Involvement of Cyclic GMP and Protein Kinase G in the Regulation of Apoptosis and Survival in Neural Cells

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Key Words

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Abstract

Our current understanding of nitric oxide (NO), cyclic GMP (cGMP) and protein kinase G (PKG) signaling pathways in the nervous systems has its origins in the early studies conducted on vascular tissues during the late 1970s and early to mid-1980s. The pioneering research into the NO/cGMP/PKG pathway in blood vessels conducted by the laboratories of Drs. Ferid Murad, Louis Ignarro and Robert Furchgott ultimately led to the awarding of the 1998 Nobel Prize in Physiology or Medicine to these three scientists. On the basis of further pioneering studies by Drs. John Garthwaite, Solomon Snyder, Steven Vincent and many other neuroscientists during the late 1980s and throughout the 1990s, it became recognized that NO serves as a neurotransmitter/neuromodulator in the central and peripheral nervous systems and that certain neural cells possess a cGMP signaling path-

way similar to that in vascular smooth muscle cells. Although NO (at high concentrations) is toxic and thought to participate in neuronal cell death during stroke and neurodegenerative diseases (e.g. amyotrophic lateral sclerosis, Alzheimer's disease, HIV dementia and Parkinson's disease), recent evidence suggests that NO at low physiological concentrations can act as an antiapoptotic/prosurvival factor in certain neural cells (e.g. PC12 cells, motor neurons and neurons of dorsal root ganglia, hippocampus and sympathetic nerves). The antiapoptotic effects of NO are mediated, in part, by cGMP and a downstream target protein, PKG. Other cGMP-elevating factors (e.g. atrial and brain natriuretic peptides) and direct PKG activator (e.g. 8-bromo-cGMP) also have antiapoptotic effects which have been quantified by the new capillary electrophoresis with laser-induced fluorescence detector technology. Inhibition of soluble guanylyl cyclase and lowering of basal cGMP levels cause apoptosis in unstressed neural cells (NG108-15 and N1E-115 cells). The cGMP/PKG pathway appears to play an essential role in preventing activation of a proapoptotic pathway, thus promoting neural cell survival.

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During the late 1970s and throughout the 1980s, the biological roles of nitric oxide (NO) in the cardiovascular system and the involvement of the cyclic GMP (cGMP) signal transduction pathway in vascular smooth muscle cells were discovered [1–4]. This led to the awarding of the 1998 Nobel Prize in Physiology or Medicine to three scientists, Drs. Ferid Murad, Louis Ignarro and Robert Furchgott, for their pioneering work in this field. These early studies showed that certain clinically used vasodilators, called ‘nitrovasodilators’ (e.g. sodium nitroprusside, nitroglycerin and isosorbide dinitrate), could generate NO in a variety of tissues, including vascular smooth muscle cells. NO was found to bind to the heme moiety of soluble guanylyl cyclase (sGC), an enzyme that synthesizes cGMP from GTP, increasing its catalytic activity and elevating cGMP levels. This signaling pathway was proposed to mediate relaxation of vascular smooth muscle cells in blood vessels, leading to vasodilation.

In 1980, Furchgott [4] discovered that the endothelial cells lining blood vessels could release a substance that he called endothelium-derived relaxant factor (EDRF), which diffused to the underlying smooth muscle cells and caused vasorelaxation. In 1982, Dr. Murad’s research group showed that EDRF activated sGC and elevated tissue levels of cGMP, a response that was identical to that caused by NO [for reviews, see ref. 1, 2]. This provided early evidence that EDRF and NO were very similar chemicals and appeared to have identical pharmacological/physiological properties.

In addition to sGC, a membrane-bound form of guanylyl cyclase, called particulate guanylyl cyclase (pGC), has been found in vascular smooth muscle cells [5], as well as other tissues, such as adrenal cortex and medulla [6] and kidney [5]. The pGC in these tissues was found to be activated by the newly discovered cardiac hormone atrial natriuretic factor (now called atrial natriuretic peptide; ANP), leading to elevations of cGMP levels in the tissues. These data provided the first evidence that ANP-induced vasodilation, diuresis and natriuresis, now recognized as the classic physiological actions of ANP, were all mediated by activation of pGC and cGMP elevations in blood vessels and kidney.

Biochemical studies during the 1970s and early 1980s identified a protein kinase, called cGMP-dependent protein kinase (cGMP-kinase, cG-kinase) or protein kinase G (PKG), that could be activated by cGMP (in the test tube), and it was proposed that this enzyme may be involved in the cGMP-mediated relaxation of smooth muscle [7]. However, during the 1970s and early 1980s, it was not known whether this enzyme could actually be activated by cGMP in intact cells. The early attempts at measuring PKG activation in intact cells or intact tissues, using assays primarily based on the established assay for cAMP-dependent protein kinase (protein kinase A), had been unsuccessful in showing PKG activation by NO, in spite of elevation of intracellular levels of cGMP in the tissue. At the time, it was thought that intracellular cGMP may not be able to activate PKG, thus potentially excluding PKG as the physiologically important downstream target of cGMP. However, our studies, conducted in the laboratory of Dr. Steven E. Mayer at the University of California, San Diego, Calif., USA, in 1982 and 1983, showed that the inability to measure NO-induced activation of PKG in the early studies was due to technical problems in preserving the activation state of PKG during the homogenizing and assaying procedures [8]. Unlike protein kinase A, PKG does not dissociate into catalytic and regulatory subunits upon activation, making it much more difficult to assay PKG without loss of its elevated activation state. During tissue homogenization and assaying procedures, the activated PKG was found to lose its activation because of the dissociation of cGMP from the allosteric/regulatory sites of PKG [8]. Thus, assaying PKG in the traditional kinase assay had greatly underestimated the intracellular activation of the enzyme.

