SNP induces calpain and caspase-mediated photoreceptor apoptosis

Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6.

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Running Title: SNP induces calpain and caspase-mediated photoreceptor apoptosis

Keywords: Apoptosis, retina, antioxidant, ROS

1 The abbreviations used are: SNP, sodium nitroprusside; CR-6, 3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2H)-benzopyran; ROS, reactive oxygen species; zVAD-fmk, (zVal-Ala-Asp-fluoro-methyl ketone); RP, retinitis pigmentosa; RNS, reactive nitrogen species; NOS, nitric oxide synthase; BHT, butylated hydroxytoluene; NAC, N-Acetyl-L-
Acknowledgements: This study was supported by funds from the European Union, Fighting Blindness Ireland and the Higher Education Authority of Ireland.
SNP induces calpain and caspase-mediated photoreceptor apoptosis

SUMMARY

A critical role for reactive oxygen species (ROS) in photoreceptor apoptosis has been established. However, the exact molecular mechanisms triggered by oxidative stress in photoreceptor cell death remain undefined. The present study delineates the molecular events occurring after treatment of the photoreceptor cell line 661W with the nitric oxide donor SNP. We show an increase in cytosolic calcium levels during photoreceptor apoptosis, which leads to the activation of the calcium-dependent proteases calpains. Furthermore, we demonstrate that caspases activation also occurs following SNP insult. However, treatment with the pan-caspase inhibitor zVAD-fmk (zVal-Ala-Asp-fluoromethyl ketone), while inhibits caspase activity per se in SNP-treated 661W cells, does not prevent apoptosis. On the other hand, we demonstrate that CR-6 (3,4-dihydro-6-hydroxy-7-methoxy-2,2-dimethyl-1(2H)-benzopyran) acts as a scavenger of ROS and reduces 661W photoreceptor apoptosis induced by SNP by preventing the activation of a pathway in which calpains have a key role. In summary, we report for the first time that both caspases and calpains are involved in 661W photoreceptor apoptosis and that calpain activation can be prevented with the ROS scavenger CR-6.
SNP induces calpain and caspase-mediated photoreceptor apoptosis

INTRODUCTION

The cell death process of apoptosis is characterised by a series of morphological and biochemical changes, including membrane blebbing, loss of plasma membrane asymmetry, chromatin cleavage and DNA fragmentation (1,2). Apoptosis plays a central role in tissue modelling during development and together with the cell generating process of mitosis is responsible for the maintenance of cell numbers in multi-cellular organisms. Deregulation of apoptosis has been well documented in several human pathologies including cancer, neurodegenerative diseases and AIDS (3,4). Apoptosis also appears to be responsible for the cell loss seen in several disorders of the retina including Retinitis Pigmentosa (RP) (a heterogeneous group of inherited disorders), glaucoma and macular degeneration (5-8). Experiments aimed at unravelling the signalling pathways of apoptosis have identified several distinct mechanisms and it has largely been accepted that caspases play a key role in both the initiation and execution pathways of apoptosis. However, the involvement of caspases does not seem to be clear-cut in some tissues systems. For example, there is still considerable controversy as to whether caspases play a role in retinal cell death (9-12). There is also some uncertainty of the role of caspases in neurodegenerative conditions (13-15). Recent work from our laboratory has indicated that photoreceptor death in animal models of RP proceeds in the absence of caspase activity suggesting a caspase independent mechanism of cell destruction (11,12,16). The exact mechanisms operating in photoreceptor death are still unclear but may involve calpains rather than caspases as the executing enzymes. This work also suggested a key role for reactive oxygen species and reactive nitrogen species (RNS) since inhibitors of
nitric oxide synthase (NOS) blocked the cell death seen. These results are quite interesting since several studies have indicated that the eye is particularly sensitive to oxidative stress and therefore, modifications of the cellular redox state of the eye have been reported to play an important role in retinal degeneration processes (17-20). Additional support for the involvement of ROS and oxidative stress in photoreceptor apoptosis comes from several studies in which antioxidants appear to retard or inhibit the degenerative pathology (21,22,23). However, the mechanisms of action of these anti-apoptotic molecules are unclear and further work is necessary to resolve whether oxidative stress acts as a common mediator of retinal degeneration in RP.

The retina is composed of several different cell types and this complicates any studies aim at delineating the underlying mechanism of photoreceptor apoptosis. The production and characterisation of the photoreceptor cell line 661W by Al-Ubaidi and co-workers has greatly facilitated work in this area (24). The cell line expresses several markers of photoreceptors and has proved useful for in vitro studies investigating photoreceptor apoptosis (19,20,25,26). In the context of the current work we have used this cell line to investigate the role played by oxidative stress in photoreceptor apoptosis induced by the nitric oxide donor SNP. This constitutes a direct extension of previous work from this laboratory in which we showed that nitric oxide and ROS played a key role in driving photoreceptor apoptosis in vivo. In the present study, we show that SNP induces ROS production in the mitochondrion and this in turn triggers apoptosis with both calpains and caspases playing a role. Treatment with the pan-caspase inhibitor zVAD-fmk inhibits the activation of caspases, but does not appear to be a useful strategy to prevent oxidative
SNP induces calpain and caspase-mediated photoreceptor apoptosis

stress-induced apoptosis in 661W photoreceptor cells. On the other hand, in the context of the current work we show that CR-6 (a vitamin E analogue that has been shown to prevent glutamate neurotoxicity in cultured neurons due to its role as a nitric oxide scavenger (27)), interferes with oxidative stress-induced apoptosis in 661W cells by preventing the activation of the calpain-mediated apoptotic pathway.

**EXPERIMENTAL PROCEDURES**

*Drugs, Reagents and Antibodies.* SNP was purchased from Sigma Chemical Co. (Poole, UK). The synthesis of CR-6 has been described elsewhere (28). Cell Signaling Technology (Beverly, MA) provided the following antibodies: PARP (poly(ADP-ribose)polymerase) (#9542), caspase-3 (#9662), -9 (#9504) and -12 (#2202). Calpain-1 (#208753) and calpain-2 (#208755) antibodies were purchased from Calbiochem (La Jolla, CA), calpastatin (#sc-7561) and calpain reg (C-20) (#sc-7528) from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin from Sigma. Secondary antibodies anti-rabbit, goat or mouse, peroxidase-conjugated, were obtained from Dako (Denmark). The broad-spectrum caspase inhibitor zVAD-fmk was purchased from Bachem (UK) Ltd. (Meyerside, UK). Alexis Co. (Läufefingen, Switzerland) provided the caspase-3 substrate Ac-DEVD-pNA (Acetyl-Asp-Glu-Val-Asp-p-NitroAniline).

