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cAMP-responsive element binding protein mediates a cGMP/protein kinase G-dependent anti-apoptotic signal induced by nitric oxide in retinal neuro-glial progenitor cells.

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Abstract

Nitric oxide (NO) is cytoprotective to certain types of neuronal cells. The neuroprotective ability of NO in the retina was reportedly mediated by the cyclic GMP (cGMP) to protein kinase G (PKG) pathway. Cyclic AMP-responsive element binding protein (CREB) plays an essential role in the NO/cGMP/PKG-mediated survival of rat cerebellar granule cells. We tested whether CREB transduces the NO/cGMP/PKG anti-apoptotic cascade in R28 neuro-glial progenitor cells. Apoptosis was induced in R28 cells by serum deprivation for 24h. Varying concentrations of two NO donors, sodium nitroprusside (SNP) and nipradilol, were added to medium with or without an NO scavenger, a soluble guanylyl cyclase inhibitor, or a PKG inhibitor. The cells were immunostained against activated caspase-3 and counterstained with Hoechst 33258. Apoptosis was quantified by counting activated caspase-3 positive or pyknotic cells. SNP and nipradilol rescued R28 cells from apoptosis in a dose-dependent manner, at an optimal concentration of 1.0 μ M and 10 μ M, respectively. Higher concentrations were cytotoxic. The NO scavenger and the inhibitors decreased the anti-apoptotic effect of the NO donors. Intracellular cGMP levels were increased after exposure to SNP and nipradilol. Western blotting showed that both NO donors increased CREB phosphorylation, which was blocked when

pre-exposed to the inhibitors. Transfection with the dominant negative CREB construct defective of phosphorylation at Ser-133 interfered with the anti-apoptotic activity of SNP. These results indicate that CREB at least in part mediates the cGMP/PKG-dependent anti-apoptotic signal induced by NO in R28 cells.

Keywords

nitric oxide, CREB, retinal progenitor cells, cytoprotection, NO/cGMP/PKG pathway, nipradilol

1. Introduction

Nitric oxide (NO) is implicated in both the pro- and anti-apoptotic signaling pathway depending on cell types and conditions. NO at high concentrations induces neuronal cell death by forming cytotoxic peroxynitrite during ischemic injury or neurodegenerative diseases (Beckman and Koppenol, 1996; Dawson and Dawson, 1998; Wink and Mitchell, 1998; Boje., 2004; Kang et al., 2004; Keynes and Garthwaite, 2004; Duncan and Heales, 2005). At relatively low concentrations, NO protects neuronal cells, such as PC12 rat pheochromocytoma cells, SK-N-BE human neuroblastoma cells, and rat sympathetic neurons (Farinelli et al., 1996; Kim et al., 1999; Ciani et al., 2002a), from apoptosis. This neuroprotective ability of NO is mediated at least in part by a cyclic GMP (cGMP)/protein kinase G (PKG) pathway (Farinelli et al., 1996; Kim et al., 1997, 1999; Fiscus, 2002). NO activates soluble guanylyl cyclase (sGC), an enzyme that catalyzes synthesis of cGMP from GTP. Elevated cGMP levels then activates PKG, which eventually exerts the anti-apoptotic ability through still unidentified mechanisms.

Cyclic AMP-responsive element binding protein (CREB) is a transcription factor that plays an important role in development, memory function, plasticity, and survival of the nervous system (Finkbeiner et al., 1997; Lonze and Ginty, 2002; Lee et al., 2004). Upon

phosphorylation at Ser-133, which is located within a kinase-inducible domain, CREB forms a stable complex with the CREB binding protein. The formation of this complex facilitates the transcriptional ability of CREB, which regulates expression of a variety of genes. CREB has also been implicated in the anti-apoptotic NO signaling in SK-N-BE human neuroblastoma cells (Ciani et al., 2002a) and rat cerebellar granule cells (Ciani et al., 2002b). However, involvement of CREB in the NO-induced retinal neuroprotection through the cGMP/PKG pathway is not known.

Nipradilol (3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran) is an α 1- and β -adrenoceptor antagonist (Uchida et al., 1983; Ohira et al., 1985) containing an NO moiety (Okamura et al., 1996; Hayashi et al., 1997). Although nipradilol was originally developed as a systemic hypotensive agent (Aramaki et al., 1992; Haneda et al., 1995), it has recently been used locally, as eyedrops, to reduce intraocular pressure by suppressing aqueous humor production at the ciliary epithelium and facilitating increased uveoscleral outflow via the adrenoceptor antagonistic properties. In addition, nipradilol induces vasodilation and acts as a neuroprotectant, which are attributed to its ability to donate NO.

Nipradilol improves the survival rate of purified retinal ganglion cells (RGCs) in culture (Kashiwagi et al., 2002), and protects rat RGCs from N-methyl-D-aspartate (NMDA)-, ischemic-, or axotomy-induced damage (Mizuno et al., 2001; Nakazawa et al., 2002; Taniai

et al., 2002). Recent reports demonstrated that nipradilol rescued PC12 cells from serum starvation-induced apoptosis by caspase-3 inhibition through the NO/cGMP/PKG pathway (Tomita et al., 2002) and activated the apoptosis-related nuclear factor kappa B (NF κ B) in these cells (Ando et al., 2005). However, the precise mechanism of nipradilol-mediated neuroprotection in retinal cells was not elucidated.

The purpose of this study was to examine the protective ability of the NO donors sodium nitroprusside (SNP) and nipradilol in R28 cells, which are rat retinal glial and neuronal progenitor cells immortalized with the transfection of the adenovirus 12S E1A. The R28 cell line is a useful model for testing the cytoprotective ability of various substances and drugs (Seigel, 1996; Tezel and Wax, 1999; Nakamura et al., 2001; Nakanishi et al., 2006; Mukuno et al., 2004). Here we show that SNP and nipradilol rescue R28 cells from serum starvation-induced apoptosis, at least in part, via the cGMP/PKG pathway. We further demonstrate that CREB mediates the anti-apoptotic ability of NO in these cells.

2. Materials and methods

2.1. Reagents

Nipradilol and denitronipradilol

(3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-hydroxy-2H-1-benzopyran) were

kindly provided by Kowa Co, Ltd. (Nagoya, Japan). These substances were dissolved in 0.1

N hydrochloric acid and further diluted with phosphate buffer to concentrations between

1×10^{-7} and 10^{-3} M. SNP (Sigma; St. Louis, MO) was dissolved in Dulbecco's modified

Eagle's medium (DMEM) to the same concentrations as nipradilol.