In 1983 and 1984, we published the first reports of a successful assay for measuring PKG activation in intact tissues [8, 9]. The study utilized a unique cold-temperature assay technique that minimized the dissociation of cGMP from PKG, thus preserving PKG activation during the assay procedure. Using this modified technique, we showed that PKG in intact strips of tracheal smooth muscle could be activated by NO or by a cholinergic agonist, methacholine [8]. This report provided the first solid evidence that PKG could serve as a key downstream target of cGMP in airway smooth muscle cells, and likely mediated NO-induced relaxation in the airways. We further utilized this PKG assay in studies on vascular smooth muscle conducted in the laboratory of Dr. Ferid Murad at

Stanford University, starting in 1983 [9]. The PKG activation state in intact strips of rat aorta was found to be significantly elevated following exposure to either a nitrovasodilator, the NO donor sodium nitroprusside, or acetylcholine, an endothelium-dependent vasodilator known to trigger the release of EDRF from endothelium. Thus, the data provided the first direct evidence that PKG was a key downstream target of cGMP in vascular smooth muscle cells and that this pathway likely mediated the NO- and EDRF-induced vasodilations. The data also provided further support for the growing concept that NO and EDRF were very similar (and perhaps identical) chemicals.

In 1985, we published the first report of PKG activation by a natriuretic peptide, atriopeptin II (a biologically active fragment of ANP) [10]. This report showed that atriopeptin II increased the PKG activation state in blood vessels with a concentration dependency and time course that matched the vasorelaxant response. These data further established PKG as the key downstream target protein in the cGMP-mediated vasodilatory response. Details of our development of the PKG assay, including potential pitfalls and unique technical problems, are given in a chapter in *Methods in Enzymology* [11]. Reports by Fiscus [12] and Lincoln and Cornwell [7] provide a comprehensive review of the role of cGMP and PKG in vasodilation and the possible substrate proteins of PKG in smooth muscle cells.

Figure 1 illustrates the original cellular model of ANP-, EDRF- and NO-induced vasodilation in blood vessels, based on the early data from Dr. Murad's laboratory [9, 10]. This model was presented at the Federation of American Societies for Experimental Biology (FASEB) meeting (Experimental Biology –1985) in Anaheim, Calif., USA, in April of 1985. Literally hundreds of similar models, showing the cellular mechanism of NO or ANP in blood vessels, have been constructed since 1985 and have appeared in numerous reviews and books on the biochemistry, physiology, pharmacology and pathology of the cardiovascular system.

Identification of EDRF as NO and the Discovery of Other Actions of NO in Blood Vessels

In 1987, the laboratories of Drs. Louis Ignarro [13] and Salvatore Moncada [14], using a variety of pharmacological and chemical techniques, eventually identified EDRF as NO. Thus, an endogenous form of NO exists within the vascular system. Endogenous NO is now recognized to play an important physiological role in the human body,

Fig. 1. Original cellular model, based on the early data from Dr. Murad's laboratory at Stanford University from 1983 to 1985 [1, 2, 9, 10], showing the proposed role of sGC, pGC, cGMP and PKG in the vasorelaxant effects of three classes of vasodilators: (1) endothelium-dependent vasodilators [represented by acetylcholine (ACh)], (2) NO generated by nitrovasodilators, and (3) atrial natriuretic factor (ANF; now called ANP). This model is based on the early model first presented at the FASEB meeting (Experimental Biology –1985) in Anaheim, Calif., USA, in April of 1985. Rec = Receptor.

mediating a variety of functions, such as regulation of arterial blood pressure, inhibition of blood clotting, regulation of immune and inflammatory responses and prevention of the migration and proliferation of vascular smooth muscle cells (thus slowing the onset of atherosclerosis in the arterial wall). These topics have been expertly and extensively reviewed by Dr. Paul Vanhoutte [15], whose laboratory has been another major contributor to this field of research.

More recently, our laboratory has shown that NO also participates in the vasorelaxant responses of two physiologically important neuropeptides, i.e. calcitonin gene-related peptide (CGRP) and substance P [16, 17]. CGRP and substance P are recognized as major participants (i.e. as vasodilators) in the increased blood flow occurring during inflammation, and it is believed that NO mediates at least part of this neurogenic vasodilation, resulting in the hyperemia of inflammation. Our laboratory has further shown that NO synergistically enhances the vasorelaxant and cyclic AMP (cAMP)-elevating responses to CGRP [18]. Moreover, we have shown that these responses are mediated by NO-induced elevation of cGMP and subse-

quent inhibition of the type III phosphodiesterase (PDE3) in vascular smooth muscle cells [18–20]. These data suggest that PDE3, in addition to PKG, may be an important downstream target of cGMP in the cGMP signaling pathway, regulating cellular functions in those types of cells that possess the PDE3 isoform of phosphodiesterase. Other factors that elevate cGMP levels in vascular smooth muscle cells, such as brain natriuretic peptide (BNP) [21] or those that specifically inhibit PDE3 (e.g. the PDE3 inhibitors SKF94120 and quazinine), have also been found to synergistically enhance the vasorelaxant and cAMP-elevating responses to CGRP [19, 20, 22], thus further establishing PDE3 as a major downstream target protein of cGMP in smooth muscle cells.

The production of and/or vascular responses to NO are progressively lost during the aging process [23]. This leads to an increased risk of developing hypertension, because of the diminished vasodilatory response of the endogenous NO, and of developing atherosclerosis, leading to coronary heart disease, kidney disease and stroke, because of the diminished NO-induced inhibition of smooth muscle cell migration and proliferation. Diabetes mellitus accelerates the loss of NO and further diminishes the actions of NO in the vasculature, resulting in an earlier onset of vascular complications [hypertension, atherosclerosis and inadequate blood perfusion of the kidneys and the extremities (i.e. diabetic foot)]. This reduction in NO production and NO-mediated responses during aging and diabetes mellitus is also likely to affect the functions of the peripheral and central nervous systems and may account, at least in part, for the diminished neural functions in diabetic patients and the elderly (see discussion below concerning the antiapoptotic/prosurvival actions of NO in neural cells and the potential role of endothelium-derived NO as a neuroprotective factor).

Early Studies of cGMP in Neural Cells and the Discovery of NO Production and Action in the Central and Peripheral Nervous Systems

Early studies of cGMP in both the central and peripheral nervous systems during the 1970s had shown that electrical stimulation or application of cholinergic agonists caused elevation of tissue levels of cGMP [24, 25]. In general, elevations of cGMP levels in neurons or addition of exogenous cGMP tended to increase membrane excitability and/or increase the rate of spontaneous firing. However, little was known about the function of the cGMP signaling pathway in the nervous systems at that time.

