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Review

The dynamics of cellular injury: transformation into neuronal and vascular protection

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Summary. Despite the immediate event, such as cerebral trauma, cardiac arrest, or stroke that may result in neuronal or vascular injury, specific cellular signal transduction pathways in the central nervous system ultimately influence the extent of cellular injury. Yet, it is a cascade of mechanisms, rather than a single cellular pathway, which determine cellular survival during toxic insults. Although neuronal injury associated with several disease entities, such as Alzheimer's disease, Parkinson's disease, and cerebrovascular disease was initially believed to be irreversible, it has become increasingly evident that either acute or chronic modulation of the cellular and molecular environment within the brain can prevent or even reverse cellular injury. In order to develop rational, efficacious, and safe therapy against neurodegenerative disorders, it becomes vital to elucidate the cellular and molecular mechanisms that control neuronal and vascular injury. These include the pathways of free radical injury, the independent mechanisms of programmed cell death, and the downstream signal transduction pathways of endonuclease activation, intracellular pH, cysteine proteases, the cell cycle, and tyrosine phosphatase activity. Employing the knowledge gained from investigations into these pathways will hopefully further efforts to successfully develop effective treatments against central nervous system disorders.

Key words: Apoptosis, Cell cycle, Cysteine proteases, DNA fragmentation, Endonucleases, Intracellular pH, Phosphatidylserine, Tyrosine phosphatase

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Anoxic injury and free radical induction of neuronal and vascular cell degeneration

One of the initial inciting events that may precipitate both neuronal and vascular injury is the loss of oxygen to the cellular environment. The absence of oxygen to neuronal or vascular cells is not an "all or none" process, but may occur in various degrees with each level of oxygen loss capable of precipitating cellular injury. The individual levels of oxygen loss can be described as anoxic anoxia, anemic anoxia, and ischemia. Anoxic anoxia consists of reduced arterial oxygen content and tension. This may be secondary to decreased oxygen in the environment or an inability for oxygen to enter the circulatory system such as during loss of cardiac function. Anemic anoxia consists of low oxygen content in the blood secondary to decreased hemoglobin content. Ischemic anoxia describes a state of insufficient cerebral blood flow. Such decreased flow states may result from cardiopulmonary collapse or during increased cerebral vascular resistance, such as in migraine or stroke.

Interestingly, a "window of opportunity" for either the prevention or reversal of ischemic disease to the nervous system exists. For example, the therapeutic window for the treatment of cerebral ischemia is narrow, usually less than six hours, and requires the rapid reversal of toxic cellular events (Maiese et al., 1993b; Yang et al., 2000). Although the core of an ischemic insult suffers from loss of cerebral blood flow and metabolism, the region surrounding the ischemic core is characterized by decreased blood flow and secluded regions of hypermetabolism (Back, 1998). Pharmacological manipulation with agents such as 3-nitropropionic acid (Brambrink et al., 2000), nicotinamide (Lin et al., 2000), and imidazole receptor binding agents (Maiese et al., 1992) have been shown to reduce the extent of ischemia within the penumbral zone. In addition, muscarinic agonists have been demonstrated to possibly influence neuronal plasticity (Maiese et al., 1994a).

Yet, subsequent neuronal and vascular degeneration

during anoxia appears to be linked to the nitric oxide (NO) pathway. One of the initial reports linking NO to cerebral anoxic injury illustrated a significant reduction in middle cerebral artery stroke size during the pharmacological inhibition of NO production (Nowicki et al., 1991). During a global cerebral ischemic insult, production of NO also has been demonstrated to be increased in non-neuronal and non-vascular cells. In these experiments, neurons in the hippocampal CA1 region normally express the enzyme nitric oxide synthase (NOS) as evidenced by NADPH diaphorase staining and by mRNA probes for NOS-I (neuronal NOS), but astrocytes do not express the NOS-I enzyme (Endoh et al., 1994a,b). If these animals are then subjected to transient global ischemia in a four vessel occlusion model, the expression and distribution of NOS is altered. Over a course of one to thirty days post an ischemic insult, a population of reactive astrocytes in the CA1 region of the hippocampus express the inducible form of NOS (NOS-II) (Endoh et al., 1994a). These studies suggest that the hippocampus is capable of expressing both NOS-I in the neurons of CA1 and NOS-II in the surrounding astrocytic layer. The work also suggests that this induction of NOS-II in the astrocytes may be detrimental to the neurons in the sensitive hippocampal CA1 layer during an ischemic insult.

Work from other laboratories has further refined the individual contribution of the NOS system during cerebral ischemia. Each isoenzyme of NOS, such as neuronal NOS (NOS-I), endothelial NOS (NOS-III), and inducible NOS (NOS-II), may differentially modulate neuronal survival. For example, mutant mice deficient in NOS-I and NOS-II have been shown to experience reduced infarct volumes (Panahian et al., 1996; Loihl et al., 1999), while variations in penumbral NOS activity may contribute to neuronal injury sensitivity (Ashwal et al., 1998). Further investigations have demonstrated that inhibition of NO production in neuronal and vascular cell culture systems during anoxia is cytoprotective (Maiese et al., 1993a,b; Grammas et al., 1998; Demerle-Pallardy et al., 2000). For example, absence of NOS-II activity significantly increases cerebral vascular endothelial cell (EC) viability during anoxia, suggesting that NO generation through NOS is cytotoxic to rat cerebrovascular ECs (Maiese et al., 2000a) (Fig. 1). Other studies have demonstrated a relationship between delayed neuronal death and NO exposure. Delayed neuronal death seven days post global cerebral ischemia in the gerbil is reduced during inhibition of NOS activity (Kohno et al., 1996) and it appears that the constitutive neuronal NOS may play a dominant role during this process (Ferriero et al., 1995).

