic composition can have a repressive effect on the apoptotic process.

Although cell shrinkage during apoptosis occurs under normotonic conditions, Maeno et al. (2000) recently reported in HeLa and U937 cells that loss of cell volume during programmed cell death may share some of the ionic mechanisms that participate in inherent volume regulatory responses, thus linking AVD to RVD. Like the RVD response, what exactly triggers AVD during programmed cell death is not completely known. However, cytoplasmic factors, such as kinases, phophatases, and small GTPase have been shown to strongly modulate the level of intracellular potassium, specifically through the regulation of potassium channel activity (Yi et al., 2001). Therefore, similar signaling pathways may be employed for both RVD and AVD.

4. The complex nature of nitric oxide and AVD

Nitric oxide (NO) has been shown to have variable consequences in relation to either promoting or preventing apoptosis, likely due to its many interactions with other biological molecules (Kim et al., 2001). Additionally, the dose and source of NO, along with the specific cell type employed in any given study may play a role in the unpredictable effects of this molecule. For example, in Jurkat T cells, NO can either activate or inhibit apoptosis, depending on the apoptotic stimulus and the presence or absence of the CD95 (APO-1/Fas) receptor. The NO donor glycerol trinitrate was shown to induce apoptosis in Jurkat cells that were sensitive to CD95-mediated cell death, but had no apoptotic-inducing effect in a CD95-resistant Jurkat subclone (Chlichlia et al., 1998), suggesting that the presence of the Fas receptor is required for NO-induced cell death. In contrast, Dimmeler et al. (1998) showed in Jurkat cells that apoptosis triggered directly through the APO-1/Fas receptor was blocked in the presence of exogenous NO donors, as well as with NO endogenously derived via inducible NO synthase. The differing results in these two studies that employ the same cell type are likely due to the mechanisms activating apoptosis, either through a death receptor pathway or directly via NO. While the APO-1/Fas receptor clearly plays an important role in both cases, the effects of NO on apoptosis depends upon whether the receptor is directly or indirectly activated. Recently, Beltrán et al. (2002) showed that treatment of Jurkat cells with a pro-apoptotic anti-Fas antibody resulted in an early burst of NO that prevented mitochondrial respiration and hyperpolarized the mitochondrial membrane potential, the latter suggestive of a protective mechanism against cell death. However, continual monitoring of these anti-Fas stimulated Jurkat cells showed an eventual loss of the mitochondrial membrane potential and caspase activation resulting in apoptosis. Therefore, even for a single cell type and given stimulus, NO may have opposing effects, highlighting the complex nature of NO during the apoptotic process.

Equally complex is the mechanism of AVD or cell shrinkage during apoptosis, despite being a fundamental and universal characteristic of the programmed cell death process. Initially, AVD was thought to be a passive event during apoptosis; a sheer consequence of other biochemical events associated with the programmed process. However, as outlined above, cell shrinkage has been shown to play a key regulatory role during cell death. Specifically, without the proper movement of ions, optimal activation of the death machinery does not occur. While potassium efflux has been shown to be a common occurrence during apoptosis, the exact pathways for this ionic loss are not completely known. To date, no universal potassium channel blocker has been shown to be effective in preventing apoptosis regardless of the cell type or stimulus. The inability to use a single potassium channel inhibitor to prevent apoptosis under all conditions implies that either the characteristics of the channel may change during the cell death process rendering the inhibitor ineffective, or that multiple complimentary pathways exist that facilitate the loss of this ion (Bortner and Cidlowski, 2002). Interestingly, inhibition of potassium channels has also been shown to activate the cell death process, as 4-aminopyridine and clofilium, known potassium channel blockers, induced apoptosis in HepG2 and HL-60 cells, respectively (Kim et al., 2000; Choi et al., 1999). Therefore, the complexity and multifaceted nature of AVD during apoptosis rivals that of NO.