In 1987, our laboratory found that two established neural cell lines, C6 glioma and PC12 pheochromocytoma cells, showed increases in both intracellular and extracellular levels of cGMP when exposed to the NO donor sodium nitroprusside or the natriuretic peptide ANP [26]. Thus, these two neural cell lines were shown to have both types of guanylyl cyclase, sGC and pGC, as well as functional receptors for ANP, and thus we believed that they could provide very useful cell culture models for studying cGMP signaling pathways in glial and neuron-like cells. However, because there were no known biological functions for either NO or ANP in neural cells at the time, our further studies of the NO/cGMP and ANP/cGMP pathways in neural cells were set aside for a number of years.

In 1988, endogenous production of NO in the central nervous system was discovered by Dr. John Garthwaite [27], and during the following several years, studies by many research laboratories, notably the laboratories of Drs. Solomon Snyder, Steven Vincent and John Garthwaite, established NO as a new class of neurotransmitter in the brain [27–29]. Excitatory amino acids such as glutamate were found to stimulate the neuronal synthesis of a diffusible substance that had all of the characteristics of NO (EDRF), i.e. a relatively short half-life (a few seconds in high-oxygen-perfused tissues), protection by superoxide dismutase, the ability to stimulate cGMP synthesis and elevate tissue levels of cGMP, inhibition of its actions by hemoglobin and the ability to relax smooth muscle [27]. NO, as a very small, gaseous molecule, was indeed a new class of neurotransmitter that could readily diffuse from one neural cell to another and, because of its lipid solubility, could rapidly penetrate cell membranes to activate intracellular receptors (e.g. sGC) without the need to bind to a cell surface receptor. NO is now thought to participate as a messenger in long-term depression in the cerebellum and as a retrograde messenger in long-term potentiation (LTP) in the hippocampus [28, 30]. Two other diffusible substances, arachidonic acid and carbon monoxide, both of which activate sGC in a manner similar to its activation by NO, have also been proposed to serve as retrograde messengers during LTP in the hippocampus [30].

Localization and Function of PKG in Central and Peripheral Nervous Systems

During the early 1980s, reports from the laboratories of Drs. Paul Greengard, Suzanne Lohmann and Ulrich Walter had shown that PKG was localized to specific neurons in the brain, primarily Purkinje cells in the cerebel-

lum [31]. Activation of PKG in these neurons resulted in phosphorylation of a 23,000-dalton protein (which they called G-substrate), which was also highly expressed in Purkinje cells [32]. Although these early studies had suggested that PKG was localized only to Purkinje cells in the brain, recent studies have shown that PKG is more widely distributed than first thought. For example, immunohistochemical evidence has shown the presence of PKG (type I) in medium-sized spiny neurons of the striatum of the brain [29] and also within a specific population of dorsal root ganglion neurons that also express the neuropeptides CGRP and substance P [33]. Studies using in situ hybridization with cloned PKG (type I) showed labeling of pyramidal cells, granule cells and several other cells in the rat hippocampus [34]. Thus, improvements in the technology for detecting and localizing PKG and its gene expression have now shown that PKG is distributed in a number of types of peripheral and central neurons.

Activation of PKG in hippocampal neurons has been shown to participate in LTP induced by NO and carbon monoxide [35]. Currently, it is not known which proteins serve as the substrates of PKG in the LTP response. However, more than 40 proteins in rat brain have been identified as endogenous substrates of PKG [36], and likely some of these proteins are involved in PKG-mediated actions in the brain.

In all of the above-mentioned studies of PKG in the nervous systems, it has been assumed that the type I isoform of PKG (either PKG I α or PKG I β) was involved. However, recent studies have shown that another isoform of PKG, called PKG II, exists within the mammalian brain [37–39]. This brain protein was found to be identical to the PKG II originally identified and studied in the intestinal mucosa. High levels of PKG II were found to exist in certain areas of rat brain, such as the thalamus and cerebral cortex, which typically have low (or undetectable) levels of PKG I [38]. Thus, in those neural cells not expressing PKG I, PKG II may serve as the downstream target of cGMP and mediate the biological actions of NO, natriuretic peptides and other agents/factors that elevate cGMP levels.

Interestingly, PKG II has been shown to effectively phosphorylate a peptide corresponding to the phosphorylation site of the cAMP-regulatory element binding protein (CREB) [39] and to be involved in the activation of *c-fos* gene expression in neuronal and glial cells [40]. It is tempting to speculate that PKG II, via phosphorylation of transcription factors like CREB, is involved in some of the physiological actions of cGMP in cells of the nervous systems.

NO Has Both Toxic and Prosurvival Actions in Various Mammalian Cells

NO can cause either toxicity, leading to apoptotic or necrotic cell death, or protective (antiapoptotic) effects in cells of the cardiovascular and nervous systems of mammals, depending on the type of cells and/or the circumstances of the NO exposure [23, 41]. In a healthy cardiovascular system, NO is continuously produced by endothelial cells, and the released NO acts continuously on other cells (e.g. vascular smooth muscle cells, platelets and immune cells), preventing the development of hypertension and atherosclerosis, thus helping to preserve the health of the cardiovascular system, as mentioned above. These physiological protective effects of NO normally occur at a relatively low concentration (submicromolar concentration) of NO, which represents the concentration of NO needed for activation of sGC and elevation of cellular levels of cGMP [15, 41].

Excess production of NO, as can occur during certain pathological conditions such as ischemia or inflammation, can cause toxic reactions within cells, leading to cellular damage and ultimately to apoptotic or necrotic cell death [23, 41]. The excess NO formed from increased expression of either the inducible form of NO synthase (iNOS) in response to elevated levels of cytokines like interleukin-1 and tumor necrosis factor or the neuronal form of NO synthase (nNOS) in response to trauma can combine with superoxide anion to form peroxynitrite, an extremely effective and potent prooxidant [23, 41–43]. Peroxynitrite can then oxidize proteins, lipids and DNA within cells, causing damage to cellular functions and resulting in either apoptosis or necrosis, depending on the level of the oxidative stress. This NO/peroxynitrite-induced oxidative stress is thought to contribute to the apoptotic cell death of neurons during the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [23, 42, 43].