It is important to note that not all studies for neuronal or vascular ECs demonstrate a detrimental role for NO. Some experimental models have argued for the protective effects of NO (Buras et al., 2000). Increased production of NO, under some circumstances, has been shown to decrease rather than increase infarct size during either neuronal or vascular injury (Dalkara et al., 1994; Guo et al., 1999; Kanno et al., 2000). Although these results have been attributed to improved cerebral perfusion to the ischemic penumbra, other studies have illustrated that improved cerebral perfusion alone to the ischemic zone is insufficient to sustain neuronal survival (Maiese et al., 1992). In addition, endothelial production of NO has been linked to the preservation of the antiapoptotic protein Bcl-2 through the down regulation of cytosolic MAP kinase phosphatase MAP kinase phosphatase-3 (MKP-3) (Rossig et al., 2000). It is unclear why certain environmental conditions may predispose NO to function as a protectant rather than a toxin. Several factors appear to contribute to these divergent observations and involve such parameters such as the experimental model, external environmental conditions, duration of the insult, age of the neuronal or vascular system, and the resultant NO species that is generated (Marks et al., 1996; Chiueh, 1999).

The role of programmed cell death during neuronal and vascular injury

Programmed cell death (PCD) is a significant component of several pathophysiological conditions that lead to neuronal and vascular degeneration. Neuronal and vascular PCD can be induced by a variety of toxic insults to the nervous system, such as during cerebral ischemia (Love et al., 2000), excitotoxicity (Didier et al., 1996), angiogenesis signalling (Martini et al., 2000), and NO exposure (Palluy and Rigaud, 1996; Ishikawa et al., 1999; Vincent and Maiese, 1999b). Although PCD is important during the development of the nervous system (Lo et al., 1995), excessive induction of PCD in neurons or ECs without effective modulation may lead to

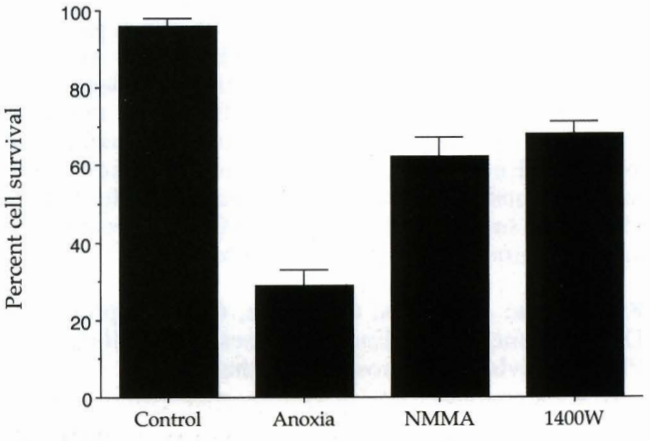

Fig. 1. Anoxia is toxic to cerebral vascular endothelial cells through the production of NO. To determine whether NO production during anoxia is cytotoxic to EC cultures, the effect of inhibition of NOS on EC viability was examined. Inhibition of EC NOS was performed in an oxygen-free environment with the NOS inhibitors 1400W (100 μM) or L-NMMA (1000 μM). Each NOS inhibitor was added directly to the culture media 1 hr prior to anoxia exposure. EC survival was determined 24 hr later following indicated time periods of anoxia by using a 0.4% trypan blue dye exclusion assay. Data represents the mean and SE.

disturbed cellular function and eventually precipitate disorders such as atherogenesis (Dimmeler et al., 1997; Galle et al., 1999).

PCD is considered to be an active, directed process that can rapidly lead to the destruction of a cell. In contrast to necrosis, PCD is characterized by the preservation of membrane integrity and internal organellae structure, chromatin condensation with nuclear fragmentation, and the budding of cellular fragments known as "apoptotic bodies". In most cellular systems, the end result of PCD is termed apoptosis (Kerr et al., 1972). In some cases, PCD requires *de novo* gene expression with subsequent protein synthesis (Ellis et al., 1991). The detailed understanding of the cellular mechanisms that modulate PCD may provide the basis for novel therapeutic strategies to prevent or reverse neuronal loss.

Neuronal and vascular PCD is believed to proceed through two dynamic, but distinct pathways that involve both DNA fragmentation and the loss of membrane asymmetry with the exposure of membrane phosphatidylserine (PS) residues (Vincent and Maiese, 1999a; Lin et al., 2000; Maiese and Vincent, 2000b). These processes are considered to be functionally independent determinants of PCD. The internucleosomal cleavage of genomic DNA into fragments may be a late event during PCD and ultimately commit a cell to its demise (Fig. 2). In contrast, the redistribution of membrane PS residues can be an early event during PCD (Martin et al., 1995; Rimon et al., 1997) that usually precedes DNA fragmentation and may serve to later "tag" injured cells for phagocytosis (Savill, 1997).