5. The relationship between NO and AVD

A major question of interest is whether there is a connection between nitric oxide and the universal morphological trait of AVD during apoptosis. While few

studies have been reported that directly address this question, Hortelano et al. (2002) recently examined the relationship between NO and AVD with regard to changes observed in the mitochondrial membrane potential in macrophages. Treatment of macrophages with a variety of apoptotic stimuli, including NO, staurosporin, etoposide, and camptothecin, resulted in an increase in the mitochondrial membrane potential, thought to be a protective response to cell death, that preceded AVD, cytochrome c release, caspase activation, DNA fragmentation, and eventual apoptosis. This increase in the mitochondrial membrane potential in apoptotic macrophages was in marked contrast to the decrease in mitochondrial membrane potential observed in NO or staurosporin-treated HeLa or Jurkat cells, suggesting release of pro-apoptotic factors from the mitochondria independent of specific changes in the organelle's membrane potential. Interestingly, the presence of phloretin and SITS, known inhibitors of volume-sensitive Cl^- channels, blocked AVD triggered by either staurosporin or NO in both macrophages and HeLa cells (Hortelano et al., 2002). However, this block of AVD did not prevent NO triggered apoptosis in these cells, suggesting that NO can induce programmed cell death in macrophages in the absence of AVD.

This observation of apoptosis in the absence of AVD is consistent with recent results from our laboratory demonstrating an uncoupling of AVD from other apoptotic characteristics (Bortner and Cidlowski, 2003). In this study, we sought to determine the importance of an observed early increase in intracellular sodium during apoptosis that depolarized the plasma membrane potential, by substituting sodium in the extracellular media with either choline or NMDG. Anti-Fas treatment of Jurkat cells under these sodium-substituted conditions resulted in cellular swelling, but the cells exhibited chromatin condensation, externalization of phosphatidylserine, caspase activity, and internucleosomal DNA fragmentation characteristic of apoptosis. The activation of the apoptotic process was shown to occur in response to a decrease in intracellular potassium in the swollen cells, consistent with previous reports showing the importance of potassium during cell death. These data suggested that the morphological feature of cell shrinkage is not the critical event, but that the underlying movement of ions is a key factor during apoptosis, with sodium controlling cell size, while potassium controls the cell death machinery.

6. A possible role for the actin cytoskeleton in NO-induced AVD

The actin cytoskeleton is known to play an important role in the regulation of cell volume (Pedersen et al., 2001). For example, in Ehrlich ascites tumor cells (EATC) cell shrinkage is associated with an increase and cell swelling with a decrease in F-actin content (Hoffmann, 1978; Pedersen et al., 1999; Mills et al., 2002). Additionally, agents, such as cytochalasin B and D, colchicines, and nocodazole have been shown to inhibit RVD by disrupting microtubules and actin filaments (Downey et al., 1995; Pedersen et al., 2001). Thus, it is not surprising that cytoskeletal changes would also occur during apoptosis.

Early studies have shown that plasma membrane alterations and cytoskeletal changes occur in human lung cancer and neuroblastoma cells upon olomoucine- and rescovitin-induced apoptosis (Van Engeland et al., 1997). Recently, alterations in the integrity of the cytoskeleton, including cleavage of intermediate filament proteins, have also been shown to occur during apoptosis (Mashima et al., 1999; Stegh et al., 2000; Maruyama et al., 2000). Reorganization of cytoskeletal proteins, such as F-actin, vimentin, and tubulin, was observed in K-562 and HL-60 leukemia cells treated with cytostatic drugs concomitant with features of apoptosis (Grzanke et al., 2003). Interestingly, the authors of this study also suggested that actin might be involved in chromatin remodeling during apoptosis. A strong correlation between the death receptor pathway and the cytoskeleton is evident from studies linking actin to the CD95 (Fas/APO-1) receptor (Parlato et al., 2000; Kulms et al., 2002). Additionally, physical disruption of the cytoskeleton itself has been shown to induce accelerated apoptosis (Rao et al., 1999; Suria et al., 1999; Yamazaki et al., 2000).