A key question has been whether the cGMP/PKG signaling pathway is involved in these toxic effects of NO. Early studies had suggested that cGMP and PKG were involved in the proapoptotic actions of NO in vascular smooth muscle cells [44], cardiac myocytes [45], a pancreatic B cell line [46] and vascular endothelial cells [47]. A recent study using colon tumor cells has further suggested that cGMP and PKG may be involved in the induction of apoptosis [48]. Thus, in some cells, the cGMP/PKG pathway appears to contribute to NO-induced toxicity.

Fig. 2. Comparison of DNA laddering using the conventional method of agarose gel electrophoresis and the new ultrasensitive CE-LIF method. Apoptotic DNA fragmentation in PC12 cells, represented by bands (agarose gel) or peaks (CE-LIF electropherograms) at approximately 180, 360, 540 and 720 bp, etc., is shown. The level of apoptosis was greatly increased by the removal of serum for 4.5 h (-S), as compared to the control in the presence of serum (+S). BNP at 1, 10 and 100 nM caused substantial protection against the onset of apoptosis. M = Markers.

In contrast, many mammalian neural cells, including hippocampal neurons, PC12 cells, NG108-15 cells, sympathetic neurons, motor neurons, dorsal root ganglion neurons and cortical neurons, do not show toxicity during elevation of cGMP levels. Rather, they show enhanced survival (see next section). For example, studies from our laboratory have shown that activation of the cGMP/PKG pathway in neurons and neuron-like cells causes neuroprotection (i.e. increased survival and inhibition of entry into the apoptotic pathway; see below).

Involvement of cGMP and PKG in the Antiapoptotic/Prosurvival Effects of the Soluble Form of Amyloid Precursor Protein, Natriuretic Peptides and NO in Neural Cells

In 1995, in collaboration with Dr. Mark Mattson's laboratory at the University of Kentucky, we showed that the soluble form of the amyloid precursor protein (APP^S) causes lowering of cytosolic calcium concentrations and improvement of neuronal survival during glutamate toxicity or glucose deprivation via the elevation of cGMP

Fig. 3. Protection against the onset of apoptosis by 8-bromo-cGMP in serum-deprived PC12 cells determined by DNA laddering by both agarose gel (bands) and CE-LIF (peaks) techniques. +S = Serum present; -S = no serum present; M = Markers.

levels in rat hippocampal neurons [49]. Our study further showed that inhibition of PKG activity in the hippocampal neurons could partially block the prosurvival effects of APPS [49], suggesting that cGMP, via activation of PKG, was mediating the neuroprotective effect of APPS.

More recently, we have used PC12 cells to study the potential involvement of the cGMP/PKG pathway in the prevention of neural cell death. A reason for selecting this cell line was because of our previous studies in 1987 that had shown the presence of both sGC and pGC in PC12 cells and had further shown clear cGMP-elevating responses to NO and ANP [26]. Thus, either NO or ANP could be used as a research tool to elevate cGMP levels and study the function of cGMP in this neural cell line. However, because of the known toxic actions of NO described above, we selected ANP (as a more pure cGMP-elevating agent) to test the potential protective effects of cGMP in PC12 cells, without the complications of the NO-induced toxicity. We found that ANP increases the survival of PC12 cells and specifically inhibits the onset of

apoptosis (measured by DNA laddering using both agarose gel electrophoresis and capillary electrophoresis; see below) caused by serum removal [50]. Another natriuretic peptide, BNP, which activates pGC and elevates cGMP levels in PC12 cells, also increases cell survival and prevents the onset of apoptosis (see next section; fig. 2). Furthermore, addition of a cell-permeable analog of cGMP (8-bromo-cGMP) that directly activates PKG was found to have similar protective effects (see next section; fig. 3). Recently, we confirmed the antiapoptotic effects of ANP in serum-deprived PC12 cells using another technique, i.e. TUNEL labeling [51]. Thus, elevation of cGMP and activation of PKG in PC12 cells by natriuretic peptides causes antiapoptotic/prosurvival effects.

Addition of exogenous NO in the form of NO donors (sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine) has also been shown to protect against the onset of cell death in serum-deprived PC12 cells as well as in nerve growth factor-deprived sympathetic neurons in primary culture [52, 53]. Interestingly, these neuroprotective ef-

fects occurred only when the NO donors were used at lower concentrations (below 100 μM), which likely generated local NO concentrations in the submicromolar or low micromolar concentration range. NO at this concentration would be capable of activating sGC and elevating intracellular levels of cGMP, but probably would not be capable of efficiently combining with superoxide anion to form the toxic peroxynitrite. At higher concentrations (i.e. above 100 μM) of these NO donors, NO would likely form peroxynitrite and cause concurrent toxic effects that could cover up the potential protective effects of NO. Nevertheless, the data from these studies [52, 53], along with our data showing the antiapoptotic effects of ANP- and BNP-induced elevations of cGMP [50, 51], suggest that the cGMP pathway in neurons may be an important protective mechanism that may serve as a useful counterbalance to the proapoptotic actions of NO. This protective mechanism may be especially important when neurons are exposed to intermediate concentrations of NO, as, for example, during brain ischemia, inflammation or trauma. Without the protective effects of the cGMP elevation in neurons exposed to elevated levels of NO, these cells may be especially susceptible to the toxic actions of NO.

cGMP and PKG may also serve as an important pro-survival pathway in other types of neural cells. For example, an early paper had shown that dibutyryl-cGMP, a cell-permeable analog of cGMP, could prevent cell death in chick embryo motor neurons [54]. In rat embryonic motor neurons, increased cell survival caused by brain-derived neurotrophic factor (BDNF) was shown to be dependent on constitutive expression of the 'so-called' endothelial form of NO synthase (eNOS), now known to be expressed in certain neurons, and on the subsequent elevation of cGMP levels [55]. Rat motor neurons require continuous low-level synthesis of NO and cGMP elevation in order for BDNF to function as an effective survival factor. Likewise, cultures of rat dorsal root ganglion neurons were shown to require the endogenous production of NO and elevation of cGMP levels to avoid cell death in culture [56]. Neuroprotective effects of NO and cGMP have also been shown in rat cerebellar granule cells [57] and rat cortical cells [58].