One of the most central issues surrounding PCD focuses on whether this process, once initiated, is committed in nature to lead to cellular death or is reversible to the extent of preventing further neuronal injury. In order to assess the reversiblity of PCD, assays that could monitor the induction of PCD in living cells required development. Current techniques employed to assess PCD, such as terminal deoxyUTP nick end labelling (TUNEL) or transmission electron microscopy, are useful to identify the extent of PCD induction in fixed tissue (Vincent et al., 1997; Fiorucci et al., 1999). Yet, these procedures lack the ability to assess dynamic changes in PCD in individual cells. As an alternative, a recently developed technique can now monitor the induction and change in PCD in individual living cells over a period of time. The method employs the reversible labelling of annexin V to exposed PS residues of cells undergoing PCD (Vincent and Maiese, 1999a; Maiese and Vincent, 2000b). By exploiting the dependence of annexin V on extra-cellular calcium to bind to exposed membrane PS residues, the technique can reversibly label individual cells. During the induction of PCD, such as following NO exposure, progressive externalization of membrane PS residues occurs that is independent of the loss of cellular membrane integrity (Fig. 3).

Studies employing cytoprotectants, such as the application of either trophic factors (Kiprianova et al., 1999), metabotropic glutamate receptor agonists (Vincent et al., 1999a; Maiese et al., 2000b; Vincent and Maiese, 2000), benzothiazole compounds (Maiese and Vincent, 2000a,b), Bcl-2 expression (Fabisiak et al., 1997), or nicotinamide (Ayoub et al., 1999; Lin et al., 2000; Mokudai et al., 2000) have shed some light in support of the concept of reversible injury during PCD. For example, since cytosolic and nuclear changes associated with PCD are evident within the first hour of NO exposure (Vincent et al., 1997; Adayev et al., 1998), the signal transduction mechanisms of the metabotropic glutamate system may reverse early steps in PCD. It is conceivable that these cytoprotective agents maintain membrane PS asymmetry through the modulation of

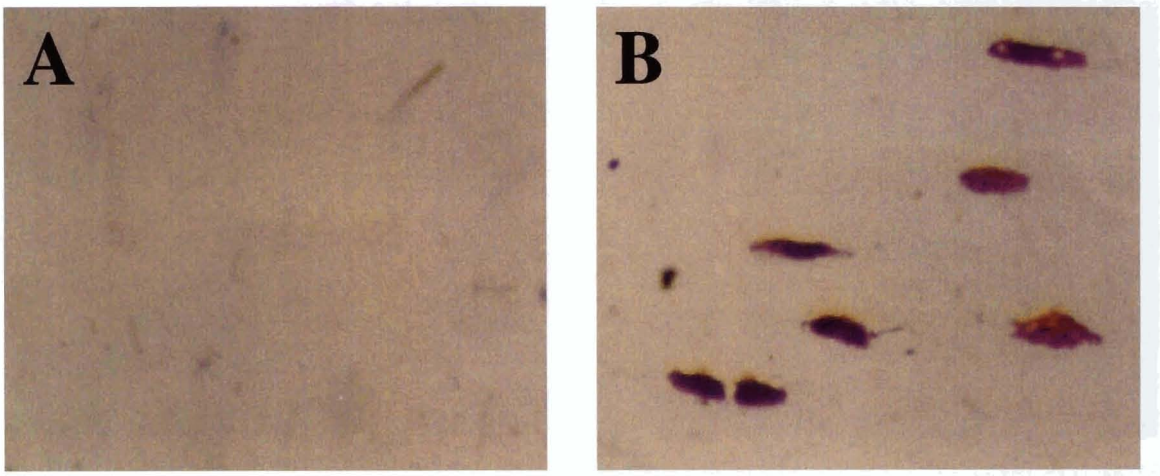

Fig. 2. NO exposure leads to the induction of DNA fragmentation in cerebral vascular endothelial cells. Representative fields of ECs illustrate the effect of NO on DNA fragmentation in EC cultures. DNA fragmentation was determined by TUNEL staining 24 hr following NO exposure (NOC-9, 1000 μM). In contrast to untreated control cultures **(A)**, a significant increase in the number of TUNEL-positive ECs (brown stained cells) is seen in cultures treated with the NO donor **(B)**. x 200

random "flip-flop" membrane phospholipids (Bratton et al., 1997). Alternatively, they may maintain cellular energy metabolism since exposure of membrane PS residues on the membrane surface is an active process facilitated by an ATP-dependent membrane translocase (Verhoven et al., 1999).

In light of the independence of PS externalization and DNA degradation during PCD, one of the critical issues concerns the biological role of PS externalization. In other systems, PS externalization can signal for the phagocytosis of cells (Rimon et al., 1997; Savill, 1997). In the nervous system, cells expressing externalized PS also may be removed by microglia (Savill, 1997). An additional role of PS externalization in vascular cell systems is the activation of coagulation cascades. The externalization of PS in platelets or ECs can promote the formation of a procoagulant surface (Bombeli et al., 1997). Therefore, in addition to the prevention of the phagocytosis of neurons, the ability to prevent and reverse PS externalization may avert vascular injury in disorders such as stroke and Alzheimer's disease. Thus, the ability of a specific cytoprotectant to prevent PS externalization may provide greater overall cellular protection through both maintenance of genomic DNA integrity and the prevention of PS externalization that can signal phagocytosis.