*Cell Culture.* As described elsewhere (20), 661W cells were routinely grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat inactivated fetal calf serum (both from Sigma) and 1% penicillin/streptomycin, at 37°C in a humidified 5%
SNP induces calpain and caspase-mediated photoreceptor apoptosis

CO₂ atmosphere. SNP was used to induce apoptosis at the doses indicated; SNP stock was prepared at a 100 mM concentration in phosphate-buffered saline (PBS, pH 7.4) buffer. Pre-treatment of 661W photoreceptors with CR-6 was done for 15 min at 37°C in the conditions described above. A 100 mM CR-6 stock was prepared in dimethyl sulfoxide (DMSO). Treatments carried out in 6-well plates (Nalge Nunc International, Hereford, UK) were as follow: 75 x 10⁴ cells per well were seeded and allowed to attach for 20 hours at 37°C. Following insult cells were detached with a trypsin/EDTA solution (Sigma) and collected together with their supernatants for flow cytometric analysis. Cells for western blot analysis were grown in 75 cm² flasks (Sarstedt AG & Co., Nümbrecht, Germany). Initially, cells (8 x 10⁵ /flask) were seeded and allowed to attach before treatment. Samples were collected 24 or 48 hours post-insult.

Analysis of generated RNI: Griess Reaction. Determination of the presence of RNI was done by means of the Griess reaction (29). Briefly, cells (5 x 10⁴) were incubated for 15 min in the darkness at room temperature with 40 µl of the Griess reagent (Alexis Corporation). Nitrites present in the samples react with sulfanilic acid and N-(1-naphthly)-ethylenediamine dihydrochloride in the presence of phosphoric acid, which produces a colored azo dye that can be measured at 548 nm.

Analysis of Intracellular ROS Generation. Measurement of superoxide anion levels was carried out as previously described (30). Briefly, cells were loaded with 10 µM hydroethidine (DHE, Molecular Probes, Leiden, The Netherlands), prepared from a 10 mM stock in Me₂SO, for 15 min at 37 °C. Superoxide anion oxidizes DHE intracellularly
SNP induces calpain and caspase-mediated photoreceptor apoptosis

to produce ethidium bromide, which fluoresces upon interaction with DNA. Monitoring
the fluorescence due to ethidium bromide in a Becton-Dickinson FACScan flow
cytometer with excitation and emission settings of 488 and 590 nm, respectively,
assessed superoxide anion levels. CellQuest software was used for data analysis and
10,000 events/sample were acquired.

Cell Death Measurement. Propidium iodide (PI; Sigma) was employed for quantification
of cell death. Treated cells were collected as described above, washed once with ice-cold
PBS buffer and resuspended to a final concentration of 1 x 10^5 cells/ml. Propidium iodide
was added (50 µg/ml) immediately before flow cytometric analysis. Fluorescence was
measured in Fl-2 (590 nm) and 10,000 events/sample were acquired.

Measurement of Intracellular Free Ca^{2+}. Intracellular Ca^{2+} levels were determined using
the intracellular Ca^{2+} probe, Fluo-3 AM (acetoxymethyl ester; Molecular Probes), which
binds Ca^{2+} with a 1:1 stoichiometry. After trypsinisation, cells were washed once with
PBS and resuspended in fresh buffer. Cells were incubated in the darkness with Fluo-3
(250 nM), prepared from a 500 µM stock for 30 min at 37ºC. Fluorescence was measured
at FL-1 (530 nm) in a Becton-Dickinson FACScan flow cytometer with excitation at 488
nm and the software CellQuest was employed for subsequent data analysis. At least
10,000 events/sample were acquired.

Western Blot Analysis. After exposure to drug, whole cell extracts were obtained and
resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Briefly,
harvested cells were washed twice with ice-cold PBS, resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na$_3$VO$_4$, 1 mM NaF, 1 mM EGTA, 1% NP40, 0.25% sodium deoxycholate) containing antipain (1 µg/ml), aprotinin (1 µg/ml), chymostatin (1 µg/ml), leupeptin (0.1 µg/ml), pepstatin (1 µg/ml) and 0.2 mM AEBSF and incubated on ice for 20 minutes. Supernatants were recovered by a 10-minute centrifugation (10,000 x g) at 4ºC and protein concentration was determined with the Bio-Rad assay (Hemel Hempstead, UK), using bovine serum albumin (BSA) as standard.

Proteins (20-40 µg) were diluted in 2X sample buffer (10% sodium dodecyl sulfate, 100 mM dithiothreitol, glycerol, bromophenol blue, Tris-HCl) and resolved on 6-12% SDS-PAGE gels. Then proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and the blots were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline buffer/0.1% Tween-20 (TBS/T), for 1 hour at room temperature. Membranes were incubated at 4ºC overnight, with the appropriate dilution of primary antibody (1:5000 anti-m and µ-calpain, 1:1000 all others). After three 5-minute washes with TBS/T, blots were incubated with the corresponding peroxidase-conjugated secondary antibody (dilution 1:1000) for 1 hour at room temperature. Then they were washed again three times with TBS/T, rinsed briefly with PBS, and developed with the enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Buckinghamshire, UK). Detection of β-actin (1:5000) was used as control for equal loading of protein.

**Determination of Ac-DEVD-pNA cleavage.** 661W cells were grown in 75 cm$^2$ flasks (8 x 10$^5$) and pre-incubated at 37°C with the caspase inhibitor zVAD-fmk (50 µM) for 1 hour prior to insult with 0.3 mM SNP. Untreated and 0.3 mM SNP-treated 661W cells were
SNP induces calpain and caspase-mediated photoreceptor apoptosis

used as negative and positive controls, respectively. After 24-hour incubation, cells were collected as described above and centrifuged at 500 x g for 5 min. The pellet was resuspended in 1 ml of ice-cold PBS 1X and transferred to a microfuge tube. Subsequently, the pellet was resuspended in 50 µl of ice-cold lysis buffer (100 mM HEPES, pH 7.4, 1 M NaCl, 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 1M DTT, 10mM EDTA, 1% NP-40) and incubated in ice for 10 min. Following a 20-second sonication, cell lysates were centrifuged for 10 min at 12,500 x g. The protein content of each sample was determined by Bio-Rad protein assay using BSA as standard. An equal quantity of protein (50 µg) was loaded into each well of a microtiter plate and the final volume was made up to 90 µl with assay buffer (as lysis buffer, minus 1% NP-40). Lysates were incubated with 02 mM caspase-3 substrate Ac-DEVD-pNA at 37°C 20 hours. Cleavage of the peptide substrate DEVD-pNA was monitored by liberation of the chromogenic pNA in a SpectraMax-340 plate reader (Molecular Devices, Menlo Park, CA) by measuring absorption at 405 nm.