1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) (Sigma) and KT5823 (Calbiochem; San

Diego, CA, USA) were dissolved in dimethyl sulfoxide (DMSO).

2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (c-PTIO) (Dojindo, Tokyo, Japan)

was dissolved in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

N²,2'-O-Dibutyrylguanosine 3', 5'-cyclic monophosphate sodium salt hydrate

(dibutyryl-cGMP) (Sigma) was diluted in distilled water. The rabbit polyclonal antibodies

against CREB and phospho CREB (Ser133) were acquired from Cell Signaling Technology

(Beverly, MA, USA). LipofectamineTM 2000 was purchased from Invitrogen (San Diego, CA,

USA). pCMV-CREB and pCMV-CREB133 vectors and pZsGreen1-N1 reporter vector were

from BD Biosciences (Palo Alto, CA, USA).

2.2. Cell culture

R28 cells were seeded on glass coverslips at a density of 2×10^5 /cm² and grown to 70% confluency in cAMP-free DMEM containing 5 mM glucose supplemented with 10% newborn bovine serum (Life Technologies, Inc. Rockville, MD, USA), as previously described (Seigel, 1996, Seigel et al., 2000; Tezel and Wax, 1999, 2000; Barber et al., 2001; Nakamura et al., 2001; Reber et al., 2002; Mukuno et al., 2004). At the start of each experiment, the cells were rinsed in phosphate buffered saline (PBS) and then incubated for 24 h in fresh serum-fed medium or serum-deprived medium with or without various concentrations of SNP, nipradilol, denitronipradilol, or 100 μ M dibutyl-cGMP. In a different series of experiments, 1.0 μ M c-PTIO, an NO scavenger, 20 μ M ODQ, an sGC inhibitor, 0.2 μ M KT5823, a PKG inhibitor, and their vehicles, DMSO or HEPES, was added to the cell cultures 10 min prior to NO donor treatment.

2.3. Apoptosis quantification

The cells were fixed in 1% paraformaldehyde and then subjected to immunocytochemistry against activated caspase-3 in combination with Hoechst nuclear staining, as previously described (Barber et al., 2001; Nakamura et al., 2001; Mukuno et al., 2004; Nakanishi et al.,

2006). In brief, the cells were blocked in PBS containing 0.1% Triton (PBST) and 10% goat serum (block solution) at room temperature for 1 h, then incubated in block solution with a rabbit polyclonal antibody against activated caspase-3 (CM-1, 1:1000, Idun Pharmaceuticals, La Jolla, CA) at room temperature for 1 h. The cells were washed three times with PBST and incubated in block solution with tetramethyl rhodamine-conjugated goat anti-rabbit IgG (1:2000, Jackson Immunologicals, West Grove, PA, USA) at room temperature for 1 h. During the secondary antibody incubation, the cells were simultaneously counterstained with bisbenzimidazole (Hoechst dye 33258, 0.5 µg/ml, Sigma). Immunostaining with exclusion of the primary antibody was done as a negative control. The above experiments were done in triplicate. Cells were viewed using X 40 objective of an Olympus PROVIS fluorescence microscope mounted with a SONY 3CCD video camera attached to a SONY personal computer running an image analysis software (Viewfinder Lite, Japan). Five visual fields were randomly sampled from each coverslip, and all the cells stained with Hoechst dye in each field were counted manually. The total number of pyknotic cells with condensed or fragmented nuclei was calculated in the five sampled regions and expressed as percentage of apoptosis per coverslip = (total number of pyknotic cells/total number of cells) × 100 (Barber et al., 2001; Nakamura et al., 2001; Mukuno et al., 2004). Similarly, the percentage of CM-1 immunoreactive cells per coverslip was calculated.

2.4. Quantification of cGMP

R28 cells were grown to 80% confluence. The initial culture medium was replaced with serum-free medium. One μM SNP or 10 μM nipradilol was added to the medium and the cells were incubated for an additional 5, 10, 20, and 30 min. The cells were lysed in 2.2 ml 0.1N hydrochloric acid. Fifty μl aliquots of the cell lysates were transferred into 96-well plates and subjected to enzyme immunoassay (EIA) for intracellular cGMP quantification using a colorimetric cGMP EIA Kit (Cayman Chemical Company, Ann Harbor, MI), according to the manufacturer's instructions. Optical absorbance in each well was measured on a microplate reader (Bio-Rad Laboratories, CA, USA) at a wavelength of 415 nm. The intracellular cGMP concentrations at specific time points after SNP or nipradilol exposure were normalized to the average control value without SNP or nipradilol treatment. The experiment was done in triplicate and repeated for three times.

2.5. Western Immunoblotting

The cells were harvested in the following lysis buffer: 10 mM HEPES, 42 mM KCl, 5 mM

MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM benzamidine, 1% Triton, and 1 protease inhibitor tablet/10 ml (Complete Mini, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany), as previously described (Barber et al., 2001; Nakamura et al., 2001; Nakanishi et al., 2006). After centrifugation at 15,000 rpm for 10 min, the supernatant was collected and protein concentrations were calculated using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories). Fifty µg of protein from each sample was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Following 1 h blocking with 5% non-fat milk in wash buffer (10 mM Tris-Cl [pH 7.2] containing 100 mM NaCl and 0.1% Tween 20; TBST), the membranes were incubated for 1 h at room temperature with primary antibodies against CREB and phospho CREB (Ser133), each diluted at 1:1000. After washing, the membranes were incubated in 1:4000 horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Protein bands were visualized with enhanced chemiluminescence reagents (ECL plus; Amersham, Arlington Heights, IL, USA) and exposed to instant films using an ECL-mini-camera (Amersham). Relative intensities of bands were quantified by densitometric analysis using the NIH image software. Phosphorylation of protein was calculated as the ratio of the level of expression of phospho relative to total CREB content.

