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Authors: Khalil H, Rosenblatt N, Liaudet L, Widmann C

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The role of endogenous and exogenous RasGAP-derived fragment N in protecting cardiomyocytes from peroxynitrite-induced apoptosis

Hadi Khalil[1], Nathalie Rosenblatt[2], Lucas Liaudet[3], and Christian Widmann[1]

[1]Department of Physiology, Biology and Medicine Faculty, University of Lausanne, Switzerland, [2]Division of Pathophysiology and [3]Department of Intensive Care Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland.

Short title: Protective role of fragment N in cardiomyocytes

Keywords: Apoptosis; peroxynitrite; caspase-3; RasGAP; Akt

Correspondence to Christian Widmann, Department of Physiology, Bugnon 7, 1005 Lausanne, Switzerland, Phone: +41 21 692 5123, Fax: +41 21 692 5505, E-mail: Christian.Widmann@unil.ch.

Highlights

- Peroxynitrite is a potent nitrating and oxidizing agent
- Peroxynitrite is generated during pathological situations affecting the heart
- The caspase-generated RasGAP fragment N has anti-apoptotic properties
- Peroxynitrite induces fast cleavage of fragment N and no associated cell protection
- A cleavage-resistant form of fragment N inhibits peroxynitrite-induced death

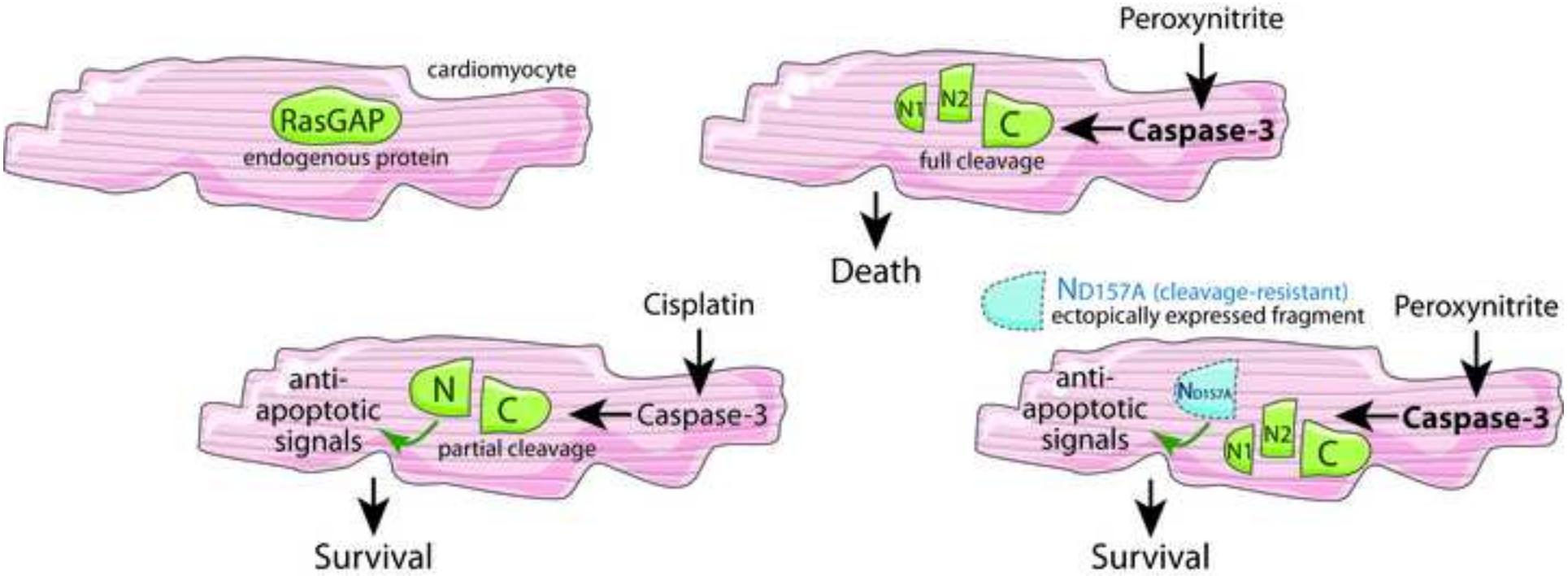
Abstract

Peroxynitrite (PN) it is a potent nitrating and oxidizing agent generated during various pathological situations affecting the heart. PN negative effects result, at least in part, by its ability to activate caspases and apoptosis. RasGAP is a ubiquitously expressed protein that is cleaved sequentially by caspase-3. At low caspase-3 activity, RasGAP is cleaved into an N-terminal fragment, called fragment N, that protects cells by activating the Ras/PI3K/Akt pathway. At high caspase-3 activity, fragment N is further cleaved and this abrogates its capacity to stimulate the anti-apoptotic Akt kinase. Fragment N formation is crucial for the survival of cells exposed to a variety of stresses. Here we investigated the pattern of RasGAP cleavage upon PN stimulation and the capacity of fragment N to protect cardiomyocytes. PN did not lead to sequential cleavage of RasGAP. Indeed, PN did not allow accumulation of fragment N because it induced its rapid cleavage into smaller fragments. No situations were found in cells treated with PN where the presence of fragment N was associated with survival. However, expression of a caspase-resistant form of fragment N in cardiomyocytes protected them from PN-induced apoptosis. Our results indicate that the anti-apoptotic pathway activated by fragment N is effective in inhibiting PN-induced apoptosis (as seen when cardiomyocytes express a capase-3-resistant form of fragment N) but because fragment N is too transiently generated in response to PN, no survival response is effectively produced. This may explain the marked deleterious consequences of PN generation in various organs, including heart.