Cellular mechanisms of neuronal and vascular programmed cell death

Induction of endonuclease activation

The orderly cleavage of genomic DNA into nucleosomal or oligonucleosomal lengths is considered to be one of the hallmarks of PCD. Exclusive of the nervous system, a variety of enzymes responsible for chromatin degradation have been differentiated based on their ionic sensitivities to zinc (Walker et al., 1994) and magnesium (Sun and Cohen, 1994). Interest also has focused on the calcium/magnesium-dependent endonucleases such as DNase I (Madaio et al., 1996), the acidic, cation independent endonuclease (DNase II) (Torriglia et al., 1995), cyclophilins (Montague et al., 1997), and the 97 kDa magnesium-dependent endonuclease (Pandey et al., 1997). Yet, no clear consensus exists concerning the ability of any one or combination of endonucleases to invoke PCD, especially in the central nervous system.

Through the use of gel electrophoresis, electron microscopy, and cytochemical staining, active DNA degradation is believed to be a significant component of cellular PCD following free radical injury with NO toxicity (Fehsel et al., 1995; Maiese, 1998; Ishikawa et al., 1999). These studies have been extended to demonstrate that modulation of endonuclease activity directly influences neuronal survival in the presence of NO (Vincent and Maiese, 1999b; Vincent et al., 1999b). Employing *in vitro* assays of endonuclease activity, three endonucleases have been characterized, each with specific pH and divalent cation requirements, that are necessary for the induction of NO induced PCD. In addition, the activities of these endonucleases are physiologically dependent upon the intracellular pH changes induced by NO.

Three separate endonuclease activities are present during NO induced PCD. They are a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium dependent endonuclease (Vincent and Maiese, 1999b). In non-neuronal systems, endonucleases similar to the constitutive acidic cation-independent endonuclease and the constitutive

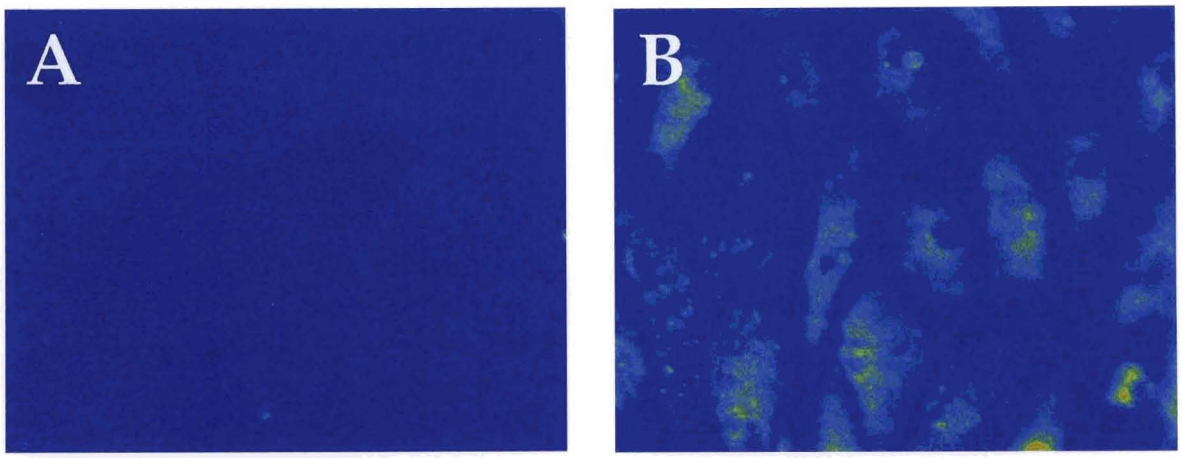

Fig. 3. NO leads to the development of membrane PS residue exposure in cerebral vascular endothelial cells. A representative field of ECs illustrates the effect of NO on the exposure of membrane PS residues in EC cultures. Annexin V staining was performed 12 hours following NO exposure (NOC-9, 1000 μM). The ECs were imaged by fluorescent light in untreated control cultures **(A)** and in cultures exposed to NO **(B)** using 490 nm excitation and 585 nm emission wavelengths to locate the annexin V-phycoerythrin label. NO-induced membrane PS exposure is visualized on the membrane surface of the ECs in panel B. x 200

calcium/magnesium-dependent endonuclease have been proposed as potentially having a role in PCD (Barry and Eastman, 1993; Montague et al., 1997; Pandey et al., 1997).

The inducible magnesium-dependent endonuclease may be unique for the nervous system (Vincent and Maiese, 1999b). Although the physiologic characteristics of the magnesium dependent endonuclease, such as a pH range of 7.4-8.0, a dependence on magnesium, and a molecular weight of 95-108 kDa, are consistent with a recently described constitutive 97 kDa endonuclease in non-neuronal tissues, the endonuclease in the nervous system is inducible during NO exposure rather than constitutive. Yet, it is also conceivable that the recently identified inducible magnesium-dependent endonuclease is a cleavage product of the constitutive calcium/magnesium-dependent endonuclease. This has not been previously reported, but may clarify the discrepancy of whether multiple endonucleases can function in concert or require the existence of a multi-functional endonuclease to cleave DNA in a particular cellular system. For example, some investigators have described sequential DNA degradation by a calcium/magnesium-dependent endonuclease that may become cleaved to yield a magnesium-dependent endonuclease (Cain et al., 1995).