Annexin V assay. A combined staining with annexin V-FITC/propidium iodide was performed as a measure of apoptosis. Harvested cells were washed once in Ca²⁺ binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and resuspended in 100 µl of the same buffer containing annexin V FITC-conjugated (IQ Products, Groningen, NL). After a 15-minute incubation in the darkness at room temperature, cells were diluted with 400 µl of binding buffer and propidium iodide was added before flow cytometry analysis. Fluorescence was measured as described above.
RESULTS

SNP induces ROS production and cell death in the photoreceptor cell line 661W. Oxidative stress has been reported to play an important role in photoreceptor cell death (18,20). Previous work carried out in this laboratory demonstrated that nitric oxide mediated retinal degeneration in vivo and that ROS significantly contributed to photoreceptor cell death (11). Despite of these findings, the mechanisms leading to photoreceptor apoptosis are still not fully understood and little is known about oxidative pathways involved in retinal death. Therefore, in order to gain a better understanding of the events triggered by RNI and ROS in photoreceptors, the 661W cell line was treated with increasing concentrations of the nitric oxide donor SNP (0.1 to 0.5 mM) for 20 h. Previous studies have shown that SNP can trigger apoptosis in neurons (31). The production of nitric oxide metabolites was detected with the Griess reaction (29). In this method, detection of the concentration nitrites is done by a colorimetric reaction. Therefore, the value of the resulting absorbance is proportional to the amount of RNI present in the samples. As illustrated in Fig. 1A, an increase of NO metabolites was observed in 661W photoreceptor cells up to treatment with 0.3 mM SNP. Higher concentrations of SNP did not significantly modify the concentration of RNI in the cells.

In parallel with this study, superoxide anion formation was monitored using the probe DHE (Fig. 1B). Contrary to results observed for RNI, treatment with the lowest concentrations of SNP (0.1-0.2 mM) did not alter significatively the levels of superoxide
anions present in 661W cells and increased levels of ROS were not detected until treatment with 0.3 mM SNP. Incubation with 0.5 mM SNP increased superoxide anion level up to 73%, indicating a modification of the redox state in 661W cells.

Quantification of cell death was performed by flow cytometry analysis. Propidium iodide was used to quantify the population of cells in which membrane integrity was lost. As expected, treatment of 661W cells with dose-dependant concentrations of SNP induced cell death, with only 29% of cells surviving post-insult with 0.5 mM SNP (Fig. 1C). Nevertheless, a concentration of 0.3 mM SNP, which gave approximately 50% cell death, was chosen for further studies. Apoptotic cell death was then assessed by detection of DNA fragmentation by DNA gel electrophoresis after treatment with 0.3 mM SNP. The presence of the DNA ladder was detected 48 hours post-insult (data not shown). In conclusion, these results confirm and extend previous observations showing the involvement of ROS in photoreceptor cell death.

661W photoreceptor cell death induced by SNP is mediated by an elevation in intracellular Ca\(^{2+}\) levels and calpain activation. Oxidative stress induces in the cell a Ca\(^{2+}\) influx from the extracellular environment and efflux from intracellular stores leading to an increase in cytoplasmic Ca\(^{2+}\) levels, which has been associated with apoptosis in diverse \textit{in vivo} and \textit{in vitro} systems (32). It has also been shown that elevated cytosolic Ca\(^{2+}\) plays a role in rod photoreceptor apoptosis (11,33,34). In the present study, intracellular Ca\(^{2+}\) levels were determined using the fluorescent probe Fluo-3 AM. To ensure that SNP treatment of 661W photoreceptor cells increased [Ca\(^{2+}\)], calcium
SNP induces calpain and caspase-mediated photoreceptor apoptosis

levels in 661W untreated cells were compared with those incubated with 0.3 mM SNP for 20 hours. As depicted in the histogram of Fig. 2A, treatment of 661W cells with 0.3 mM SNP produced an increased Fl-1 fluorescence, indicating an increased concentration of intracellular calcium.

Activation of calcium-dependant proteases, such as calpains, is thought to play an important role in certain models of oxidative stress-induced apoptosis (35-37). In addition, several studies have shown that calpains can be a mayor contributor to neuronal death (38-40) and calpain inhibitors have been used to block apoptosis (41-43). However, to date little is known about the involvement of calpains in photoreceptor cell death. Previous work by this laboratory has reported the activation of calpains during light-induced retinal degeneration (12). To verify the activation of calpains in 661W cells following increased levels of intracellular Ca\(^{2+}\), we performed immunoblotting analyses using polyclonal antibodies against the calpain isoforms m-calpain and \(\mu\)-calpain. Moreover, we also analysed the common small regulatory subunit of calpains, which is dissociated in response to increased levels of cytosolic calcium (44). As illustrated in Fig. 2B, a decrease in the total amount of m-calpain is observed after 24 hour-treatment of 661W with 0.3 mM SNP (50% cell death). The reduction in calpain levels is even more evident after 48 hours (76% cell death). Although calpains only require an elevation in \([\text{Ca}^{2+}]_i\) to become active, the auto proteolytic cleavage further enhances their activity. Fig. 2B shows that SNP-treated 661W cells contain the autolysed form of \(\mu\)-calpain (78 kDa), indicating that this isoform is also activated by SNP. Consonant with the results of the immunoblotting analysis of the latent forms of calpains, a decrease in the intensity of
SNP induces calpain and caspase-mediated photoreceptor apoptosis

the band corresponding to the calpain small subunit (28 kDa) was observed after 24 hour-treatment whereas after 48 hours the band was not detectable any longer. Western blot analysis of the calpain substrate calpastatin was carried out to confirm the activation of calpains. The complete disappearance of the calpastatin band after 24-hour treatment strongly indicated that calpains are involved in 661W photoreceptor death induced by SNP.

661W photoreceptor cell death induced by SNP is caspase dependent. It is well documented that caspases are the central executioners of apoptosis in a wide range of cell types (45,46). Several reports have described a role for caspases during photoreceptor apoptosis (9,47). Tuohy and co-workers recently described that apoptosis of 661W photoreceptors triggered by transfecting the Fas-associated death domain is caspase dependent (26). In contrast, a lack of caspase activation has also been demonstrated during retinal cell apoptosis (10). Work performed in two in vivo models of photoreceptor apoptosis has also shown that light-induced photoreceptor apoptosis and N-methyl-N-nitrosourea-induced retinal cell apoptosis are caspase independent (11,12,16). Because oxidative stress appears to play a key role in 661W cell death induced by SNP, we determined the activation status of caspase-3, -9 and -12 by western blot analysis.