Keywords: peroxynitrite; caspases; RasGAP; Akt; apoptosis; cardiomyocytes

Abbreviations:
CI, confidence intervals; PFA, paraformaldehyde; PN, peroxynitrite

Graphical Abstract



Introduction

Peroxynitrite (PN), the product of superoxide-mediated nitric oxide oxidation, has toxic and damaging effects in cells [1]. PN-induced cellular damage is mediated by protein nitration and oxidation of tyrosine residues, anti-oxidant depletion (e.g. inactivation of glutathione peroxidase), and modifications of DNA bases [2]. This impacts on the modulation of several signal transduction pathways including the phosphoinositide 3-kinase/Akt pathway and mitogen activated protein kinase pathways [3, 4]. PN can also lead to executioner caspase activation and apoptosis [5-8]. PN generation plays an important role in oxidative and nitrosative stress in various patho-physiological situations, including myocardial injury, heart failure and cardiomyopathy [9].

RasGAP is an ubiquitously expressed, multi-domain protein that acts as a regulator of Ras and Rho GTP-binding proteins. RasGAP contains two conserved caspase-3 cleavage sites [10]. At low caspase-3 activity levels, RasGAP is cleaved at position 455. The resulting N-terminal fragment (fragment N) activates a potent anti-apoptotic-signaling pathway mediated by the Ras/PI3K/Akt pathway [11], which is crucial for cell survival in low stress conditions [12]. However, at higher caspase 3 activity, fragment N is further cleaved at position 157, abrogating its anti-apoptotic activity [13]. The fragments resulting from this second cleavage (in particular fragment N2 [RasGAP 158-455)]) regulate tumor cell death by sensitizing them to anti-cancer treatment-induced apoptosis [13]. Therefore RasGAP acts as a sensor of caspase activity that controls the balance between cell death and survival. Over-expression of an uncleavable form of fragment N *in vitro* protects various cell types against genotoxins, death receptor ligands, UV-c, staurosporine, and inflammatory cytokines [12, 14]. Moreover, transgenic mice expressing fragment N specifically in pancreatic beta cells are more resistant against pro-diabetogenic conditions and their beta cells experience less apoptosis in such situations [15, 16].

The capacity of fragment N to protect cells against protein nitration- and oxidation-induced death has not been investigated yet. Here, we have evaluated how PN modulates the formation of fragment N and how efficient fragment N was in counteracting PN-induced cell death.