The generation of free radicals, such as during the exposure of NO, has been suggested to be sufficient to directly result in the degradation of DNA (Wink et al., 1991). Yet, in some systems, DNA degradation following NO exposure required a downstream mediator, such as endonuclease activation (Vincent and Maiese, 1999b; Vincent et al., 1999a). As a result, the greatest potential for the treatment of neuronal or vascular degeneration appears to lie with the investigation of the subsequent cellular and molecular mechanisms that determine cellular survival. In this respect, modulation of the activity of either the constitutive or inducible pH dependent endonucleases may prevent or reverse cellular injury. Elucidating the role of specific endonucleases in cellular PCD will hopefully provide further insight into the molecular mechanisms that mediate free radical neuronal injury.

Free radical modulation of intracellular pH

As described during the induction of PCD, multiple endonucleases may be responsible for the destruction of a particular cell's genome (Torriglia et al., 1995; Madaio et al., 1996; Pandey et al., 1997; Vincent and Maiese, 1999b). Yet, what appears to be physiologically relevant for each of the endonuclease activities is the intracellular pH during PCD (Meisenholder et al., 1996). Present work has suggested that intracellular acidification may be both necessary and sufficient for the induction of PCD during free radical injury and identifies the modulation of intracellular pH as a possible therapeutic target against neuronal degeneration (Vincent and Maiese, 1999b; Vincent et al., 1999a,b).

Reactive oxygen species have been postulated as a potential mechanism for the induction of acidosis-induced cellular toxicity (Shen et al., 1995). Although NO toxicity can invoke multiple mechanisms to induce cellular degeneration, such as protein kinase C or protein kinase A activity (Maiese et al., 1993b; Maiese and Boccone, 1995), cellular calcium release (Maiese et al., 1994b; Clementi et al., 1996), and endonuclease activation (Vincent and Maiese, 1999b), NO injury is also clearly linked to intracellular acidification (Ito et al., 1997; Vincent et al., 1999b; Maiese et al., 2000b). NO employs rapid but transient intracellular acidification as a downstream mediator of neuronal degeneration. Prevention of NO induced intracellular acidification markedly, but not completely, improves neuronal survival, illustrating the pathophysiological contribution of NO induced intracellular acidification during neuronal degeneration (Vincent et al., 1999b). In addition, cellular injury and PCD during either NO or intracellular acidification are partially dependent upon endonuclease activation. Enhanced activity of the acidic-dependent endonuclease is believed to contribute to genomic DNA destruction during the abrupt intracellular acidification by NO. To a similar degree, the calcium/magnesium-dependent and the magnesium-dependent endonucleases may participate in cellular injury during the secondary biphasic intracellular alkalinization induced by NO (Vincent and Maiese, 1999b; Vincent et al., 1999a,b). Thus, free radical NO toxicity, at least in part, is dependent upon the initial acidification of the neuronal intracellular environment.

Cysteine proteases mediate genomic DNA degradation and membrane PS exposure

Some of the pathways that are responsible for the generation of genomic DNA degradation and membrane PS exposure during neuronal PCD result from the activation of a family of cysteine proteases. The cysteine proteases (caspases) are mammalian homologues of the *C. elegans* cell death (CED) genes (Ellis and Horvitz, 1986). Each of the aspartate-specific cysteine proteases is synthesized as a proenzyme that is proteolytically cleaved to subunits that form catalytically active heterodimers during development or injury (Martin and Green, 1995). Investigations with the cysteine proteases caspase 1 and caspase 3 have been implicated to lead to the induction of neuronal PCD (Du et al., 1997; Krohn et al., 1998; Maiese and Vincent, 1999). Caspase 1-like proteases may promote DNA degradation through the activation of other proteins, such as protein kinase C (Emoto et al., 1995) and caspase 3 (Enari et al., 1996). In some cellular systems, caspase 3-like proteases can cleave fodrin and focal adhesion kinase to induce membrane PS residue exposure during PCD (Levkau et al., 1998). In addition, the caspase 3-like proteases have been directly linked to the development of DNA fragmentation (Enari et al., 1998).

The subsequent downstream pathways that mediate

NO-induced PCD appear to be closely linked to the modulation of cysteine protease activity, but continue to require further investigation. Generation of NO in neurons can elicit cysteine protease activity and directly stimulate caspase 1 and caspase 3-like activities (Brune et al., 1999; Maiese et al., 2000b) (Fig. 4). This signal transduction system of cysteine protease activation by NO also appears to be maintained in ECs (Maiese et al., 2000a). In addition, activation of the metabotropic glutamate system mediates protection against PCD in neurons and in ECs through the direct inhibition of both caspase 1 and caspase 3-like activities (Maiese and Vincent, 1999; Maiese et al., 2000b).

Further work has examined the ability of caspase 1-like and caspase 3-like activities to directly modulate genomic DNA degradation following NO exposure. Exposure to NO can directly lead to the induction of both caspase 1-like and caspase 3-like activities over a period of twenty-four hours (Brown and Borutaite, 1999; Maiese and Vincent, 1999; Maiese et al., 2000b; Yabuki et al., 2000; Yamaguchi et al., 2000). This level of activity is sustained over a 24 hour period of time. In addition, NO-induced PCD is dependent, at least in part, upon the generation of cysteine protease activity since inhibition of either caspase 1 or caspase 3 activity can prevent either neuronal or vascular EC injury. It is believed that caspase 1 and caspase 3 may sequentially activate each other, since the individual inhibition of either cysteine protease can prevent genomic DNA degradation to a similar degree (Lin et al., 2000; Maiese et al., 2000b). A cascade of caspase activation has been demonstrated for various members of the caspase family (Marks and Berg, 1999). In particular, caspase 1-like activity may lead directly or indirectly to the induction of caspase 3-like activity (Enari et al., 1996).