2.6. Transfections

R28 cells were seeded on glass coverslips and grown to 80% confluency. Following replacement of the initial culture medium with one without antibiotics, the cells were co-transfected with either 1.2 µg pCMV-CREB or pCMV-CREB133 and 0.4 µg pZsGreen1-N1 per well in 12-well plates using Lipofectamine™ 2000, according to the manufacturer's recommendations. pCMV-CREB is a parental vector with a cytomegalovirus promoter, which expresses the human wild-type (WT) CREB protein and used as a control, whereas pCMV-CREB133 is a vector containing the CREB133 (CR133) construct, a mutant CREB defective of phosphorylation at Ser-133 (Amorino et al., 2003). pZsGreen1-N1 is a reporter plasmid expressing ZsGreen1 *Zoanthus* sp. fluorescent protein (Matz et al., 1999). After a 24 h incubation period, the cells were deprived of serum and incubated for an additional 24 h with or without 1.0 µM SNP, followed by CM-1 immunohistochemistry, as in the previous experiments. Cells were viewed using X 20 objective of a fluorescence microscope (Keyence, Tokyo). Five visual fields under fluorescence microscope were randomly sampled from each coverslip, and the total number of green-fluorescent cells, which represented the successfully transfected cells, and the number of CM-1

immunoreactive cells in each field was counted. The ratio of cells co-expressing ZsGreen and CM-1 relative to those expressing ZsGreen fluorescence was calculated. These experiments were done in triplicate and repeated twice.

2.7. Statistic analyses

ANOVA with Bonferroni/Dunn test (StatView Software, SAS Institute Inc.) was used for statistical comparisons. A *P*-value of < 0.05 was considered significant.

3. Results

3.1. Dose-dependent anti-apoptotic effects of SNP and nipradilol in R28 cells

To test whether NO exerts an anti-apoptotic effect in R28 cells, they were deprived of serum for 24 h with or without various concentrations of SNP or nipradilol. Hoechst nuclear staining and activated caspase-3 immunostaining confirmed that 24 h serum deprivation induced apoptosis in R28 cells, as previously reported (Seigel, 1996; Tezel and Wax, 1999; Nakamura et al., 2001; Mukuno et al., 2004) (Figs. 1B and 2A; *P* < 0.001).

The effect of SNP treatment on R28 cells is dose-dependent, where it is cytoprotective at concentrations up to 1.0 μ M, then cytotoxic at higher concentrations (Figs. 1C and 2A; $P < 0.02$). At 1000 μ M, SNP significantly increased the number of apoptotic cells (Figs. 2A; $P < 0.0001$). The effect of nipradilol treatment was also dose-dependent, preventing apoptosis at an optimal concentration of 10 μ M and promoting apoptosis at higher concentrations (Figs. 1D and 2B). Treating R28 cells with hydrochloric acid, the vehicle used for nipradilol, did not have a significant effect on R28 cell survival (data not shown).

To evaluate whether NO donation mediates the cytoprotective effect of SNP and nipradilol, two experiments were performed. First, various concentrations of denitronipradilol, a nipradilol metabolite without the nitroso moiety, were used to treat R28 cells, as in the above experiments. As shown in Fig. 2C, the percentage of apoptotic R28 cells was not significantly changed with exposure to 0.1 to 100 μ M denitronipradilol. Secondly, to evaluate the effect of scavenging NO, 1.0 μ M c-PTIO was added to the serum-free medium 10 min before treatment with 1.0 μ M SNP or 10 μ M nipradilol. Hoechst staining and activated caspase-3 immunostaining were performed, as above. c-PTIO significantly blocked the cytoprotective effect of SNP ($P < 0.001$; Figs. 1E and 3A). The addition of c-PTIO or its vehicle alone did not enhance the R28 cell death ($P = 0.87$ and $P = 0.56$, respectively; Fig. 3A). The cytoprotective effect of nipradilol was also blocked by c-PTIO ($P < 0.001$; data not

shown). These results indicate that NO donation is essential for the SNP- and nipradilol-induced cytoprotection of R28 cells.

3.2 Cytoprotection of R28 cells by NO donors via the sGC/cGMP/PKG cascade

Inhibitors to sGC, cGMP, and PKG were used to determine whether the NO-induced cytoprotective effects of SNP and nipradilol are mediated by this pathway. Twenty μM ODQ, an sGC inhibitor, or 0.2 μM KT5823, a PKG inhibitor, was added to the serum-free media 10 min prior to treatment with 1.0 μM SNP or 10 μM nipradilol.

ODQ significantly attenuated the cytoprotective effect of SNP ($P < 0.02$), whereas ODQ alone did not change the number of apoptotic cells ($P = 0.74$; Figs. 1F and 3B). Similarly, the cytoprotection afforded by SNP was negated by KT5823 ($P < 0.001$), whereas KT5823 alone had no influence on R28 cell viability ($P = 0.33$; Figs. 1G and 3C). The cytoprotective effect of nipradilol was also reduced by ODQ and KT5823 treatment ($P < 0.001$ and $P < 0.02$, respectively; data not shown).

Dibutyl- cGMP , a membrane-permeable cGMP analog, was used to evaluate whether cGMP enhances R28 cell survival. As shown in Fig. 1H, addition of 100 μM dibutyl- cGMP into the serum-free media significantly reduced the pyknotic as well as the activated

caspase-3 immunoreactive R28 cells ($P < 0.001$; data not shown). These results demonstrated that the sGC/cGMP/PKG cascade is involved in the NO-induced cytoprotection of R28 cells.

3.3. Elevation of cGMP levels in R28 cells induced by SNP and npradilol

To confirm if SNP or npradilol increases cGMP levels in R28 cells, cytoplasmic cGMP concentrations were quantified. Following a 3 h incubation in serum-free medium, R28 cells were treated with 1.0 μ M SNP or 10 μ M npradilol for specific time periods then lysed. Whole cell lysates were subjected to ELISA. cGMP levels were transiently, but significantly, elevated 5 min after addition of SNP (Fig. 4A; $P < 0.01$) and 20 min after addition of npradilol (Fig. 4B; $P = 0.036$).

3.4. NO/cGMP/PKG-dependent CREB phosphorylation in R28 cells induced by SNP and npradilol

A previous study demonstrated that CREB was essential in the cGMP/PKG-mediated anti-apoptotic activity of NO in rat cerebellar granule cells (Ciani et al., 2002b). To evaluate

the involvement of CREB in the NO signaling pathway in R28 cells, CREB phosphorylation was quantified after treatment with SNP or nipradilol. Culture medium was replaced by serum-free medium 3 h before cell harvest. Cells were incubated with 1.0 μ M SNP or 10 μ M nipradilol for the indicated time periods, and 50 μ g portions of whole cell lysates were electrophoresed and probed for total and phospho CREB (Ser-133). The ratio of phospho relative to total CREB content was densitometrically quantified. CREB phosphorylation was significantly increased 10 min after the addition of SNP (Fig. 5A; $P = 0.024$) and 30 min after the addition of nipradilol (Fig. 5B; $P = 0.013$). This demonstrates that SNP and nipradilol activates CREB in R28 cells.