Caspase-12 is predominantly found as a proenzyme in the ER (endoplasmic reticulum) and it has been recently demonstrated that this caspase can mediate an ER-specific apoptotic pathway (48). The activation of caspase-12 results from agents or insults that affect ER homeostasis and Nakagawa and co-workers have recently shown that activation
SNP induces calpain and caspase-mediated photoreceptor apoptosis

of m-calpain is required for pro-caspase-12 processing (35). In view of the fact that we had observed m-calpain activation in SNP-treated 661W cells, we investigated whether caspase-12 was active. Fig. 3A demonstrates the presence of two fragments approximately at 38-kDa in 661W cells treated with 0.3 mM for 24 hours. The level of these fragments is increased at 48 hours and also an additional fragment appears at 30/33-kDa at this time point. Moreover, an increase in the full-length caspase-12 level can be observed in both time points. Although the molecular weight of the active subunits of caspase-12 has not been precisely described, previous work has referred to protein fragments at approximately 35 kDa as indication of caspase-12 activation by m-calpain (35). Therefore, our results indicate that caspase-12 is active in SNP-treated 661W photoreceptor cells.

Caspase-9 is activated in response to stimuli that lead to cytochrome c release from mitochondria and is essential for the initiation of the caspase cascade, involving caspase-2, -3, -6 and -7 (49). Nitric oxide has been reported to induce mitochondria permeability transition, which is supposed to cause the liberation of apoptogenic factors from mitochondria (50,51). In our case, the elevated ROS levels detected in SNP-treated 611W cells suggested a mitochondrial dysfunction. Therefore, to determine whether caspase-9 was active or inactive in our system, 661W photoreceptor cells were incubated with 0.3 mM SNP over 24 and 48 hours prior to western blot analysis. 661W cells treated with 100 nM staurosporine for 24 hours were used as positive control. The tyrosine kinase inhibitor staurosporine has shown to induce apoptosis in 661W cells following a mitochondrial pathway in which caspase-3, -7 and -9 are active (Gomez-Vicente, V. and
SNP induces calpain and caspase-mediated photoreceptor apoptosis

Cotter, T.G., unpublished results). The concentration of staurosporine was chosen in order to give a similar percentage cell death as seen for 0.3 mM SNP. Caspase-9 is synthesised as a 45-kDa inactive proenzyme and is cleaved to generate active subunits of 39 and 37-kDa. The presence of the 39 and 37-kDa caspase-9 fragments after 24-hour incubation of 611W photoreceptor cells with 0.3 SNP demonstrated the activation of caspase-9 (see Fig. 3B).

Caspase-3 is considered the main executioner of apoptosis and the involvement of this enzyme in photoreceptor apoptosis has been reported. (47,52,53). Caspase-3 is synthesised as a 32-kDa proenzyme that requires cleavage by caspase-9 to its 17-19-kDa active subunits (49). As it can be seen in Fig. 3B, the presence of caspase-3 cleaved fragments is not detected in 661W cells after 24-hour treatment with 0.3 mM SNP. At this time point, the western blot only reveals a 29-kDa band under the procaspase-3 unit. This fragment has been recently identified as the cleaved product of procaspase-3 by m-calpain (54), which supports our previous observations that suggest that calpains are active in SNP-induced photoreceptor apoptosis. After 48 hours, the 17-kDa active subunit of caspase-3 is visible in the blot, indicating classical caspase-3 activation. This result demonstrates that cleavage of procaspase-3 is a late event in the signalling cascade triggered by SNP. Western blotting analysis suggests that activation of calpains and caspase-9 takes place 24 hours earlier before detecting processing of the executioner caspase. To confirm caspase-3 activation, cleavage of the caspase-3 substrate PARP was analysed by western blot. PARP is a polymerase that can be cleaved by either calpains or caspase-3. A polyclonal antibody was used to detect both PARP and the 85-kDa fragment
SNP induces calpain and caspase-mediated photoreceptor apoptosis

resulting from caspase-3 cleavage (55). According to results obtained for caspase-3, the 85-kDA inactive subunit of PARP was only present after 48-hour treatment with SNP (see Fig. 3B). Summarising, the results obtained in the western blot analysis demonstrate that signalling mediated by both calpains and caspases may be involved in SNP-induced 661W photoreceptor apoptosis. This is the first demonstration that both caspases and calpains are involved in photoreceptor apoptosis.

The broad range caspase inhibitor zVAD-fmk cannot inhibit SNP-induced apoptosis in 661W photoreceptor cells. Caspase inhibitors have been employed to block apoptosis in cells systems in which caspases have a central role. In view of the fact that SNP-induced apoptosis in 661W cells is caspase-dependent, we examined the effect of zVAD-fmk on photoreceptor apoptosis. Therefore, 661W cells were pre-incubated with zVAD-fmk prior to treatment with 0.3 mM SNP and subsequently, caspase-3 activity was analysed by cleavage of the colorimetric substrate Ac-DEVD-pNA. As depicted in Fig. 4A, pre-incubation of 661W cells with caspase inhibitor zVAD-fmk completely prevented caspase-3 activation due to SNP-treatment. However, a parallel determination of cell death in those samples (Fig. 4B) revealed that despite zVAD-fmk inhibition of caspase-3, the pan-caspase inhibitor was unable to prevent SNP-induced apoptosis in 661W photoreceptor cells. Moreover, the cell death percentage estimated for zVAD-fmk pre-treated 661W cells was significativelly higher than the one exhibited by those photoreceptors that had not been pre-incubated with the caspase inhibitor (61 and 53% respectively). We have demonstrated that that both caspases and calpains are involved in photoreceptor apoptosis. Therefore, our findings suggest that inhibition of caspases in
SNP induces calpain and caspase-mediated photoreceptor apoptosis

SNP-treated 661W photoreceptor cells activates a more harmful death pathway, not necessarily apoptotic, in which calpains could be the main executioners. According to this hypothesis, immunoblotting analysis of m-calpain and its small subunit in SNP-treated samples revealed that the pan-caspase inhibitor zVAD-fmk does not inhibit calpains activity, on the contrary a slight increase in m-calpain activation was observed under these conditions compare to samples only treated with SNP, whereas no difference was observed in the dissociation of the small regulatory subunit of calpain (Fig. 4C). As a view of these results, other alternatives different from caspase inhibitors have to be found to inhibit apoptosis in 661W photoreceptor cells.