However, in certain other neural cells, e.g. mouse hippocampal HT22 cells [59] and rat cerebellar neurons [60], cGMP has been reported to promote cell death. Thus, cGMP-mediated protection against cell death appears not to be universally observed in all types of neurons. Further studies with direct side-by-side comparisons among the various neural cells will be needed to determine the exact

role of cGMP and PKG in the initiation or prevention of apoptosis in different types of neural cells.

Use of Capillary Electrophoresis with Laser-Induced Fluorescence Detector as a New, Ultrasensitive, Quantitative Technology for Measuring Apoptotic DNA Fragmentation in Neural Cells

Internucleosomal fragmentation of DNA is a hallmark of apoptosis in neural cells. Unfortunately, the conventional technique for measuring apoptotic DNA fragmentation, i.e. DNA laddering by agarose gel electrophoresis, is a relatively insensitive method that typically requires 1–10 million cells per sample for the detection of apoptosis. Furthermore, DNA laddering on agarose gels is not quantitative, making it difficult to objectively analyze the effects of pro- and antiapoptotic agents.

Recently, our laboratory has developed an ultrasensitive quantitative technique for quantifying apoptotic DNA fragmentation in neural [50, 61] and epithelial [62] cells using capillary electrophoresis (CE) coupled with the new, ultrasensitive laser-induced fluorescence detector (LIF). This new CE-LIF technology provides a more than 1,000-fold improvement in the sensitivity of measuring DNA fragmentation, as compared to the conventional agarose gel technique. Furthermore, we have used both internal and external DNA standards as well as computer analysis of CE-LIF electropherograms to accurately quantify the levels of apoptotic DNA fragmentation. This allows us to accurately determine the -fold increase in the levels of apoptotic DNA fragmentation caused by proapoptotic agents/factors as well as the percentage of inhibition of apoptosis caused by antiapoptotic agents/factors.

Figure 2 shows a comparison between the agarose gel and CE-LIF techniques for determination of the antiapoptotic effects of BNP in serum-deprived PC12 cells. Apoptotic DNA fragmentation was measured 4.5 h after initiation of apoptosis by removal of the serum, with or without the addition of BNP at the time of serum removal, as in our previous study [50]. Each sample represented 5 million PC12 cells. Furthermore, each sample (at the cell lysis step) was determined to have the same amount of total DNA (measured by Picogreen™ quantitation) before beginning the phenol-chloroform extraction procedure (with ethanol precipitation) to partially purify the apoptotic DNA fragments for analysis on agarose gels or CE-LIF, as described in previous publications [50, 62]. Nine tenths of each of the fragmented DNA extracts were

used for the agarose gels and one tenth was used for the CE-LIF. The band density on the agarose gels or the peak area on the CE-LIF of the apoptotic DNA fragments represent the relative levels of apoptosis in each sample.

BNP had substantial protective effects, inhibiting the onset of apoptotic DNA fragmentation in the PC12 cells, but this effect was difficult to see in the agarose gel because of the poor sensitivity (5 million cells per lane were used!). In contrast, CE-LIF electropherograms, representing only one tenth of the DNA fragment extract, provided clear images of the onset of apoptotic DNA fragmentation induced by serum removal and of the protective effects of BNP. Using computer analysis to integrate the area under the 360- or 540-bp peak and determination of the levels of apoptotic DNA fragments using DNA standard curves, BNP at 1, 10 and 100 nM was found to inhibit apoptotic DNA fragmentation by 47.7, 98.4 and 91.5%, respectively. TUNEL labeling analysis gave similar levels of antiapoptotic effects [51].

Figure 3 illustrates the effects of the PKG activator 8-bromo-cGMP on apoptotic DNA fragmentation in serum-deprived PC12 cells, analyzed by both agarose gel electrophoresis and CE-LIF. The new CE-LIF technology showed that 8-bromo-cGMP at 0.1 and 1.0 mM inhibited apoptotic DNA fragmentation by 86.2 and 88.7%, respectively.

The new CE-LIF technology allows accurate and quantifiable measurements of entry into the apoptotic cell death pathway in neural cells. A clear advantage of using CE-LIF is that the resulting data can be analyzed by objective methods using modern statistical techniques. Furthermore, because of the exquisite sensitivity of the CE-LIF technology, which requires fewer than 1,000 cells for analysis of apoptotic DNA fragmentation, large numbers of treatment conditions and replications of these conditions can be tested with a total cell population considerably smaller than a million cells. Also, this technology allows, for the first time, accurate measurement of apoptotic DNA fragmentation in experiments using limited, small numbers of cells, such as primary cultures or extremely small punch biopsies.

Basal Levels of cGMP May Be Necessary for Preventing Entry into the Apoptotic Pathway in Unstressed Neural Cells

In 1988, Garthwaite and Garthwaite [63] showed that exposure of young rat cerebellar slices to agents known to inhibit sGC, such as N-methylhydroxylamine and meth-

ylene blue, caused progressive destruction of differentiating cells. The destructive effect could be reversed by coadministration of a cGMP analog. The data suggested that basal levels of cGMP may have protective effects in neural cells. However, methylene blue is known to have other actions, such as generation of oxygen free radicals, particularly superoxide anion [64–66], which could have contributed to the destruction of the neural cells by a mechanism independent of cGMP depletion. More recent studies using a more potent and selective inhibitor of sGC, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) [67], have shown that sGC inhibition decreases the survival of rat motor neurons cultured in the presence of BDNF [55], as well as rat dorsal root ganglion neurons maintained in culture [56].