Materials and Methods

Cells

H9C2 cells, a rat heart clonal cell line (ATCC, Manassas, VA) [17], and HEK 293 cells (ATCC, Manassas, VA catalog n°CRL-1573) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, catalog n°61965) supplemented with 10% fetal calf serum (Invitrogen, catalog n° 41Q2174K), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, catalog n° P0781). HeLa cells were maintained in RPMI 1640 (GIBCO catalog n° 61870-10) containing 10% fetal calf serum. All these cells were cultured at 37°C and 5% CO2.

Primary culture of neonatal mouse cardiomyocytes

Hearts were isolated from 1-2 days old new born mice killed by decapitation and placed on ice in ADS buffer [1.1 M NaCl, 1 M Hepes, 0.48 M KCl, 0.1M NaH2PO4 , 0.12 M MgSO4·7H2O, 0.55 M Glucose). Ventricles without atria were digested in ADS containing 0.45 mg/ml collagenase (Worthington Biochemical Corp.) and 1 mg/ml pancreatin (Invitrogen Corp.) as described [18]. Briefly, the tissue homogenates were incubated for 15 minutes at 37 ºC rocking at 700 rpm. The supernatants were collected and the pellets were subjected to two additional rounds of digestion in 1.5 ml ADS containing collagenase and pancreatin. The supernatants collected out from the three digestion rounds were mixed and plated in 10 cm-culture dishes (10 ventricles per dish) for two rounds of 45 minute-differential plating at 37 ºC and 10% CO2. Cells in the supernatants of the first differential plating were collected and replated for second differential plating. The cells in the supernatants of the second plating were collected, washed once in 5 ml of a 3:1 mixture of DMEM and Medium 199 (Invitrogen) supplemented with 10% horse serum (Oxoid), 5% Fetal Calf Serum (Invitrogen), 100 U/ml penicillin, and 0.1 µg/ml streptomycin centrifuged for 10 minutes at 192 *g* at room temperature. Seventy-five thousand cells were seeded onto 0.1% gelatin-coated 24 well-plates.

Chemicals and Antibodies

Peroxynitrite was supplied as a 50 mM stock in 0.3 M sodium hydroxide, (Cayman Chemicals; catalog n°81565). Hoechst 33342 was from Roche (catalog n°H-1399). It was diluted in water at a final concentration of 10 mg/ml and stored at 4°C in the dark. The anti-RasGAP antibody was from Enzo Life Science (catalog number ALX-210-860-R100) and is directed at the fragment N2 moiety of the human protein (amino acids 158-455). The anti-phospho serine 473-Akt rabbit polyclonal IgG antibody as well as anti caspase-3 monoclonal antibodies were from Cell Signaling Technology (catalog n°9271 and 9665 respectively). The mouse polyclonal IgG antibody recognizing sarcomeric alpha-actinin was from Sigma (catalog n°A78811). The monoclonal antibody specific for the HA tag was purchased as ascites from Babco (Richmond,CA; catalog n°MMS-101R). This antibody was adsorbed on HeLa cell lysates to decrease non-specific binding as previously described [10]. Secondary antibodies for Western blot were donkey anti-mouse fluorescein-conjugated antibody and donkey anti-rabbit Cy3-conjugated antibody (Jackson ImmunoResearch, catalog n°715-095-1507 and catalog n°711-165-152, respectively). The secondary antibody used in the immunocytochemistry was Cy3-conjugated AffiniPure Goat anti-Mouse IgG (H+L) from Jackson Immuno Research Laboratories (catalog n°115-165-146). Alexa Fluor 680 goat anti-rabbit IgG (H+L) (Molecular Probes; catalog n°A21109) and IRDye 800-conjugated affinity purified anti-Mouse IgG (H+L) (Rockland catalog n°610-132-121) were the secondary antibodies used on Western blots. HRP-goat anti-mouse and HRP-goat anti-rabbit antibodies were from Jackson Immuno Research (catalog n°211-035-146 and 211-035-109, respectively). The chemiluminescent HRP substrate was from Witec AG (catalog n°K-12043-D20)