CR-6 acts as a scavenger of ROS and reduces cell death in 661W photoreceptor cells induced by SNP. CR-6 could be envisaged as a simple tocopherol analogue. In addition to the phenol moiety, its structure contains two non-substituted and highly activated aromatic positions that confer the molecule the ability to react with radical species. Thus, the capacity of CR-6 to react with nitrating species such as NO or ONOO⁻ has already been demonstrated; moreover, CR-6 has shown to prevent glutamate neurotoxicity in primary cultures of cerebellar neurons by scavenging NO (27). Therefore, CR-6 appealed to us as an worthwhile candidate to study the effect that pre-treatment with an antioxidant molecule could have in 661W cell death induced by SNP, since we had already observed that RNI and ROS were involved in 661W cell apoptosis. 661W cells were pre-incubated for 15 min with increasing concentrations of CR-6 (from 0.2 to 100 µM). Then, 0.3 mM SNP was added to the wells and incubation continued for 20 hours. A significant reduction of cell death was observed when 661W cells were pre-treated with high
SNP induces calpain and caspase-mediated photoreceptor apoptosis

centrations of CR-6 (100 to 12.5 µM). Thus, 15 minute-incubation with 25 µM of
CR-6 prevented cell death in a 63%. Moreover, the protecting capacity exhibited by CR-6
in 661W cells was superior to other assessed antioxidants (butylated hydroxytoluene,
resveratrol, ascorbic acid and N-acetyl-L-cysteine) and similar to α-tocopherol’s (data not
shown). As a result of this study, a concentration of 25 µM CR-6 was chosen for further
assays. We then tested whether CR-6 prevented cell death when 661W cells are incubated
with dose-dependent concentrations of SNP. We also assessed whether CR-6 acted as a
scavenger of radical species. As previously described, the presence of nitric oxide
metabolites was determined colorimetrically by means of the Griess reaction, while the
generation of superoxide anions was monitored using the probe DHE. Fig. 5A illustrates
the results of these experiments. As it can be seen in Graph a, preincubation of 661W
cells with 25 µM of CR-6 significantly prevented cell death for all concentrations of SNP
studied. It should be noted that the percentage of cell death was reduced to basal levels in
those samples pre-treated with CR-6 and preincubated with 0.1 and 0.2 mM SNP.
Therefore, CR-6 confers complete protection against apoptosis induced by low
concentrations of SNP in 661W photoreceptor cells under the conditions tested. In the
rest of the samples, the protection achieved varied from 70% to 50% when cells were
given an insult of 0.3 and 0.5 mM SNP, respectively. Graph b illustrates the reduction of
superoxide anion levels in all the samples assayed which had been pre-treated with CR-6,
although the decrease in the levels of ROS is more significantly detected at high
concentrations of SNP (46 and 40% reduction for samples pre-treated with 25 µM CR-6
and incubated with 0.4 and 0.5 mM SNP, respectively). The ability of CR-6 to scavenge
superoxide anions has never been previously described. However, previous work has
demonstrated that CR-6 exhibits a potent inhibitory activity of lipid peroxidation induced on rat liver microsomes, which could constitute an evidence of ROS scavenging by CR-6 (56). Moreover, it has been suggested that scavenging of ROS could have been a contribution to the protective effect exhibited by CR-6 in primary cultures of cerebellar neurons (27). Graph c illustrates the presence of nitrites in control 661W cells treated with increasing concentrations of SNP and in those pre-treated with CR-6. Surprisingly, an increase in nitrites was detected in all cases. Initially, a decrease in RNI would have been expected if CR-6 had trapped these radicals. However, despite of the increased levels of nitrites observed, a scavenging of RNI by CR-6 cannot be discarded, since the increase in nitrite concentration could be the result of the reaction between CR-6 and nitric oxide or peroxynitrites.

Annexin V staining of 661W cells clearly shows that treatment with 0.3 mM SNP for 20 hours is enough to increase phosphatidylserine exposure at the surface of the cell compared with untreated cells (Fig. 5B). Dot blots from the FACS analysis of annexin V-staining cells are divided in four different quadrants. The bottom panels show those cells not stained with propidium iodide, i.e. with intact membranes. Cells on the left bottom panel correspond to the annexin V-negative propidium iodide-negative population, while the right bottom panel shows the annexin V-positive propidium iodide-negative cell population. The top panels display cells that have lost membrane integrity and have become propidium iodide-positive. The percentages of cell counts in the two lower quadrants are indicated with numbers. Thus, in dot blot a (untreated cells) annexin V-negative propidium iodide-negative cell population counts for 89% of the whole cells,
SNP induces calpain and caspase-mediated photoreceptor apoptosis

while only a 5% results annexin V staining-positive. Treatment with 0.3 mM SNP for 20 hours shifts annexin V-positive population to a 15.2% (dot blot b). Nevertheless, a 15 minute-incubation of 661W cells with 25 µM of CR-6 is enough to completely reverse the annexin V-positive percentage to a 4.6% (dot blot c). Therefore, under these conditions, CR-6 confers substantial protection against SNP induced phosphatidylserine exposure.

CR-6 can reduce elevation in intracellular calcium levels and prevent m-calpain activation in 661W photoreceptor cells treated with SNP. We have previously demonstrated that oxidative stress in 661W cells due to treatment with SNP leads to an increase in cytosolic Ca$^{2+}$ levels, which subsequently activates calpains. Since CR-6 traps ROS reducing the oxidative stress in SNP-treated cells, we considered worthwhile investigate the downstream effects that scavenging of ROS by CR-6 could have in SNP-treated 661W photoreceptors. Thus, the cytosolic Ca$^{2+}$ levels were measured in control cells (cells treated with 0.3 mM SNP during 20 hours) and in those pre-treated with 25 µM CR-6 for 15 minutes. As expected, CR-6 can partially inhibit the increase observed in intracellular Ca$^{2+}$ levels induced by SNP treatment in all the concentration analysed (Fig. 6A). Although oxidative stress has been described as one of the causes of increased [Ca$^{2+}]_i$ in the cell, other agents can generate an increase in intracellular Ca$^{2+}$ concentration, as well. For example, Bcl-2 family proteins in addition to other apoptotic factors, such as activated caspases, may also have a control over the Ca$^{2+}$ levels in cells (57,58). This fact suggests that other agents could be involved in the increased calcium concentration observed in SNP-treated 661W cells and could explain why CR-6
SNP induces calpain and caspase-mediated photoreceptor apoptosis

scavenging of ROS cannot reduce completely the intracellular calcium concentration to its basal level, at least at the lowest concentrations of SNP treatment.