Our own studies have shown that ODQ (40 μ M, 24 h), by itself, triggers the onset of apoptosis in two neural cell lines, NG108-15 [68, 69] and N1E-115 [69], as determined by DNA laddering on both agarose gels and the new CE-LIF technology. Both neural cell lines were cultured under normal growing conditions (i.e. in the presence of serum and without exposure to any other toxic substances). ODQ (40 μ M) completely blocked the cGMP synthesis stimulated by *S*-nitroso-N-acetylpenicillamine (up to 0.5 mM) in NG108-15 cells, illustrating that ODQ at 40 μ M can completely block sGC activity in this cell line. Furthermore, ODQ (40 μ M) substantially lowers the basal levels of cGMP (from basal levels of about 0.30 to about 0.04 pmol/million cells in NG108-15 cells). To test whether this reduction in basal cGMP levels was responsible for the induction of apoptosis, we determined if elevating cGMP levels with a pGC activator, i.e. ANP or BNP, could prevent ODQ-induced apoptosis. Both ANP and BNP provided nearly complete protection against the proapoptotic effects of ODQ, thus indicating that the lowering of basal cGMP levels was indeed responsible for the onset of apoptosis caused by ODQ. Addition of the direct PKG activator 8-bromo-cGMP also provided nearly complete protection against ODQ-induced apoptosis.

These data suggest that low, basal levels of cGMP and low-level activation of PKG may play an essential role in preventing the activation of a proapoptotic pathway in unstressed neural cells. Further experiments will be needed to determine the exact role of basal cGMP levels and PKG activity in the regulation of the onset of apoptosis in different types of neural cells.

Fig. 4. Proposed cellular model of antiapoptosis/prosurvival effects of APPS, ANP, BNP and NO in neurons. NPR-A represents the natriuretic peptide receptor type A, which is a transmembrane protein with pGC activity in its intracellular domain. Not all neurons have receptors for ANP or APPS and thus may not respond to these agents with neuroprotection. However, most and perhaps all neurons have sGC, the intracellular receptor for NO, and thus would be expected to show similar activation of the cGMP signaling pathway. A key question for future experiments is whether all neurons have PKG.

Proposed Cellular Model of the Antiapoptotic/Prosurvival Effects of APPS, ANP, BNP and Therapeutic and Endothelium-Derived NO in Neurons

Figure 4 illustrates our current cellular model of the antiapoptotic/prosurvival effects of cGMP-elevating agents, including APPS, ANP, BNP and NO in neurons. Because even low, basal levels or slightly elevated levels of cGMP can activate this neuroprotective mechanism, it seems likely that the low, physiological levels of NO surrounding central and peripheral neurons under normal physiological conditions may play an essential role in protecting the neurons. Although only a small percentage of neurons can synthesize their own NO (unless stimulated by cytokines or trauma), all neurons in the central and peripheral nervous systems are within a few microns of vascular endothelial cells, which continuously release low levels of NO, sufficient to activate sGC in nearby cells. Thus, under normal physiological conditions, endothe-

lium-derived NO may be an important antiapoptotic/prosurvival factor for neurons in the central and peripheral nervous systems.

Because aging and diabetes mellitus are known to lower NO production in endothelial cells and reduce the ability of NO to activate sGC in nearby cells [23], this neuroprotective effect of endothelium-derived NO may become impaired in diabetic patients and the elderly. Thus, some of the neurological problems associated with diabetes mellitus and aging may be related, in part, to the diminished neuroprotective effects of endothelium-derived NO.

Many endogenous vasodilatory factors, such as acetylcholine, arachidonic acid, ATP, bradykinin, histamine and increased shear stress (because of increased blood flow past the surface of the endothelium), as well as the neuropeptides CGRP, substance P and vasoactive intestinal peptide, are all known to trigger the release of NO from endothelial cells [12, 15]. For this reason, these endogenous vasodilatory factors may have neuroprotective effects via two pathways: (1) NO-induced vasodilation and increased blood flow to the neural tissues, thus providing better delivery of oxygen and nutrients, and (2) NO-induced inhibition of apoptosis and increased survival of neurons via the neural cGMP/PKG signaling pathway. Likewise, a number of therapeutic agents (e.g. isosorbide dinitrate, nitroglycerin and sodium nitropruside), as well as nutritional supplements and additives (e.g. sodium nitrite) and health food/herbal medicines (e.g. ginseng and *Ginkgo biloba*), are all known to cause vasodilations either by triggering the release of NO from endothelial cells or generating NO independent of the endothelium. These therapeutic, nutritional, herbal-medicinal agents would be predicted to have beneficial effects in the nervous systems, potentially inhibiting apoptosis via the neural cGMP/PKG pathway and thus prolonging the survival of the neural cells.

Neurotrophic factors, such as APPS in hippocampal neurons [49] and BDNF in motor neurons [57], have been shown to protect against neuronal cell death by elevating intracellular levels of cGMP and activating PKG. In the case of BDNF, this neurotrophic factor stimulated the gene expression of eNOS in motor neurons, providing a basal intracellular production of protective NO, which in turn elevated sGC activity, leading to cGMP elevations in these neurons. Further studies in this field are needed to determine if the protective effects of other neurotrophic factors involve, in part, activation of the neural cGMP/PKG pathway.

The protein substrates of PKG mediating the anti-apoptotic/prosurvival effects in neural cells are currently unknown and therefore are not shown in the model in figure 4. However, some clues concerning the nature of the PKG substrate(s) have recently emerged. For example, the laboratories of Drs. Young-Myeong Kim, Richard L. Simmons and Timothy R. Billiar have shown that NO and 8-bromo-cGMP inhibit the release of cytochrome c from the mitochondria as well as the activation of caspase-3 in serum-deprived PC12 cells [53]. Their data suggest that PKG may activate a key apoptosis-regulating protein(s) associated with the mitochondria. Interestingly, a very recent report has shown that cGMP, via activation of PKG, inhibits the mitochondrial permeability transition pore in isolated mitochondria of rat brain [70]. The authors suggested that cGMP/PKG blocks apoptosis in neural cells by preventing the reduction of mitochondrial membrane potential and the release of cytochrome c.