Plasmids

HA-hRasGAP[1-455](D157A).lti (#353) (previously called N-D157A.lti) is a lentiviral vector encoding the uncleavable form of fragment N [14]. **HA-hRasGAP[1-455].lti** (#769) is a lentiviral vector encoding the HA-tagged wild-type form of fragment N. It was constructed by sub-cloning the 1423 bp fragment isolated from the BamHI/XhoI digestion of HA-hRasGAP[1-455].dn3 (#14) (previously named HA-GAPN.dn3 and described in [10]) into the TRIP-

PGK-ATGm-MCS-WHV (#349) lentiviral vector opened with the same restriction enzymes. **Myr-mAkt1-HA.cmv** (#249), which encodes a constitutively active form of Akt that bears a Src myristoylation sequence at its N-terminus and an HA tag at its C-terminus, was described earlier under the name myr-Akt.cmv [11]. Plasmid **hIkB alpha delta N2.cmv** (#11) encodes the human IkBa protein with the DN2 deletion (i.e. amino acid 3–71); this constructs cannot be phosphorylated by IkB kinases and degraded by the proteasome and therefore functions as an inhibitor of NF-κB. It has been described before under the name of IkBαΔN2 [11]. The **pEGFP-C1** (#6) plasmid encoding the green fluorescent protein (GFP) is from Clonetech. **Stag.dn3** (#763) was generated by sub-cloning the following two oligonucleotides into pcDNA3 (#1; Invitrogen) opened with HindIII and XbaI: oligo # 806 [AGCTT (N2-N6 of HindIII site), CCCGGG (SmaI), CCACC (Kozak; N6 of SmaI provides the first nucleotide of the Kozak sequence), ATG (start codon), AAA GAA ACC GCT GCT GCT AAA TTC GAA CGC CAG CAC ATG GAC AGC (S·tag), TAA (stop codon), T (N1 of XbaI site)] and oligo # 807 [CTAGA (N5-N1 of XbaI site), TTA (stop codon), GCT GTC CAT GTG CTG GCG TTC GAA TTT AGC AGC AGC GGT TTC TTT (S Tag), CAT (start codon) GGTGG (Kozak sequence), CCCGGG (SmaI), A (N6 of HindIII site)]. **Stag-hRasGAP[158–455].dn3** (#754) encodes the S-tagged form of fragment N2 (S·tag-N2) [19]

Lipofectamine transfection

H9C2 cells were co-transfected with a GFP-encoding plasmid in combination with the indicated plasmids using lipofectamine 2000 (Life Technologies; catalog n°11668-019). After transfection, the cells were incubated in serum-containing medium for 48 hours at 37°C, 5% CO_2. At that time, the percentage of GFP-positive H9C2 cells is about 30%.

Apoptosis scoring based on nuclear morphology

Apoptosis was determined by scoring the number of cells displaying pycnotic nuclei as described previously [20].

Apoptosis scoring based on quantitation of cytoplasmic histone-associated-DNA fragments

Apoptosis was monitored by quantifying the cytoplasmic histone-associated-DNA fragments using cell death detection ELISA plus from Roche (catalog n°1-774-425-001). After the indicated treatments described in Fig. 5, cells in 24 well-plates were washed once with ice-cold PBS and then lysed in 200 µl of the kit lysis buffer for 30 minutes on ice. The lysates were centrifuged at 200 g for 10 minutes. Supernatants contain the cytoplasmic fraction while pellets contain the nuclei and high molecular weight non-fragmented DNA. Twenty µl of the supernatants were transferred to streptavidin-coated 96-well microplate to which 80 µl of an immuno-reagent mixture containing two antibodies, a biotin-labeled anti-histone antibody which binds to H1, H2A, H2B, H3 and H4 histones and that will therefore retain histone proteins in the wells through biotin-streptavidin binding, and a HRP-labeled anti-DNA-POD antibody which reacts with single or double stranded histone-associated DNA. The plates were then incubated for 2 hours at room temperature with gentle shaking (300 rpm). After this incubation period the wells were washed 3 times with 250 µl of the incubation buffer, 100 µl of HRP substrate were added, and the plate was incubated at room temperature for an additional 15-20 minute period with gentle shaking. The reaction was stopped by adding 100 µl of stop solution. Absorbance was measured at 405 nm in triplicates. In each experiment a background control (incubation buffer only mixed with the ABTS stop solution) and a negative control (untreated cells) were performed. The background value was subtracted to all measurements. An enrichment factor, corresponding to the level of mono- and oligo-nucleosomes released to the cytoplasm, was calculated by dividing the corrected absorbance from the experimental conditions with the corrected negative control value.