To investigate whether CR-6 could prevent calcium-mediated calpain activation, control cell samples treated with 0.3 mM SNP during 24 and 48 hours were compared to those incubated with 25 µM CR-6 for 15 minutes by western blot analysis. Fig. 6B shows that CR-6 pre-treatment of 661W cells prevents m-calpain activation, since 661W photoreceptors incubated with CR-6 do not show the 58 kDa band corresponding to active m-calpain. To ensure that CR-6 prevents calpain activation, we studied the extent of calpastatin cleavage in 661W cells pre-treated with CR-6 before insult with SNP. As illustrated in the blot, CR-6 can completely abolish the calpastatin cleavage when 661W photoreceptors have been treated with SNP for 24 hours. At 48 hours, CR-6 can only partially attenuate the calpastatin cleavage. These results correlate very well with previous observations. At 24-hour treatment with 0.3 mM SNP 661W cell death percentage is 50%. Pre-treatment of photoreceptors with CR-6 prevents cell death in a 70%. However, at the 48-hour time point the cell death percentage is comparable with that of 0.5 mM SNP treatment and under these conditions, cell death can only be prevented in a 49%.

CR-6 prevents caspase-12 processing while partially inhibits caspase-3 and -9 cleavage.

The observation that CR-6 can prevent m-calpain cleavage led us to assay whether caspase-12 processing was inhibited as well. Immunoblots were performed with 661W control photoreceptors (0.3 mM SNP insult for 24 and 48 hours) and with those pre-
SNP induces calpain and caspase-mediated photoreceptor apoptosis

treated with 25 μM CR-6 (see Fig. 7A). As expected, since m-calpain activates caspase-12, pre-treatment of 661W cells with CR-6 also prevents caspase-12 activation. The 33-kDa-cleavage band observed after treatment with 0.3 mM SNP for 24 hours is not visible when 661W photoreceptors have been incubated with the antioxidant CR-6. Moreover, the increased intensity of the pro-caspase-12 band at the 24 hour-time point compared to the untreated cells is completely restored to the basal levels in CR-6 pre-treated photoreceptors. At 48 hours, CR-6 can also completely prevent caspase-12 processing. Both, the 33 and 38-kDa cleavage bands are not present in the 661W cells incubated with the antioxidant. However, the basal levels of the pro-caspase-12 are not completely restored in this case, indicating that some stress is still present under these conditions.

Following the analysis of caspase-12, we proceeded with the study of the activation state of caspase-3 and -9 when SNP-treated 661W photoreceptors are pre-incubated with CR-6 before insult. Fig. 7B illustrates the results obtained. Activation of caspase-9 cannot be prevented by CR-6 and only at the 48 hour-time point a slight recovery can be observed in the levels of the pro-caspase-9 band. According to the results observed with caspase-9, caspase-3 activation is not prevented either by CR-6 pre-treatment of the 661W cells. As seen for caspase-9, only a partial inhibition of the pro-caspase-3 cleavage is observed at the 48 hour-time point. The results obtained for the caspase-3 substrate PARP agree with previous observations. The 85-kDa-cleavage band is still present at the 48-hour time point after pre-treatment of 661W cells with CR-6. These results seem to indicate that calpain mediated apoptotic pathway in 661W photoreceptors induced by SNP may run...
SNP induces calpain and caspase-mediated photoreceptor apoptosis independently from the mitochondrial pathway and that CR-6 scavenging of ROS can only prevent the events triggered in the ER (see Fig. 8).
DISCUSSION

Oxidative stress generated by the nitric oxide donor SNP induces in 661W photoreceptor cells an apoptotic programmed death in which calpains and caspases are involved. In this study we have explored the molecular events occurring after induction of oxidative stress in 661W photoreceptor cells by treatment with the nitric oxide donor SNP. Nitric oxide has been reported to inhibit cytochrome oxidase, the terminal enzyme of the mitochondrial electron transport chain, leading to the production of superoxide anions that can activate cell death mechanisms (59). For example, it has been shown that nitric oxide triggers cell death in neurons and that apoptotic events are mediated by ROS (13,60). In the same context, previous work performed in this laboratory with an animal model has also demonstrated that NO has a key role in retinal degeneration and that an early and rapid increase in intracellular reactive oxygen species accompanies retinal cell apoptosis in vivo (11). The data presented in this work are in agreement with previous observations and demonstrate that SNP induces ROS production in 661W photoreceptor cells, which activates the apoptotic cascade leading to cell death.

Elevation in cytosolic Ca$^{2+}$ levels has been reported to be a key event in apoptosis (32). Studies performed in isolated rat retinas have demonstrated that Ca$^{2+}$ overload can induce mitochondrion depolarisation and subsequently the release of apoptosis-inducing factors, such as cytochrome c, which lead to the sequential activation of caspase-9 and -3 (34). Preventing apoptosis in photoreceptors using calcium channels blockers (12,33) and calcium antagonist (61) have confirmed these observations. In the present study,
SNP induces calpain and caspase-mediated photoreceptor apoptosis

intracellular calcium levels were monitored after insult with dose-dependent concentrations of SNP. An increase in the concentration of cytosolic calcium was determined at every concentration of SNP studied (Fig. 2A and 6B). Moreover, it was noted that the increase observed in the $\text{Ca}^{2+}$ level correlated with the percentage of photoreceptor death.

Calpains are calcium-dependent proteases that have been proposed to participate in apoptosis. Calpain activation during apoptosis has been observed both in in vitro and in vivo systems (35,36,42,62). Recently, it has been demonstrated a role for calpains in PC12 cell apoptosis due to oxidative stress and increased calcium levels (36). However, to date little is known about the importance of calpains in retinal apoptosis and the molecular events they induce in photoreceptor cells have not been defined yet. The results presented in this work provide evidence of the contribution of calpains to photoreceptor apoptosis. We have shown that calcium overload activates both m- and µ-calpain in SNP-treated 661W photoreceptor cells (Fig. 2B). The dissociation of the small subunit of calpains, the processing of the endogenous calpain inhibitor calpastatin and the truncation of procaspase-3 to a 29-kDa polypeptide (54) have further supported the role of calpains in SNP-induced 661W photoreceptor apoptosis.

It has been demonstrated that photoreceptor cells from adult mice can undergo caspase-independent cell death in vivo (10,11). However, this study has illustrated that the caspase machinery is activated in 661W photoreceptor cells after insult with SNP (Fig. 3). This apparent contradiction may be explained by the fact that in adult mice there is a
SNP induces calpain and caspase-mediated photoreceptor apoptosis

significant reduction in the expression of caspases-3 and -9. Furthermore, it has been shown the protein levels of the apoptotic protease-activating factor (Apaf-1) also decrease with aging (12). This can be the key reason why caspase independent apoptosis is seen in the retina of these animals. The retina from newborn animals still expresses caspases and can undergo caspase dependent apoptosis. The 661W cell line is derived from the retina of 8 day-postnatal mice. This would reasonable be expected to retain the caspase pathway of apoptosis. In addition, it has been reported that the nature and severity of an insult determines whether apoptosis is caspase dependent or independent (63). It is reasonable to conclude then that photoreceptors are capable of caspase-dependent and independent cell death with the initial insult and aging shaping the cellular response. On the other hand, this study has shown for the first time that photoreceptor cells can undergo an apoptotic-programmed death in which calpains and caspases are involved. There is growing evidence of cross-talk between caspases and calpains and it has been reported that calpain can mediate the cleavage of caspase-3, -7, -8, -9 and -12 (35,39,54,62,64). We have shown that inhibition of m-calpain prevents caspase-12 activation. The processing of procaspase-3 by m-calpain has been suggested by the presence of the 29-kDa fragment in 661W cells 24 hour post-insult with SNP.