To test whether SNP- and nipradilol-stimulated CREB phosphorylation is dependent on the NO/sGC/PKG pathway, phosphorylation of CREB was quantified by western blotting after denitronipradilol. As shown in Fig. 6A, denitronipradilol had no significant effect on CREB phosphorylation at any time period ($P = 0.638$).

In a different series of experiments, 20 μ M ODQ or 0.2 μ M KT5823 was added to the R28 cell culture 10 min prior to 1.0 μ M SNP treatment for 10 min. Both ODQ and KT5823 significantly attenuated the effect of SNP on CREB phosphorylation (Fig. 6B; $P=0.023$ and 0.009, respectively). These results indicate that CREB phosphorylation induced by the NO in the R28 cells was sGC/cGMP/PKG-dependent.

3.5. CREB phosphorylation as an essential event in the anti-apoptotic signaling cascade of SNP in R28 cells

A dominant negative inhibition experiment was performed to confirm the involvement of CREB phosphorylation in the NO-induced anti-apoptotic effect. R28 cells were grown for 24 h on glass coverslips, which were placed onto 12-well plates, then co-transfected with pZsGreen1-N1 reporter vector (0.4 µg/ml medium) and pCMV-CREB (1.2 µg/ml medium) or pCMV-CREB133 (1.2 µg/ml medium) 24 h prior to SNP treatment. The cells were then incubated for an additional 24 h in serum-free medium with or without 1.0 µM SNP, followed by Hoechst staining and activated caspase-3 immunostaining, as described above. About 20% of the cells were successfully transfected, irrespective of the vector pairs, which were calculated by the ratio of green fluorescent cells over blue Hoechst-stained nuclei.

Approximately 17% of the cells transfected with WT-CREB were immunoreactive to activated caspase 3 in the absence of SNP, which was significantly reduced to 7% in the presence of SNP ($P < 0.0001$; Fig. 7). In contrast, approximately 13% of the cells transfected with the CREB 133 mutant were immunoreactive to activated caspase 3, regardless of SNP treatment ($P = 0.689$; Fig.7). Thus, CREB phosphorylation at the Ser-133

residue is a prerequisite for the anti-apoptotic activity of SNP in R28 cells. The results from the dominant negative inhibition and the quantification of CREB phosphorylation demonstrate that CREB is the downstream target of the NO/cGMP/PKG anti-apoptotic signaling pathway in R28 cells.

4. Discussion

The present study clearly demonstrated that relatively low concentrations of SNP and nipradilol protect R28 cells from serum deprivation-induced apoptosis, and that this cytoprotection is mediated, at least in part, by CREB phosphorylation through the sGC/cGMP/PKG pathway. Since the cytoprotective ability was abolished by an NO scavenger and by removal of the NO moiety from a nipradilol molecule, it is evident that this intracellular anti-apoptotic cascade was triggered by NO donation (Fig. 8).

On the other hand, the NO donors induced apoptosis at concentrations two to three decimal-orders higher than that optimal for cytoprotection. This concentration-dependent dual activity of NO donors in terms of cell survival and death verifies previous reports. As mentioned earlier, excess amounts of NO molecules react with superoxide anions generating highly reactive peroxynitrite molecules that damage the lipid cellular membrane

and eventually lead to apoptotic and necrotic cell death (Beckman and Koppenol, 1996; Dawson and Dawson, 1998; Wink and Mitchell, 1998; Fiscus, 2002; Boje, 2004; Kang et al., 2004; Keynes and Garthwaite, 2004; Duncan and Heales, 2005; Taguchi et al., 2006). In addition, excess NO is associated with cytotoxicity by inhibiting mitochondrial energy production (Wink and Mitchell, 1998). Thus, whether NO induces neuroprotection or neurotoxicity depends largely on the cell type and on the intracellular and extracellular milieu (Beckman and Koppenol, 1996; Wink and Mitchell, 1998; Fiscus et al., 2002; Keynes and Garthwaite, 2004).

Although the elevation of intracellular cGMP and phosphorylation of CREB induced by SNP and nipradilol were transient, the rescue effect was prolonged. This is not surprising since once the intracellular signaling cascade is initiated it is considerably amplified. Furthermore, CREB is a transcription factor that can regulate the expression of a number of genes. Thus, a transient increase in intracellular cGMP levels is sufficient for initiating the anti-apoptotic cascade, which leads to a substantial degree of cell survival. Our results showed that the increase in intracellular cGMP and phosphorylation of CREB was induced earlier with SNP than with nipradilol. This could be explained by the fact that SNP releases NO rapidly and spontaneously, whereas an intracellular enzymatic catalytic step is required by nipradilol before releasing NO.

Caspase-3, a key mediator of apoptosis in mammalian cells, is cleaved by caspase-8 or caspase-9 for activation. Cytochrome c released from the mitochondria forms the apoptosome, which activates caspase-9. Bcl-2 and phosphorylated Bcl-associated death promoter prevents this caspase-cascade by inhibiting the Bax-regulated cytochrome c release from the mitochondria. NO is known to enhance cellular survival by regulating this caspase-Bcl family interaction at several points. NO can directly inactivate caspase-3 by S-nitrosylation at cysteine residues thus preventing apoptosis of cerebrocortical neurons and hepatocytes (Kim et al., 1997; Tenneti et al., 1997). In addition, NO-stimulated cGMP production inhibits apoptosis by preventing cytochrome c release, increasing Bcl-2 expression, and decreasing Bax expression in PC12 cells (Ando et al., 2005), cerebellar granule cells (Ciani et al., 2002b), and dorsal root ganglion cells (Thippeswamy et al., 2001), respectively. Although the present study documented that reduced caspase-3 activation by SNP and nifedipine was cGMP/PKG-dependent in R28 cells, it does not entirely exclude direct inhibition of caspase-3 activity by NO.