The degradation of genomic DNA also may be directly associated with the individual induction of caspase 3-like protease activity. For example, caspase 3-like proteases cleave a constitutive endonuclease inhibitor that can degrade genomic DNA (Enari et al., 1998). Caspase 3-like proteases also cleave poly(ADP-ribose) polymerase (PARP). PARP has been shown to be required for DNA repair and functions by attaching poly(ADP-ribose) at DNA strand breaks (Cristovao and Rueff, 1996). A significant decrease in intact PARP following exposure to NO has been recently observed in neurons (Lin et al., 2000; Maiese et al., 2000b) (Fig. 5). Experimental models suggest that activation of additional proteases may further degrade the 85 kDa PARP fragment (Taylor et al., 1997; Lin et al., 2000).

In addition to the fragmentation of genomic DNA, induction of cysteine protease activity may also be responsible for the externalization of membrane PS residues. During cytokine mediated injury, the externalization of PS residues has previously been linked to the activity of caspase 1-like proteases through a mechanism that may involve the cleavage of membrane cytoskeletal proteins such as fodrin (Cryns et al., 1996; Kayalar et al., 1996). Current studies that employ visualization of PS externalization in individual cells have demonstrated that PS externalization is related to caspase 1-like activity (Maiese et al., 2000b; Vincent and Maiese, 2000). This externalization of membrane PS residues is independent of the loss of membrane integrity (Vincent et al., 1997; Maiese et al., 2000b; Maiese and Vincent, 2000b; Vincent and Maiese, 2000). Inhibition of caspase 1-like, but not caspase 3-like activity, following NO exposure can both prevent and reverse the

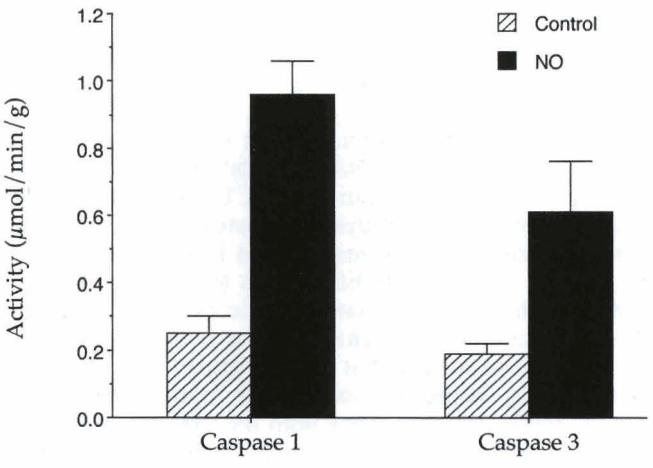

Fig. 4. NO enhances caspase 1 and caspase 3-like activities in neurons. To investigate whether NO-induced neuronal injury is linked to the generation of caspase activity, caspase 1 and caspase 3-like activities were determined by measuring the cleavage of colorimetric substrates following NO exposure. Caspase 1-like activity was determined by measuring the cleavage of Ac-YVAD-pNA at 24 hrs following NO exposure (NOC-9, 300 μM). NO exposure significantly increased caspase 1-like activity 24 hrs following NO treatment compared to untreated control cultures. Caspase 3-like activity was determined by measuring the cleavage of Ac-DEVD-pNA at 24 hrs following NO exposure (NOC-9, 300 μM). NO exposure significantly increased caspase 3-like activity 24 hrs after NO exposure compared to untreated control cultures. Data for caspase 1 and caspase 3-like activities represent the mean and SE.

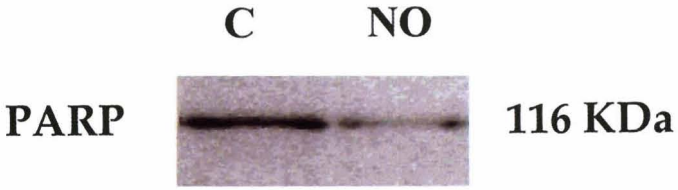

Fig. 5. NO exposure results in PARP proteolysis in neurons. A representative western blot is illustrated. Total protein extracts were prepared from either untreated control cultures or from extracts prepared 12 h following a 5 minute NO exposure. The NO donor employed was NOC-9 (300 μM). In both lanes, equal amounts of neuronal protein extracts (25 μg/lane) were separated by 7.5% SDS-PAGE and were then immunoblotted with polyclonal anti-PARP antibody. Detection was by enhanced chemiluminescence. Lane "C" (control) contained an extract from untreated rat hippocampal neuronal cultures. In lane "NO" (nitric oxide), a decrease in the amount of 116 kDa PARP occurred within 12 hr following NO exposure. The molecular weight marker is present on the right of the western blot.

externalization of PS residues.