Longer-term protection against the onset of apoptosis by the cGMP/PKG signaling pathway may involve the activation of transcription factors and increased or decreased expression of genes. For example, both PKG I [71] and PKG II [40] are known to catalyze the phosphorylation of a peptide corresponding to the phosphorylation (and activation) site (i.e. serine 133) of CREB. Interestingly, CREB has been proposed to act as a survival factor in human melanoma cells [72]. In cerebellar granule neurons, phosphorylation of CREB increases the gene expression of the antiapoptotic/prosurvival protein BCL-2, a mitochondria-associated protein known to inhibit the release of cytochrome c [73]. In PC12 cells, the HIV-1 Tat protein, thought to be responsible for promoting dementia in HIV-infected patients, has been shown to inhibit expression of CREB and cause cell death [74]. Thus, CREB and phosphorylation of this important transcription factor appear to play key roles in promoting neural cell survival. The exact involvement of CREB and phosphorylation of CREB in the cGMP/PKG-mediated inhibition of apoptosis in neural cells needs to be determined.

Other Potential Downstream Targets of cGMP

Figure 5 illustrates four classes of potential downstream targets of cGMP that may be involved in the physiological/pharmacological/pathological regulation of cell function mediated by the cGMP signal transduction pathway. Two excellent reviews from the early 1990s by Dr.

Fig. 5. Four downstream target proteins, PKG, PDE2, PDE3 and CNG cation channels, in the cGMP signaling pathway.

Michael Goy [75] and Drs. Thomas Lincoln and Trudy Cornwell [7] summarized the early considerations regarding the potential cGMP target proteins, such as phosphodiesterases, particularly types II and III (PDE2 and PDE3), and cyclic nucleotide-gated (CNG) cation channels, in addition to the various forms of PKG (PKG Iα, PKG Iβ and PKG II).

Although PKG has been considered the major downstream target of cGMP in most physiological actions of cGMP in most cells [7–12, 35–40, 75], other important targets of cGMP are known to exist in certain cell populations. Most notable are the CNG cation channels known to mediate the cGMP signaling pathway in the rods and cones of the retina and in the olfactory neurons [34, 76–80]. Although originally thought to be localized to just the retinal and olfactory cells, recent studies have shown a more widespread distribution of CNG cation channels in the mammalian brain. For example, most neurons of the rat hippocampus have been found to express both the rod form and the olfactory form of the CNG cation channels [34], suggesting that they may play a role in hippocampal function, such as NO/cGMP-mediated LTP [76, 77]. CNG cation channels have also been found in the cerebellum, cortex and basal ganglia [77]. In general, CNG cation channels are thought to participate in the regulation of neural cell development and synaptic plasticity [77].

Compared to PKG, CNG cation channels have a relatively low affinity for binding cGMP, typically requiring cGMP levels 10–100 times higher than those needed to activate PKG [7]. Thus, it seems less likely that CNG cation channels would be activated by cGMP at basal or slightly elevated levels in most neurons. (The rods of the retina, which utilize cGMP and CNG cation channels in the phototransduction pathway, are an exception, having

extremely high basal cGMP levels of around 10–40 μ M, which maintain the continuous opening of these channels during the dark current.) The neuroprotective antiapoptotic/prosurvival effects of cGMP described above are thus unlikely to be mediated by activation of CNG cation channels. However, when cGMP levels are greatly elevated, the CNG cation channel, if present, may become activated, leading to increased influx of Na^+ and Ca^{2+} followed by membrane depolarization. Potentially, this could lead to abnormal Ca^{2+} accumulation and toxic reactions within the neural cells. This type of toxic response to elevated cGMP levels may explain the degeneration of photoreceptor cells in animal models of inherited retinal diseases (e.g. retinitis pigmentosa), in which abnormal metabolism of cGMP and exceptionally high levels of cGMP have been observed [81–83]. This toxic mechanism, involving the overactivation of CNG cation channels by high cGMP levels, has also been proposed to mediate the cell death of mouse hippocampal HT22 cells induced by glutamate exposure, mentioned above [59].

The properties and localization of PDE2, a cGMP-stimulated cAMP phosphodiesterase, and PDE3, a cGMP-inhibited cAMP phosphodiesterase, have been described in two excellent reviews by Drs. Joseph Beavo, Marco Conti and Richard Heaslip [84] and Drs. William Sonnenburg and Joseph Beavo [85]. Unlike the CNG cation channels, PDE2 and PDE3 have affinities for cGMP that are in the same range as affinities for cGMP binding to PKGs. For example, the K activation for stimulation of PDE2 by cGMP is 300 nM, and the IC_{50} for inhibition of PDE3 by cGMP is 100 nM [85], similar to the K activation of cGMP-induced activation of purified PKG [7] as well as PKG in freshly prepared crude homogenates of airway [8] and vascular [9–11] smooth muscle cells. Thus, basal or slightly elevated levels of cGMP within cells could potentially regulate the catalytic activities of PDE2 or PDE3 and thereby change the intracellular levels of cAMP, as shown in figure 5.

If present within neural cells, PDE2 and PDE3 may be effective downstream targets of cGMP. Although PDE2 has been found in mammalian brain, PDE3 is generally thought to be localized primarily in other types of tissue, such as adipose tissue, heart and smooth muscle cells of both the airways and vasculature [85]. In PC12 cells, PDE2 was found to be the predominate cAMP-metabolizing enzyme, and agents that elevated cGMP levels, such as ANP and NO, were found to increase the rate of cAMP metabolism [86]. Although this pathway may be responsible for some actions of cGMP in neural cells, it does not appear to be responsible for the antiapoptotic/prosurvival

effects of cGMP in PC12 cells [50]. Further studies will be needed to elucidate the role of PDE2 and PDE3 as downstream targets of cGMP in neural cells and the potential involvement in cGMP-mediated neuroprotection.

Pathophysiological Significance of the Antiapoptotic Effects of the NO/cGMP/PKG Pathway in the Nervous Systems

NO is thought to play a key role in the neuronal damage occurring during various neurological disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, HIV dementia, Parkinson's disease and stroke [23, 43, 87]. The major toxic effects of NO in neural cells appear to involve the combination of NO with superoxide anion to form peroxynitrite, a powerful oxidizing substance that can damage proteins, lipids and DNA within cells. Either apoptotic or necrotic cell death of neural cells can occur, depending on the level of oxidative stress caused by NO/peroxynitrite. Recently, it has been proposed that NO-induced cell death in neural cells may also be mediated by the ability of NO to activate the Ras-ERK pathway [88].