This study showed for the first time that CREB phosphorylation is a crucial step in the NO-mediated cGMP/PKG-dependent anti-apoptotic pathway in retinal neuro-glial progenitor cells. Previous studies demonstrated that expression of dominant negative forms of CREB interferes with pro-survival signaling of growth factors (Riccio et al., 1999) and that CREB

null mice exhibited extensive apoptotic death of dorsal root ganglion sensory neurons and sympathetic neurons (Lonze et al., 2002). Thus, our study provides additional evidence that CREB is a convergent point in the neuronal survival-signaling pathway involving various stimuli including NO. Several reports demonstrated that CREB phosphorylation was induced in the RGC layer after exposure to constant light, Ca⁺-channel activation, and NMDA stimulation (Yoshida et al., 1995, 1998; Isenoumi et al., 2004). The enhanced phosphorylation of CREB may be a form of self-defense neuroprotective mechanism employed by the RGCs. However, the overall effect of the intrinsic CREB-dependent neuroprotective mechanism may be too small for the RGCs to overcome insults, particularly in instances of prolonged and repeated exposure. Augmenting this neuroprotective mechanism is therefore necessary and pharmaceutical intervention provides a promising option. These drugs may therefore cease or considerably retard the progression of neurodegenerative disorders, such as glaucomatous optic neuropathy and diabetic retinopathy. Both retinal diseases are known to share common pathologies, which include altered glial function, microglial activation, impaired glutamate metabolism, and the eventual increase in RGC apoptosis (Quigley et al., 1995; Kerrigan et al., 1997; Dkhissi et al., 1999; Tatton et al., 2001; McKinnon et al., 2002; Barber, 2003; Kanamori et al., 2004).

There is accumulating evidence that nipradilol not only reduces intraocular pressure via its

adrenoceptor antagonistic property but also promotes RGC survival *in vitro* and *in vivo* via the NO donation as mentioned earlier (Mizuno et al., 2001; Kashiwagi et al., 2002; Nakazawa et al., 2002; Taniai et al., 2002). The present study demonstrated the ability of nipradilol to activate CREB in retinal neuro-glial progenitor cells. This may serve as an important base for further evaluation of the role of nipradilol as a potential retinal neuroprotectant *in vivo*. It was reported that in monkeys, pharmacologically effective concentrations of topically instilled radiolabeled-nipradilol reaches the retina via the periocular route (Mizuno et al. 2001, 2002). Further studies for a more effective method of drug delivery to the retina and optic nerve head, particularly of nipradilol and other NO donors, are therefore necessary.

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Figure legends

Fig. 1. Representative pictures of immunostained R28 cell cultures. R28 cells were serum-fed (A) or serum-deprived (B to H) for 24 h in the presence or absence of 1.0 μ M SNP (C, E, F, G), 10 μ M nipradilol (D), or 100 μ M dibutyl-*c*-GMP (H). The cells were subjected to immunofluorescence against activated caspase-3 with the polyclonal antibody CM-1 (red), counterstained with Hoechst 33258 for nuclear morphology (blue). In the serum-fed condition, CM-1 immunoreactive or pyknotic cells were not detected (A). A significant number of CM-1 immunoreactive cells (arrow), as well as pyknotic cells (arrowhead), were detected in the serum-deprived cultures (B). The number of CM-1 positive and pyknotic cells was decreased in the SNP and nipradilol-treated cultures (C and D). Addition of the NO scavenger *c*-PTIO (E), the sGC inhibitor ODQ (F), and the PKG inhibitor KT5823 (G) to SNP-treated cells (E, F, G) all showed an increase in the number of CM-1 immunoreactive and pyknotic cells. Dibutyl-*c*-GMP decreased the number of CM-1 immunoreactive and pyknotic cells, similar to the SNP and nipradilol-treated cells (H).

Fig. 2. The anti-apoptotic effect of SNP and nipradilol via NO donation is dose-dependent.

Twenty-four hour serum-deprived R28 cell cultures were treated with increasing concentrations of SNP, nipradilol, or denitronipradilol. Cells were immunoreacted with the CM-1 antibody and counterstained with Hoechst 33258. The percentages of CM-1 positive and pyknotic cells per coverslip were calculated. Data represent the mean \pm SD of five randomly sampled visual fields in 3 coverslips. (A) SNP significantly reduced the percentage of apoptotic R28 cells at concentrations of 0.1 to 1.0 μ M. However, this is reversed at higher concentrations, and at 1000 μ M exceeds the serum free baseline. (B) Nipradilol significantly reduces the percentage of apoptotic R28 cells at concentrations of 0.1 to 10 μ M. The anti-apoptotic effect is lost at concentrations greater than 10 μ M. (C) Treatment with denitronipradilol does not affect the percentage of apoptotic R28 cells. *, $P < 0.001$; **, $P < 0.02$. The statistical differences presented were for the comparisons of the pyknotic cell ratio. Identical statistical values were used for the comparisons of the CM-1 immunoreactive cell ratios. The descriptions, however, were omitted for clarity.

Fig. 3. The cytoprotective ability of SNP was mediated by the NO/sGC/cGMP/PKG pathway.

R28 cell treatment with 1.0 μ M of the NO scavenger c-PTIO (A), 20 μ M of the soluble guanylyl cyclase inhibitor ODQ (B), or 0.2 μ M of the protein kinase G inhibitor KT5823 (C), 10 minutes before the addition of 1.0 μ M SNP, all resulted in blocking the cytoprotective

ability of SNP. The percentage of apoptotic R28 cells with addition of these agents, or their vehicles alone is comparable with the serum-deprived condition. *, $P < 0.001$; **, $P < 0.01$, ***, $P = 0.037$.

Fig. 4. SNP and nipradilol elevate intracellular cGMP levels in R28 cells. Cells were incubated in serum-free medium with or without 1.0 μM SNP or 10 μM nipradilol for the indicated periods and intracellular cGMP concentrations were measured by ELISA. Intracellular cGMP levels increased significantly 5 min after exposure to SNP (A) and 20 min after exposure to nipradilol (B). Bars are mean \pm SD values from three replications. *, $P < 0.01$; **, $P = 0.036$ as compared with the serum-deprived conditions.

Fig. 5. SNP and nipradilol increase CREB phosphorylation in R28 cells. Fifty μg lysates of R28 cells treated with 1.0 μM SNP or 10 μM nipradilol at different time periods were electrophoresed and probed for total CREB or phospho CREB (Ser-133). CREB phosphorylation ratios relative to total CREB content were quantified. CREB phosphorylation is increased by SNP 10 minutes after treatment (A) and by nipradilol 30 minutes after treatment (B). Bars are mean \pm SD values from four replications. *, $P = 0.024$; **, $P = 0.013$.