The cell cycle and tyrosine phosphatases: potential "up-stream" molecular targets against neuronal and vascular injury

Cell cycle induction in post-mitotic neurons and vascular ECs

Although it is clear that the processes of PCD are intimately involved during neuronal and vascular injury, the molecular mechanisms that mediate cellular degeneration require further definition. It is conceivable that modulation of the cell cycle, especially in post mitotic neuronal cells, may represent one of the cellular pathways that is ultimately responsible for cellular injury. As one of the primary modulators of the cell cycle, the retinoblastoma gene product (pRb) is a 110kDa nuclear phosphoprotein that coordinates cellular pathways of growth and differentiation.

The pRb gene was initially identified for its role in tumorigenesis (Knudsen, 1987), but subsequently was studied for its ability to suppress neoplastic growth (Yandell and Dryja, 1989). Hypophosphorylated pRb binds to and inactivates E2F transcription factors (Ludlow et al., 1990). Phosphorylation of pRb deregulates E2F activity and, depending on the cell type, can promote cell proliferation or tumorigenesis (Sherr, 1994). Although pRb shares its ability with p53 to restrict G1 cell cycle progression, p53 acts upstream from pRb by inducing the p21 CDK inhibitor to prevent pRb phosphorylation (Hansen et al., 1986). Loss of pRb function can eliminate the ability of p53 to mediate G1 arrest (Demers et al., 1994). Yet, in several cell systems, pRb can function independently of p53 to determine a cell's fate (Hara et al., 1996; Serrano et al., 1996). For example, early stages of DNA damage in proliferating cells appear to be dependent on the phosphorylation state of pRb, but independent of p53 (Dou et al., 1995).

The pRb gene product leads a dual life not only as a "inhibitor of growth", but also as a "protector against death". Cells deficient in pRb function, such as pRb-/- mouse embryos and pRb-/- fibroblasts, are prone to death (Almasan et al., 1995). The massive PCD seen in these systems has been interpreted as a response to the loss of the pRb gene and the subsequent inactivation of mechanisms that usually safeguard against uncontrolled growth. Loss of functional pRb removes the checkpoint on the E2F transcription factors and allows cells to proceed past the G1 checkpoint into the S phase. This block on E2F transcription by pRb also can be removed during E2F over expression (Johnson et al., 1993).

In the nervous system, animals that are homozygous for pRb deficiency develop until midgestation, then suffer from ectopic nervous system mitoses and succumb to extensive cell death (Lee et al., 1992). Prior to midgestation, pRb does not modulate the G1 checkpoint (Savatier et al., 1996). Thus, the pRb nullizygous animal supports the premise that pRb regulates neuronal cell cycle arrest. Yet, once neuronal cells have become committed and enter the process of differentiation, loss of pRb activity with subsequent cell cycle induction appears to result in degeneration and PCD in the nervous system. Other studies also support the premise that loss of cell cycle regulation by pRb can lead to neuronal PCD. For example, if pRb is de-activated in neuronal ectodermal cells that are destined for differentiation, loss of functional pRb triggers apoptosis (Slack et al., 1995). In addition, the expression of mitotic cyclins and their associated kinases have been reported during periods of neurodegenerative disease (Busser et al., 1998). During injury paradigms such as anoxia or NO exposure, phosphorylated pRb expression appears to promote PCD while the enhancement of hypophosphorylated pRb expression prevents the induction of PCD (Maiese and Gallant, 1997).

As described, binding of hypophosphorylated pRb to E2F prevents the induction of cell transcription. As a result, deregulation of E2F can lead to PCD in some cell systems (Nevins, 1992). In addition, excessive production of E2F can overcome pRb's ability to inhibit cell cycle progression (Lukas et al., 1996). Thus, E2F is a critical downstream target of pRb and offers a potential therapeutic target during NO induced neuronal injury and PCD induction. Recently, loss of E2F function and the subsequent ability to prevent cell transcription in post-mitotic neurons has been linked to the induction of PCD in both in vivo (MacManus et al., 1999) and in vitro systems (Shaw et al., 2000).

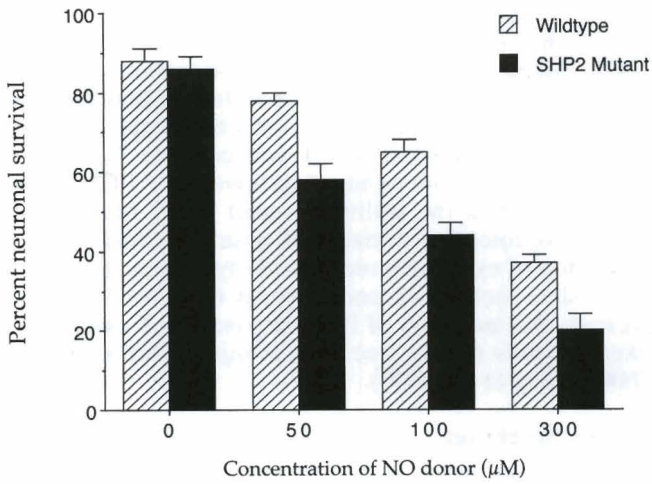

Fig. 6. SHP2 mutant neurons have increased sensitivity to NO toxicity. Increasing concentrations of a NO generator (NOC-9) for 5 minutes were applied to primary hippocampal wildtype and SHP2 mutant mouse neuronal cultures. The SHP2 mutant cultures have structurally intact SHP2 tyrosine phosphatase, but the SHP2 tyrosine phosphatase is functionally inactive. Neuronal survival was assessed 24 hours later using 0.4% trypan blue dye exclusion assay. In the absence of NO exposure, neuronal survival was approximately 86% (untreated control cultures). In each case, SHP2 mutant neurons are more sensitive to NO toxicity when compared to wildtype neurons. Data represents the mean and SE.