**CR-6 acts as a scavenger of ROS and reduces 661W photoreceptor cell death induced by SNP by preventing the activation of the calpain mediated apoptotic pathway.** This study shows that the broad spectrum caspase inhibitor zVAD-fmk, even though prevents caspase-3 activation, cannot inhibit SNP-induced 661W photoreceptor apoptosis (Fig. 4). The fact that also calpains participate in 661W cell apoptosis and are still active in the
SNP induces calpain and caspase-mediated photoreceptor apoptosis

presence of zVAD-fmk (Fig. 4C), could explain why the inactivation of caspases does not lead to an inhibition of apoptosis. On the other hand, the results presented in this work demonstrate that CR-6 reduces apoptosis induced by SNP in 661W photoreceptor cells (Fig. 5A, graph a). Our results show that incubation of 661W cells with CR-6 prior to insult results in a decrease of superoxide anion levels indicating that CR-6 behaves as a ROS scavenger in our system (Fig. 5A, graph b), which emphasises the critical role of ROS in photoreceptor apoptosis. In addition, CR-6 has demonstrated to have a very low toxicity in 661W photoreceptor cells (data not shown), which makes it an ideal candidate as a potential therapeutic agent.

The downstream effects that CR-6 could have in SNP-treated 661W photoreceptors were investigated. In this section of the study we have shown that CR-6 scavenging of intracellular reactive oxygen species leads to a reduction in the increased cytosolic calcium levels induced by SNP treatment (Fig. 6A). Sequentially, the decrease observed in intracellular Ca\(^{2+}\) concentration is reflected in m-calpain inactivation (Fig. 6B), which in turn inhibits procaspase-12 processing (Fig. 7A). Therefore, CR-6 has been demonstrated as a useful tool to reduce oxidative stress induced apoptosis in 661W cells and has allowed us to show the important role played by calpains in photoreceptor apoptosis. Despite the inhibitory effect elicited by CR-6 in the calpain mediated apoptotic pathway, CR-6 scavenging of ROS cannot prevent the activation of the caspase mediated mitochondrial pathway. Only partial prevention of procaspase processing is observed most likely as a consequence of m-calpain inhibition (Fig. 7B). In conclusion, the data presented here provides a possible mechanism by which oxidative stress mediates
SNP induces calpain and caspase-mediated photoreceptor apoptosis in 661W photoreceptor cells (Fig. 8). Moreover, our findings suggest a central role for both caspases and calpains in the apoptotic process. We have also shown that the inhibition of caspases triggers an alternative mechanism of cell death in 661W cells and that only prevention of calpain activation leads to a reduction of 661W photoreceptor apoptosis induced by SNP.
SNP induces calpain and caspase-mediated photoreceptor apoptosis

FIGURE LEGENDS

Figure 1. SNP induced RNI and ROS production and apoptotic cell death in the photoreceptor cell line 661W. 661W cells were treated with increasing concentrations of SNP (0.1 to 0.5 mM) for 20 hours. A, the presence of NO metabolites was detected colorimetrically by the Griess reaction. The error bars correspond to the standard deviation (SD) of three independent experiments done in duplicate. B, superoxide anion levels were quantified by flow cytometry using the probe DHE prior to SNP treatment (0 mM) and after treatment with 0.1 to 0.5 mM SNP. The percentage of cells displaying increased levels of ROS is shown at each SNP concentration. Results are representative of three independent experiments done in duplicate. C, cell death measurements were done by flow cytometry using propidium iodide. Dot blots show that treatment of 661W photoreceptor cells with the nitric oxide donor SNP induces oxidative stress leading to cell death. Results are representative of three independent experiments carried out in duplicate.

Figure 2. Oxidative stress in 661W photoreceptor cells induced an elevation in intracellular calcium levels and led to activation of m-calpain. A, intracellular Ca\(^{2+}\) levels were monitored using the fluorescent probe Fluo-3 AM in 661W untreated cells and those incubated with 0.3 mM SNP for 20 h. A shift in the peak to increased fluorescence in the FL-1 channel indicated an increased level of intracellular Ca\(^{2+}\) in 661W SNP treated cells. The histogram is representative of three independent experiments done in duplicate. B, immunoblot analysis of m- and \(\mu\)-calpain and their
SNP induces calpain and caspase-mediated photoreceptor apoptosis

small regulatory subunit (28 kDa) and calpastatin. Western blots were performed using 661W-cell lysates taken prior to SNP insult (UT), and 24 and 48 hours after treatment with 0.3 mM SNP. Polyclonal antibodies were used to detect activation of m- and μ-calpain. This result was confirmed by dissociation of the small subunit and detection of the calpain substrate calpastatin. After 24 and 48 hour-treatment the calpastatin band (110 kDa) can not be detected, suggesting a calpain-mediated processing. The blot was reprobed with an antibody to β-actin to demonstrate equal protein loading. Analyses were done in triplicate with similar results.

Figure 3. Caspase-3, -9 and -12 are activated during 661W photoreceptor apoptosis induced by SNP. A, cell lysates were taken from 661W cells 24 and 48 hours after treatment with 0.3 mM SNP. Untreated 661W cells were used as negative control. Caspase-12 activation is indicated by the presence of 38-kDa bands and a 30-33 kDa fragment in the blot. B, analysis of caspase-3, its substrate PARP and caspase-9 activation by western blot analysis. Untreated 661W cells and 100 nM staurosporine-treated 661W cells (24 hours) were used as negative and positive control, respectively. Procaspase-3 processing was verified after 48-hour treatment with SNP by the presence of the 17-kDa active subunit in the blot. Detection of the 85-kDa PAPR fragment confirmed caspase-3 activation. Caspase-9 activation was demonstrated by the presence of its cleaved fragments (39 and 37-kDa) after 24-hour-treatment of 661W cells with 0.3 mM SNP. All the blots were reprobed with an antibody to β-actin to demonstrate equal protein loading. A representative result of three experiments is shown in all immunoblots.
Figure 4. The caspase inhibitor zVAD-fmk inhibits SNP-triggered DEVD-like caspase-3 activity in 661W photoreceptor cells but cannot prevent 661W apoptosis.