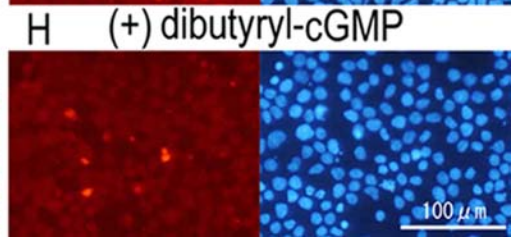
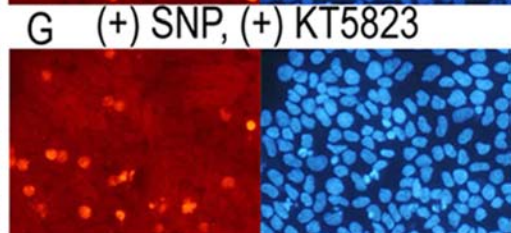
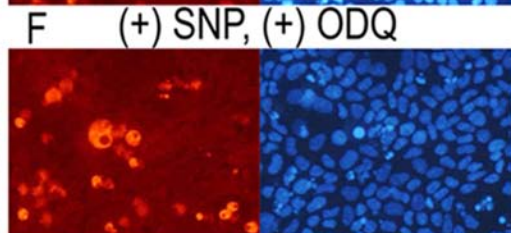
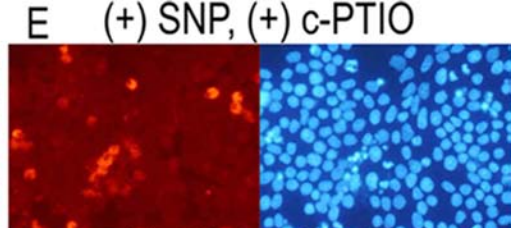
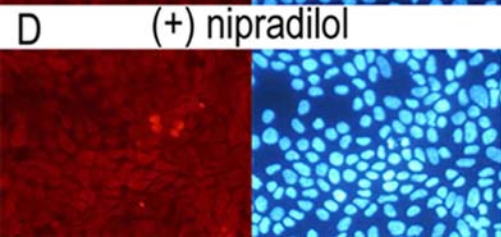
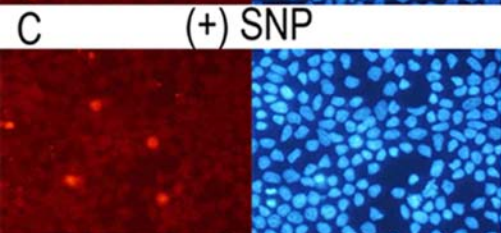
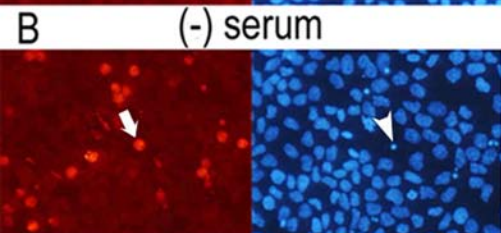
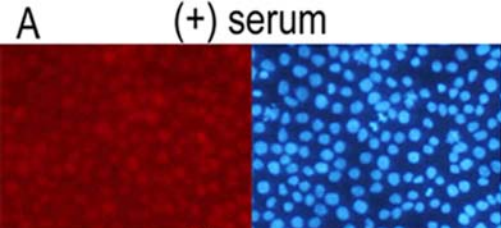
Fig. 6. SNP and nipradilol-stimulated CREB phosphorylation is dependent on the NO/cGMP/PKG pathway. R28 cells were treated with denitronipradilol (A) or with 1.0 μ M SNP in the presence or absence of 20 μ M ODQ or 0.2 μ M KT5823. Quantification of CREB phosphorylation was performed as in the Fig.5. Denitronipradilol, a nipradilol metabolite lacking in the NO moiety, does not affect CREB phosphorylation at any incubation time period tested (A). ODQ and KT5823 block the CREB phosphorylation induced by 10-min SNP treatment (B). Bars are mean \pm SD values from three replications. *, $P = 0.023$; **, $P = 0.009$.