To determine the effects of the actin cytoskeletal architecture on NO-induced apoptosis, Kim et al. (2003a) used the model system of primary rabbit articular chondrocytes to determine if cytochalasin D (CD) treatment impacted the programmed cell death process. Disruption of the actin cytoskeleton by CD not only inhibited NO-induced apoptosis, but also prevented other

cellular events, such as dedifferentiation, COX-2 expression, and prostaglandin E2 production. These authors suggested that these CD-mediated effects occurred through negative regulation of caspase-3 and NF-κB activity via PKC inhibition (Kim et al., 2003a). This study implicates the actin cytoskeleton in mediating NO-induced apoptosis in chondrocytes through the modulation of various signalling pathways that are not solely limited to the cell death process, but are also associated with other important cellular events. However, in an earlier study, NO donors were shown to breakdown actin filaments, microtubules, and nuclear lamins prior to the appearance of typical features of apoptosis in cultured cerebellar granule cells (Bonfoco et al., 1996). Although alterations in the cytoskeleton were associated with NO-induced neuronal apoptosis, a direct link of NO to the actin architecture was not examined. The authors of this study did note that disorganization of actin alone was not sufficient to produce the morphological changes associated with apoptosis in cerebellar granule cells. However, disruption of microtubules and lamins in conjunction with actin disorganization led to marked changes in cellular morphology and trigger apoptosis (Bonfoco et al., 1995).

In a different study, production of NO-induced calcium transients in human neutrophils resulted in marked reduction in F-actin content and a profound increase in cell size, suggesting that NO can induce morphological alterations (Loitto et al., 2000). Additionally, NO has been suggested to have effects on the regulation of red blood cell deformability, as NO donors and NOS inhibitors significantly increased and reduced red blood cell deformability, respectively (Bor-Kucukatay et al., 2003). Since the major determinants of cellular deformability are cell geometry and cell shape, this result implies that NO can also have important regulatory effects on the mechanical properties of a cell.

7. NO regulation of volume-related ionic pathways during cell death

In considering a link between NO and volume regulation, questions of interest include identifying the major players in the AVD/RVD process and how might NO affect these specific ionic transport mechanisms. While many of these ionic pathways have yet to be completely defined, the influx and/or efflux of potassium, sodium, and chloride is known to play an important role in both AVD and RVD processes. Specifically, the movement of potassium and chloride via the K–Cl co-transporter is known to be a major mechanism in maintaining cell volume homeostasis. NO is known to be an endogenous endothelium-derived relaxing factor that regulates vascular smooth muscle cell proliferation and cell death, such that these cells provide an attractive model system to study the role of NO in both ion transport and apoptosis. Recently, the activity of various K–Cl co-transporter isoforms was shown to increase in the presence of NO donors in vascular smooth muscle cells, with the increase in activity prevented by inhibition of the cGMP pathway, protein phosphatases, and tyrosine kinases (Lauf and Adragna, 2000; Adragna et al., 2000). While the expression of the K–Cl co-transporter-3 was shown to be regulated in primary cultures of rat vascular smooth muscle cells by protein kinase G (Di Fulvio et al., 2001a), NO was shown to regulate the expression of the K–Cl co-transporter-1 (Di Fulvio et al., 2001b), the isoform considered to be the primary transporter that maintains volume homeostasis in all cells (Lauf and Adragna, 2000). These studies suggest a strong correlation between the NO-inducible K–Cl co-transporter and the regulation of cell volume, although no direct role for the K–Cl co-transporter during AVD has been shown during apoptosis.

In pulmonary vascular smooth muscle cells, NO was shown to directly induce apoptosis through the activation of potassium channels (Krick et al., 2002). Treatment of NO-induced smooth muscle cells with the potassium channel blockers tetraethylammonia, iberiotoxin, and 4-aminopyridine significantly blocked apoptosis. Additionally, the authors identified large-conductance Ca^{2+}-activated potassium channels and increased activity of voltage-activated potassium channels in these vascular smooth muscle cells. Furthermore, independent activation of these Ca^{2+}-activated potassium channels was shown to enhance NO-induced apoptosis in this model system.