Because cGMP and PKG are known to mediate many of the physiological actions of NO [12, 15], most notably vasodilation in blood vessels and the inhibition of platelet activation, it has been speculated that cGMP/PKG may also participate in the neuronal damage caused by NO. Although cGMP/PKG has been reported to contribute to NO-induced toxicity in some cells, such as cardiac myocytes, pancreatic B cells and vascular endothelial and smooth muscle cells, most neural cells do not show toxicity when cGMP levels are elevated, at least within a normal physiological range (see the section 'NO Has Both Toxic and Prosurvival Actions in Various Mammalian Cells' above). Rather, neuroprotection (specifically, inhibition of the onset of apoptosis) occurs in many neural cells (e.g. PC12 cells, hippocampal neurons, dorsal root ganglion neurons, motor neurons, NG108-15 cells and sympathetic neurons) when cGMP levels are elevated or when PKG is directly activated [50–58, 63, 68, 69].

Future experiments will need to determine the precise role of the NO/cGMP pathway by using cells and animal models with specific deletions or overexpression of the genes for the key signaling proteins, i.e. the three isoforms of NO synthase [eNOS (NOS3), iNOS (NOS2) and nNOS (NOS1)], sGC, pGC and PKG, as well as the other potential downstream targets of cGMP (PDE2, PDE3 and CNG cation channels). Recently, our laboratory has characterized the biological actions of NO donors and neuro-

peptides (e.g. CGRP and vasoactive intestinal peptide) capable of releasing endothelium-derived NO, using the C57BL/6J mouse [89], a common wild-type control for genetically modified mice. Furthermore, we have shown that mice with deletion of either eNOS or iNOS (i.e. eNOS or iNOS knockout mice) have significant alterations in their NO-dependent responses to the neuropeptide vasoactive intestinal peptide [90], suggesting that these mice will provide useful models (as animal, tissue and primary cell culture models) in future experiments to study the involvement of endothelium-derived NO in the neuroprotection of neurons in the central and peripheral nervous systems.

Aging leads to a progressive reduction of the endothelial synthesis and biological actions of NO, resulting in an increased risk of developing hypertension and atherosclerosis, leading to coronary heart disease and stroke [23]. Aging also impairs vascular responses to important vasodilatory neuropeptides such as CGRP, partly because of the diminished NO synthesis/action in the elderly [91]. Because endothelium-derived NO may have direct neuroprotective effects on central and peripheral neurons, this reduction in endothelium-derived NO during aging may result in increased susceptibility to the onset of apoptosis in neural cells. This may contribute to neuronal damage during the development of age-related neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Currently, our laboratory is utilizing animal models of aging [91] to determine the involvement of diminished endothelium-derived NO in the increased susceptibility to neuronal apoptosis in the elderly.

Diabetes mellitus, partly because of sustained high glucose levels and excess production of oxygen free radicals in endothelial cells, accelerates the aging process in the cardiovascular system, resulting in damage to endothelial cells and dramatic reduction in the synthesis and biological actions of NO [23, 92]. This early impairment of endothelial NO in diabetic patients may contribute to increased susceptibility to damage in neurons that are normally protected by the NO/cGMP/PKG signaling pathway. This may be responsible, in part, for the polyneuropathies commonly observed in diabetic patients. In addition to using aging models, studies in our laboratory also utilize animal models of diabetes mellitus [92] to determine if this type of pathological damage to the endothelial NO system contributes to the damage to peripheral nerves, leading to the diabetic polyneuropathies.

Potential New Therapies for the Prevention and Treatment of Neural Cell Damage during Stroke and Neurodegenerative Diseases (Alzheimer's Disease, Amyotrophic Lateral Sclerosis, HIV Dementia and Parkinson's Disease)

If a clear connection can be established between endothelial damage/reduction in endothelium-derived NO during aging and diabetes mellitus and the concurrent damage to central and peripheral neurons, then it may be possible to develop new strategies for intervening in these damaging processes. The new information obtained from these studies may lead to the development of new therapeutic agents, nutritional supplements or health food/herbal medicines that specifically protect against endothelial damage and/or replace or supplement the lost NO.

The new knowledge about the cGMP signaling pathway and its potential role in preventing destruction and cell death in the brain and the peripheral nervous systems will undoubtedly provide other new avenues for intervening in the destructive processes occurring during stroke and neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, HIV dementia and Parkinson's disease. This new knowledge should facilitate the development of better, more efficacious therapies for the prevention and treatment of these diseases. Of special note is the recent development of ViagraTM for the treatment of erectile dysfunction [23]. Viagra was developed on the basis of the knowledge obtained during the early experiments with NO (EDRF), cGMP, PKG and cGMP-metabolizing enzymes in the vascular system [1–4, 7, 9–15, 84, 85]. Viagra works by inhibiting the type V (cGMP-metabolizing) phosphodiesterase in smooth muscle cells of penile arteries and corpus cavernosum penis, thus potentiating the ability of NO to cause accumulation of cGMP, activation of PKG and relaxation of the smooth muscle cells, resulting in penile erection [23]. Viagra is especially effective in those individuals who have diminished production of NO (e.g. diabetic patients and elderly men).

Future new developments for the prevention and therapy of neural cell damage during stroke and neurodegenerative diseases may take a similar route to the development of Viagra. Agents that enhance the accumulation of cGMP and/or the activation of PKG in neural cells of the brain and peripheral nerves may be especially useful in protecting against damage from stroke and neurodegenerative diseases. However, these new neuroprotective agents would need to have selectivity for the neural cells that are at risk of damage, because enhancement of the

cGMP/PKG pathway in other cells, such as vascular smooth muscle cells in the arterial system, could lead to unwanted side effects (e.g. systemic vasodilation and uncontrolled drop in the arterial blood pressure). Along this same line of reasoning, it could be predicted that some of the currently used therapeutic agents, such as Viagra and the antihypertensive drugs, particularly those that generate NO and/or enhance the cGMP signaling pathway, may have beneficial (antiapoptotic/prosurvival) effects on neural cells of the central and peripheral nervous systems. Future experiments along these lines as well as a careful reexamination of previous animal studies and clinical

trials may help to reveal the potential beneficial effects of these agents on the protection of neural cells in the brain and the peripheral nerves.

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