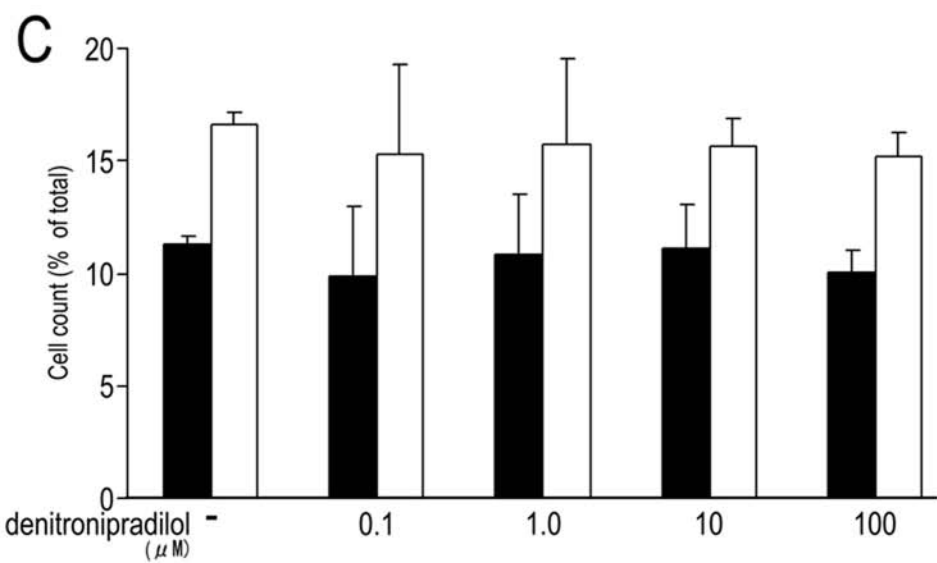
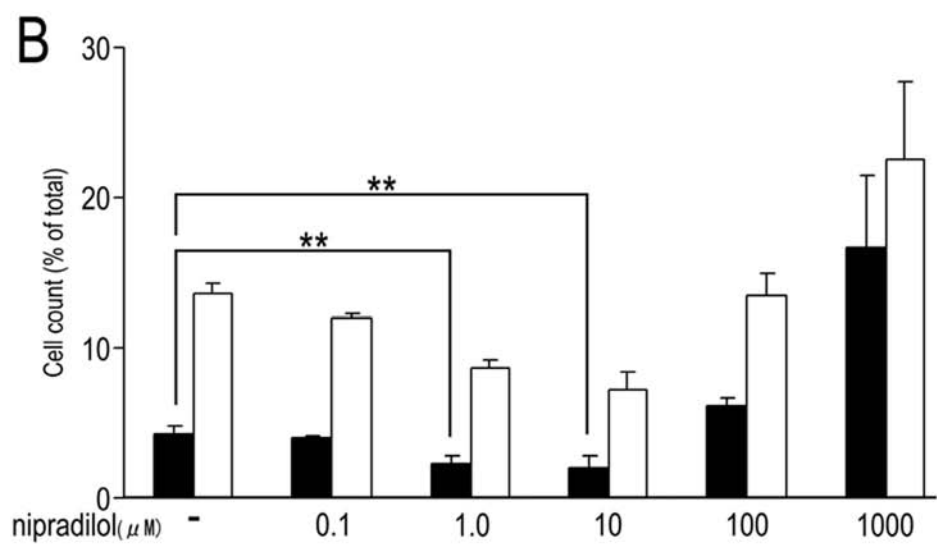
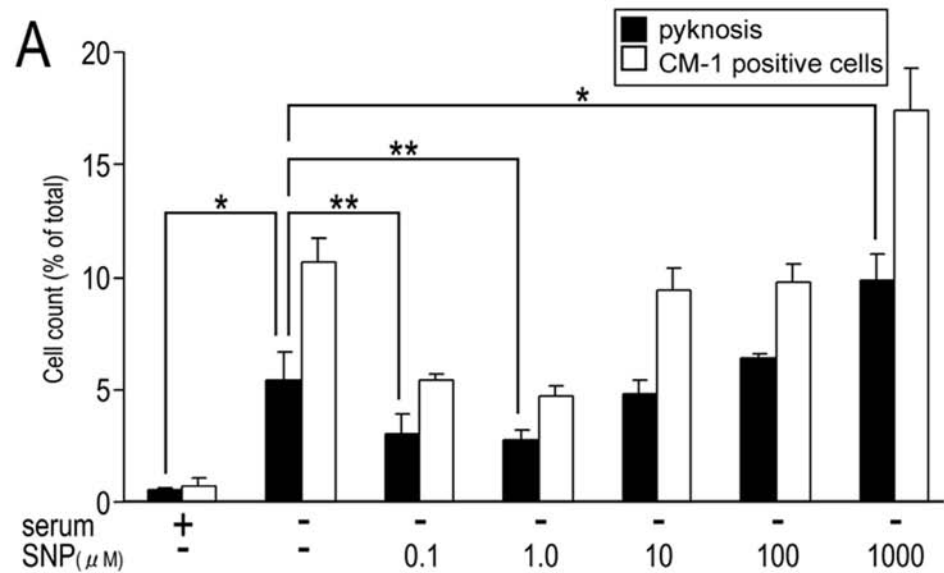
Fig. 7. CREB phosphorylation is a critical event for the NO-mediated anti-apoptotic cascade in R28 cells. R28 cells were transfected with CR133, a dominant negative CREB mutant defective of Ser-133 phosphorylation ability, or a lconstruct expressing the human wild-type (WT)-CREB. All transfected cultures were co-transfected with the pZsGreen1-N1 reporter plasmid expressing ZsGreen reef coral fluorescent protein. Cell cultures were serum-deprived 24 hours after transfection and incubated for another 24 hours with or without 1.0 μ M SNP before immunostaining. (A) A series of representative photographs, where Hoechst 33258 nuclear staining (blue), activated caspase-3 CM-1

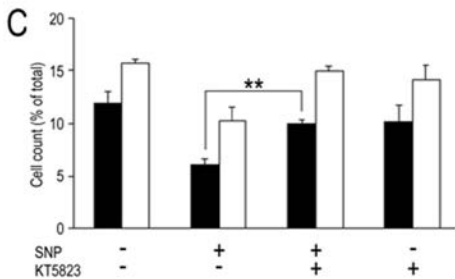
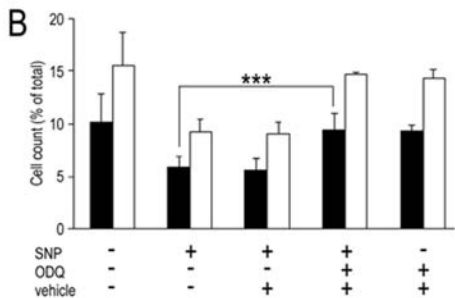
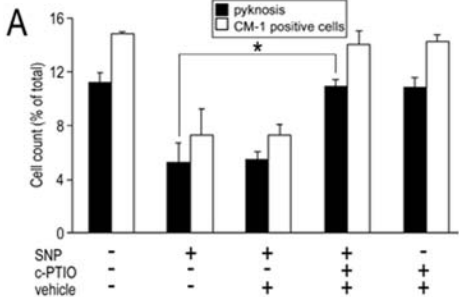
immunofluorescence (red), and transfected Zs fluorescence (green) are presented. Without SNP treatment, a similar portion of WT-CREB and CR133 transfectants was immunoreactive to activated caspase-3 CM-1. In contrast, with SNP treatment, a higher percentage of CRE133 transfectants were CM-1 positive compared with the WT-CREB transfectants. (B) Quantification of ratio of apoptotic transfectants. Approximately 17% of the cells transfected with WT-CREB were immunoreactive to activated caspase 3 in the absence of SNP, which was significantly reduced to 7% in the presence of SNP ($P < 0.0001$). In contrast, approximately 13% of the cells transfected with CREB 133 mutant were immunoreactive to activated caspase 3 regardless of SNP treatment ($P = 0.689$).