A majority of studies examining AVD during apoptosis have focused on the movement of potassium, mainly due to the high intracellular concentration of this ion in most cells. Likewise, studies involving NO and ion transport have also concentrated on the movement of potassium. However, as discussed earlier, we recently found that sodium plays an important role in

controlling AVD through an initial influx of extracellular sodium during apoptosis in anti-Fas-treated Jurkat cells (Bortner and Cidlowski, 2003). Our previous work showed that an early transient increase in intracellular sodium resulting in plasma membrane depolarization was due in part to an early inhibition of the Na^+, K^+-ATPase during anti-Fas-induced apoptosis (Bortner et al., 2001). Interestingly, exposure of interferon-γ-treated intestinal T84 cells to the NO donor SPER-NO led to a rapid inhibition of the Na^+, K^+-ATPase, followed by a decrease in the expression of the $Na^+/K^+/2Cl^-$ co-transporter (Sugi et al., 2001). Inhibition of the Na^+, K^+-ATPase in these cells resulted in an increase in intracellular sodium, an increase in cell volume, and eventual dysfunction of the epithelial cells. Although apoptosis was not observed in this model system, the data suggests that NO can directly regulate critical ion transport mechanisms shown to be involved in selected apoptotic model systems. Additionally, NO has been shown to mediate membrane depolarization-promoted survival of rat neuronal PC12 cells, although no direct effect of NO on specific ion transport mechanisms was discussed (Kim et al., 2003b).

In addition to cationic transport mechanisms, anionic pathways, specifically the volume-regulated anion channels (VRACs), have been shown to play an important role in cell volume homeostasis (Lang et al., 1998). For example, VRACs have been shown to contribute to the release of osmolytes, such as excitatory amino acid release upon neuronal damage of the neurohypophysial system (Phillis et al., 1997; Kimelberg and Mongin, 1998). Treatment of astrocytes with the peroxynitrite (OONO-) donor 3-morpholinosydomine hydrochloride (SIN-1) resulted in an increase in the volume-dependent release of excitatory amino acids through a tyrosine kinase-dependent mechanism (Haskew et al., 2002). Interestingly, treatment of the same cell type with the NO donors sodium nitroprusside and spermine NONOate did not result in this volume-activated release of amino acids, suggesting specificity of NO donors in their ability to regulate the release of excitatory amino acids. Although no direct link between apoptosis and NO-directed anion channels has been reported, the important role of these pathways in volume regulation suggests further study is warranted.

NO may also regulate key apoptotic proteins indirectly through an effect on intracellular ions. Liossia et al. (1997) showed that heat stress could stimulate pathways that enhance the susceptibility of T lymphocytes to Fas/CD95-mediated apoptosis. Kiang et al. (2003) expanded on this observation and showed in Jurkat T cells that heat stress increases constitutive NO synthase phosphorylation, thereby, inhibiting enzymatic activity and decreasing the overall production of NO. As a result of the decrease in NO, a reduction in Na^+/Ca^{2+} exchanger activity occurred that correlated with increased expression of Fas/CD95 on the cell surface. Increased cell surface expression of a death receptor in turn renders these cells more susceptible to apoptosis. Thus, NO regulation of ion transport mechanisms not directly involved in changes in cell volume can impact the programmed cell death process.

As a final point, it has recently been shown that the NO signalling pathway can be activated by volume stress, resulting in increased expression of auxiliary proteins involved in programmed cell death. In the brain, injury-induced expression of p75NTR, the common receptor for neurotrophins, was shown to be required for oligodendrocyte apoptosis (Beattie et al., 2002). After traumatic injury, neurite regeneration of damaged neuronal cells is suppressed through p75NTR signalling, and this receptor was shown to be regulated by both NO and osmotic swelling (Peterson and Bogenmann, 2003). Therefore, unbalanced osmotic homeostasis can elicit NO activity and regulate proteins that function in a pro-apoptotic manner.