The photoreceptor cell line 661W was incubated with 50 µM zVAD-fmk for 1 hour before treatment with 0.3 mM SNP. Untreated cells and SNP-treated 661W were used as negative and positive control, respectively. A, Ac-DEVD-pNA hydrolysis was monitored after 24-hour incubation at 37°C and quantified colorimetrically at 405 nm. Results are expressed in arbitrary units (absorbance of the corresponding sample between the absorbance of the untreated cells). Asterisk (*) indicates a significant (t-test, p<0.05) difference from SNP-treated cells. B, cell death measurements were done by flow cytometry using propidium iodide. Error bars correspond to SD of three independent experiments carried out in duplicate. C, immunoblotting analysis of m-calpain and its small regulatory subunit (28 kDa) shows increased calpain activity in 661W cells pre-treated with zVAD-fmk.

Figure 5. Pre-treatment of 661W photoreceptor cells with CR-6 inhibits apoptosis induced by the NO donor SNP. A, 661W control cells were treated with dose-dependent concentrations (0.1 to 0.5 mM) of SNP for 20 hours (white bars in graphs a-c). CR-6 pre-treated cells were incubated for 15 minutes with a 25 µM concentration of the antioxidant before insult (black bars). CR-6 significatively reduces cell death induced by SNP for all concentrations studied (graph a) due to the scavenging of superoxide anions (graph b). Results correspond to three different experiments done in duplicate; error bars indicate SD. B, flow-cytometry analysis of 661W untreated cells (dot blot a), 661W control cells treated with 0.3 mM SNP for 20 h (dot blot b) and 661W cells pre-treated with 25 µM CR-6 (dot blot c).

SNP induces calpain and caspase-mediated photoreceptor apoptosis
SNP induces calpain and caspase-mediated photoreceptor apoptosis

CR-6 before SNP insult (dot blot c), stained with annexin V and propidium iodide. Numbers in the corresponding quadrants indicate the percentage of cells present in the area. Reduction in the percentage of annexin V-positive population in those cells pre-treated with CR-6 indicates the protective effect conferred by the antioxidant against phosphatidylserine exposure. The FACS analysis was repeated three times with similar results.

**Figure 6. Effect of CR-6 on increased intracellular calcium and calpain activation induced by SNP in 661W photoreceptor cells.** A, intracellular Ca$^{2+}$ levels were measured using the fluorescent probe Fluo-3 in untreated cells (0 mM SNP, continuous line), in control cells treated with dose-dependent concentrations of SNP (0.1 to 0.5, dotted line in histograms on the left side) and in those pre-treated with 25 µM CR-6 for 15 min before insult with the corresponding SNP concentration (dotted line in histograms on the right side). The percentage of cells that show increased Ca$^{2+}$ levels in 661W photoreceptor cells induced by SNP treatment is displayed at each histogram on the left side. Histograms on the right side illustrate partial inhibition of intracellular Ca$^{2+}$ increase by CR-6. A representative result of three experiments is shown. B, immunoblotting analysis of m-calpain and its substrate calpastatin in 661W control cells and in those pre-treated with 25 µM CR-6. Western blots were performed using equivalent quantities of total protein from 661W-cell lysates taken prior to SNP insult (UT), and 24 and 48 hours after treatment with 0.3 mM SNP. Results illustrate that m-calpain activation can be prevented by pre-treatment with t CR-6. Results are representative of three experiments.
Figure 7. Effect of CR-6 on caspase-3, -9, -12 activation status in SNP-treated 661W photoreceptor cells. A, procaspase-12 processing can be prevented by pre-incubating the 661W photoreceptor cells with the antioxidant CR-6 before insult with SNP. Cell lysates were taken from 661W cells 24 and 48 hours after treatment with 0.3 mM SNP. 661W untreated cells were used as negative controls. B, CR-6 can only partially inhibit caspase-3 and -9 activation. 661W cells treated with 100 nM staurosporine during 24 hours were used as positive control. The presence of the caspase-9 cleaved fragments (39 and 37-kDa) when SNP-treated 661W cells have been pre-incubated with 25 µM CR-6 demonstrates the antioxidant cannot prevent procaspase-9 processing. Accordingly with these results, caspase-3 activation is not inhibited by CR-6 either. All the blots were reprobed with an antibody to β-actin to demonstrate equal protein loading. A representative result of three experiments is shown in all immunoblots.

Figure 8. Schematic representation of the hypothetical events that take place distal to treatment of 661W photoreceptor cells with SNP. The nitric oxide donor SNP induces mitochondria permeability transition, which can cause release of apoptogenic factors such as cytochrome c. Caspase-9 activation following apoptosome formation leads to processing of downstream caspases. Simultaneously, the oxidative stress generated by the SNP insult affects ER homeostasis, which is reflected as increased intracellular calcium levels. The calcium-dependent protease, m-calpain is then activated. Subsequently, caspase-12 processing occurs. Altogether, these events lead to the apoptotic death of the 661W photoreceptor cells. The activation of the calpain mediated
SNP induces calpain and caspase-mediated photoreceptor apoptosis

apoptotic pathway can be prevented by CR-6, which has been demonstrated to scavenge ROS in SNP-treated 661W photoreceptors.
SNP induces calpain and caspase-mediated photoreceptor apoptosis

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SNP induces calpain and caspase-mediated photoreceptor apoptosis


37
SNP induces calpain and caspase-mediated photoreceptor apoptosis


SNP induces calpain and caspase-mediated photoreceptor apoptosis

SNP induces calpain and caspase-mediated photoreceptor apoptosis


SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 1
SNP induces calpain and caspase-mediated photoreceptor apoptosis

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SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 2
SNP induces calpain and caspase-mediated photoreceptor apoptosis

B

Sanvicens N et al, 2004 Figure 2
SNP induces calpain and caspase-mediated photoreceptor apoptosis

A

![Caspase 12 gel blots](image)

B

![Caspase gel blots](image)

Sanvicens N et al, 2004 Figure 3
SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 4
SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 4
SNP induces calpain and caspase-mediated photoreceptor apoptosis

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SNP induces calpain and caspase-mediated photoreceptor apoptosis

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SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 6
SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens et al, 2004 Figure 6
SNP induces calpain and caspase-mediated photoreceptor apoptosis

A

Sanvicens N et al, 2004 Figure 7

B
SNP induces calpain and caspase-mediated photoreceptor apoptosis

Sanvicens N et al, 2004 Figure 8
Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6
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J. Biol. Chem. published online June 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402202200

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