Tyrosine phosphatases offer cytoprotection against NO

Src family members are tyrosine kinases that are maintained at the plasma membrane by an N-terminal myristyl group and are involved in several cellular activities, such as cytoskeletal maintenance, cell division, and cell differentiation. SHP2 (SH-PTP2, PTP1D, Syp, PTP2C, and SH-PTP3) is a cytosolic protein tyrosine phosphatase that contains two Src homology 2 (SH2) domains (Vogel et al., 1993; Feng and Pawson, 1994), a protein tyrosine phosphatase domain, and a C-terminal hydrophilic domain with tyrosyl phosphorylation sites (Bennett et al., 1994). SHP2 has been linked to a variety of signaling pathways that involve receptor tyrosine kinases (Bennett et al., 1996; Wright et al., 1997) and trophic factor signaling pathways (Milarski and Saltiel, 1994; Yamauchi et al., 1995; Saxton et al., 1997). Of particular interest is the observation that SHP2 is expressed in the brain in areas such as the cortex, cerebellum, midbrain, and the hippocampus (Suzuki et al., 1995).

Although the tyrosine phosphatase SHP2 is employed into tyrosine kinase signaling pathways as a regulator during cell growth and development, the role of SHP2 and its downstream signaling pathways during neuronal or vascular cell injury is not well understood. Recently, loss of function of SHP2 has been associated with enhanced neuronal injury and the induction of PCD (Miller et al., 2000; Zhang et al., 2000) (Fig. 6). Interestingly, cells that are deficient in SHP2 function are with overt evidence of anatomical or physiologic disability. Yet, during injury paradigms with anoxia or NO exposure, cells that are without intact SHP2 function suffer from early induction of genomic DNA degradation and membrane PS exposure (Miller et al., 2000; Zhang et al., 2000). These observations suggest that SHP2 may be cytoprotective during neuronal or vascular degeneration. Given the knowledge that the cysteine proteases caspase 1 and caspase 3 directly modulate these independent pathways of PCD, it is conceivable that the ability of intact SHP2 function to offer cytoprotection during cell injury is linked to the regulation of cysteine protease activity. Recent work has established such a link and suggests that SHP2 directly prevents the induction of both caspase 1 and caspase 3-like activities during free radical injury (Miller et al., 2000; Zhang et al., 2000).

Future directions

Neuronal and vascular injury may occur at several levels within a cell. Early studies supported the consensus that cellular injury is closely tied to excitotoxicity (Rothman and Olney, 1986) and, in several cellular environments, specific glutamate receptors play a significant role during both neuronal and vascular injury. Yet, alternate cellular pathways are increasingly being recognized as vital mechanisms that can regulate cellular injury. The free radical NO has clearly been linked to ischemic cellular injury in both animal models and cell culture systems. The final cellular pathways that lead from the generation of NO to eventual cellular death are under continual investigation, but include intracellular calcium release (Maiese et al., 1994b, 1999; Skarzynski et al., 2000), the modulation of protein kinase activity (Maiese et al., 1993b; Zhang, 2000), enhanced neuronal endonuclease activity (Vincent and Maiese, 1999b; Vincent et al., 1999a), rapid induction of intracellular acidification (Ito et al., 1997; Vincent et al., 1999b; Maiese et al., 2000b), activation of cysteine proteases (Brown and Borutaite, 1999; Maiese and Vincent, 1999; Maiese et al., 2000b; Yabuki et al., 2000; Yamaguchi et al., 2000), cell cycle control (Maiese and Gallant, 1997; DiGregorio et al., 2000), and tyrosine kinase regulation (Miller et al., 2000; Zhang et al., 2000).

Knowledge of the underlying pathways that modulate both neuronal and vascular injury serve to provide the foundation for the design of successful cytoprotective strategies. For example, by addressing the independent components of PCD which include genomic DNA degradation and membrane PS exposure, therapeutic regiments can then offer both novel and broad based cellular protection, such as demonstrated with the metabotropic glutamate system (Maiese et al., 2000b; Vincent and Maiese, 2000), the upregulation of Bcl-2 (Fabisiak et al., 1997), or the agent nicotinamide (Lin et al., 2000). In this regard, therapy can provide both immediate and long-term cytoprotection. Immediate protection is afforded through the maintenance of intact genomic DNA. Long-term protection results through the inhibition of membrane PS residue exposure which can allow cells to be recognized by phagocytes for subsequent destruction. In addition, prevention of membrane PS exposure serves to maintain a normal anticoagulant state in ECs and prevent the development of atherosclerosis.

Yet, it is clear that further investigation is required to isolate the downstream cellular and molecular mechanisms that control neuronal and vascular survival prior to the onset of PCD. Studies that focus on more conventional pathways as well as less accepted mechanisms, such as post-mitotic cell cycle regulation and cellular tyrosine phosphatase activity, can offer insight into the mechanisms that may actually reverse cellular injury once it has been initiated. Future investigations can then open new therapeutic foundations for the treatment of acute and chronic central nervous system disorders.

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