8. Conclusion

The studies outlined above suggest that among the numerous effects of NO on cellular physiology, regulation of volume-sensitive ionic pathways that promote or prevent AVD during apoptosis is a critical role played by NO. The ionic pathways involved in regulating AVD are quite diverse with no single set of channels or transporters identified that accounts for this event during apoptosis. Likewise, the role of NO during programmed cell death appears to be equally diverse, as this agent has been shown to be both pro- and anti-apoptotic depending on the stimulus and/or cell type, that is employed. NO has been shown to directly activate volume-sensitive Cl^- channels in various cells resulting in cell shrinkage during apoptosis. Additionally, NO can affect the actin cytoskeleton that may in

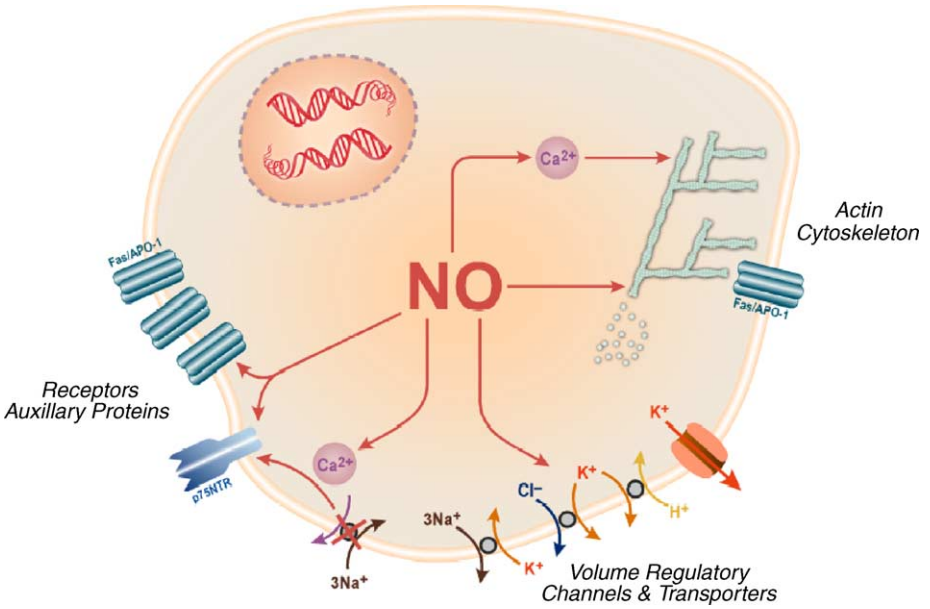

Fig. 1. Nitric oxide (NO) has been shown to function at various cellular levels that also impinge on the regulation of cell volume and the cell death process. The actin cytoskeleton can be a target of NO during apoptosis either by itself or in relation to changes in intracellular calcium, thus affecting not only cell structure and size, but also the function and regulation of death receptors. Additionally, NO can directly effect volume regulatory ionic pathways, such as the K–Cl co-transporter, the Na^+, K^+-ATPase, and various ion channels thought to play a role during AVD. Finally, NO through ionic pathways can affect auxiliary proteins/receptors, such as the p75 neurotrophin and the Fas/APO-1 receptors that can have a direct impact on apoptosis.

turn regulate AVD during apoptosis either directly or through the expression of numerous death-inducing receptors. Furthermore, NO has been shown to influence various potassium transport mechanisms, such as the K–Cl co-transporter and the Na^+, K^+-ATPase, further implicating this agent in volume regulation (Fig. 1). Clearly, the effect of NO on AVD requires further study to precisely define the role NO as it relates to AVD during apoptosis.

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