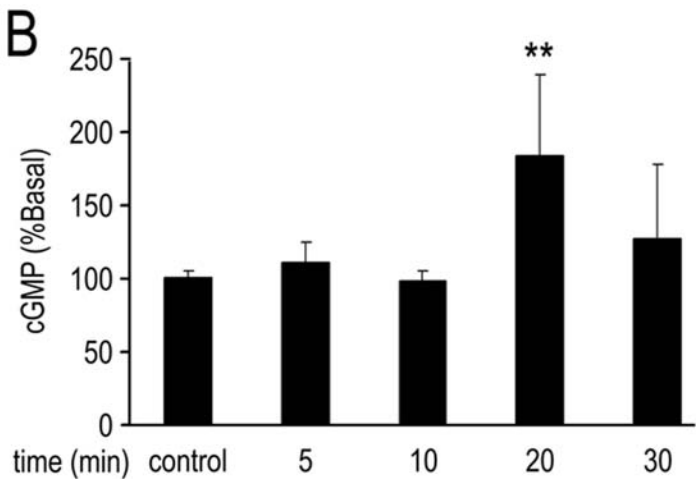
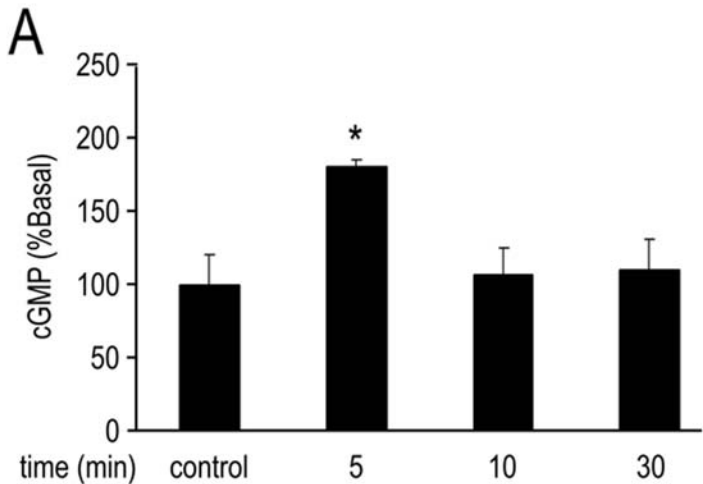
*, $P < 0.0001$; *n.s.*, not significant.

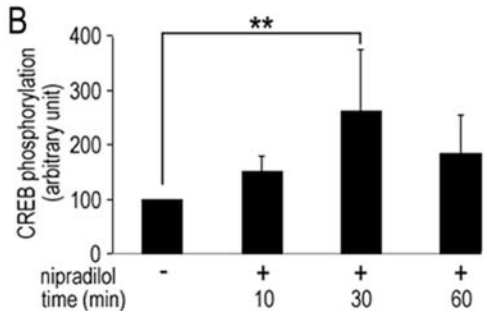
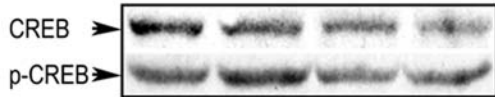
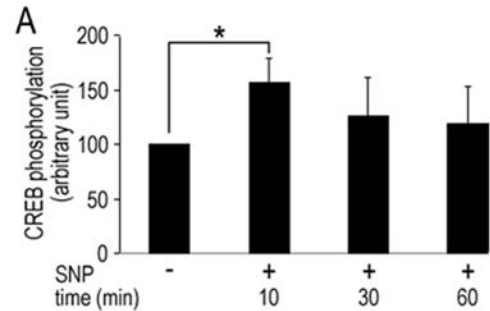
Fig. 8. Proposed cytoprotective cascade induced by NO in R28 cells.

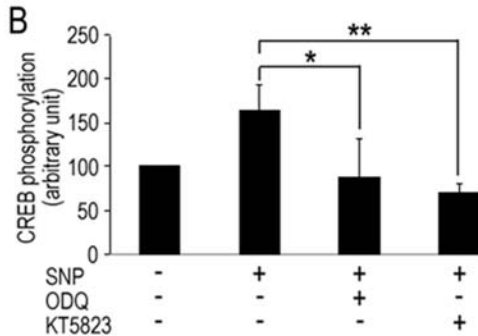
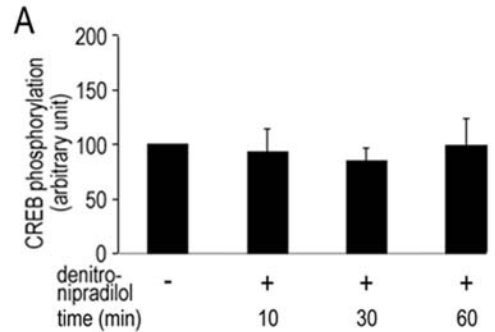


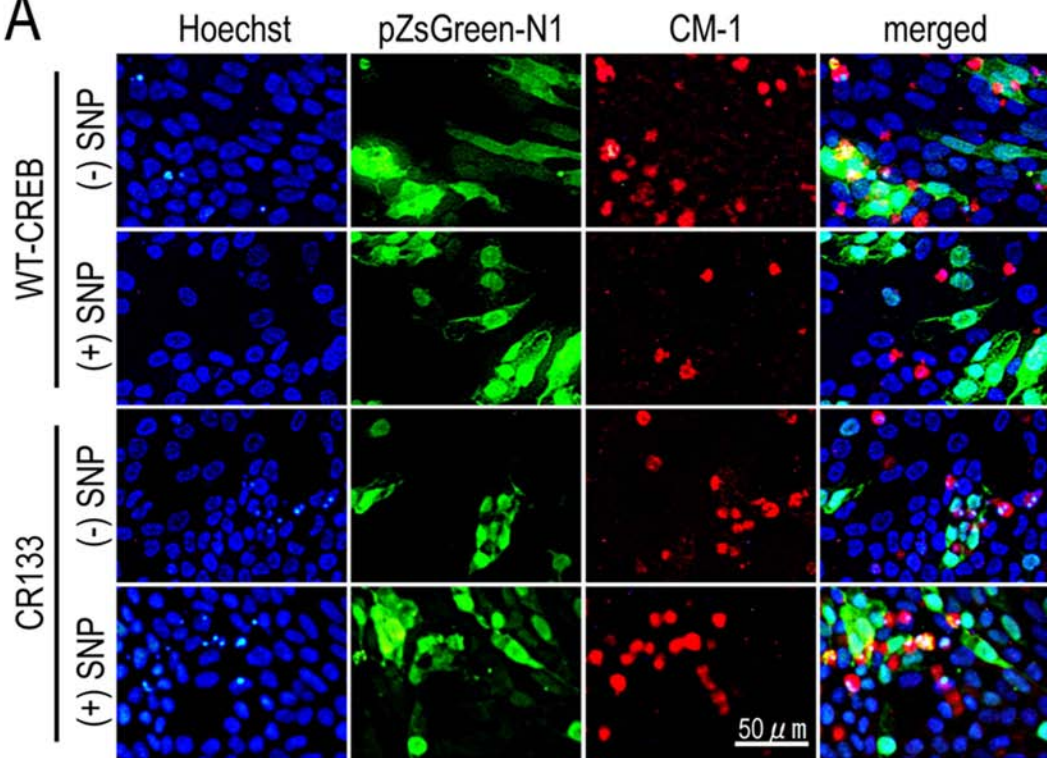










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