Stimulation with PN

PN was delivered to the cells as a single bolus at a 1:100 dilution against one side of the dish, while rapidly swirling the medium to ensure optimal exposure of the cells to PN, which was used at final concentrations ranging from 12.5 to 500 µM, which are in agreement with the concentrations of PN used in previous *in vitro* studies investigating the mechanisms of PN cytotoxicity [4,

17, 21]. Although such concentrations may appear elevated at first glance, it is noteworthy that, due to the rapid decomposition of PN, the cells are actually exposed to lower concentration of this agent. Indeed, it has been determined that bolus addition of 50-500 µM PN to cultured cells resulted in an actual exposure of 5-50 µM of the oxidant. Furthermore, the concentrations of PN used in our study are physiologically relevant, as it has been estimated that the rate of PN generation may reach up to 1 mM min^{-1} in an inflamed organ such as the lung *in vivo*.

Lentiviral infection

Recombinant lentiviruses were produced as described previously [14].

Western blot

Cells were lysed in monoQ-C buffer (70 mM β-glycerophosphate, 0.5% Triton X-100, 2 mM MgCl2, 100 mM Na3VO4, 1 mM dithiothreitol, 20 µg/ml aprotinin) and protein quantification was performed by the Bradford technique. Equal amounts of proteins were subjected to SDS PAGE and then transferred onto a nitrocellulose membrane (Biorad catalog nº162 0115). The membranes were blocked with TBS-Tween 20 0.1% containing 5% non-fat milk and incubated over night at 4°C with the specific primary antibodies. Blots were then washed with TBS-Tween 0.1%, incubated with the appropriate secondary antibody (1:5000 dilution) 1 hour at room temperature and subsequently visualized and quantified with the Odyssey infrared imaging system (LICOR Biosciences, Bad Homburg, Germany) or with the Chemidoc XRS+ system (Biorad laboratories). In some instances, the blots were stripped and reprobed. This was performed by incubating the blots at 50°C for 30 minutes in stripping buffer (62.5 mM Tris-base, 100 mM β-mercaptoethanol, 1% SDS, pH to 6.7) followed by three 20 minute-long washes at room temperature in TBS-tween 0.1%.

Immuno-cytochemistry

Cells were grown on glass coverslips, two days post-infection, the cells were fixed and immuno-cytochemistry was performed as previously described [22].

Statistical analysis

Results are shown as mean ± 95% confidence intervals. The statistical analyses used in this study were one-way ANOVAs and paired Student's test. In the latter case, the difference between the indicated conditions was considered significant when $p < 0.05/n$, where p is the probability derived from the t test analysis and n is the number of comparisons done (Bonferroni correction). Statistically significant differences are indicated by asterisks.

Ethics Statement

Experiments on the mice were carried out in strict accordance with the Swiss Animal Protection Ordinance (OPAn) and in conformation with directive 2010/63/EU of the European Parliament. The protocol was approved by the Veterinary office of the state of Vaud, Switzerland (approval reference number 2185).

Results

Pattern of PN-induced RasGAP cleavage in H9C2 cells

In order to investigate the pattern of RasGAP cleavage upon PN stimulation, we exposed H9C2 embryonic rat heart myoblast cells [23] to a dose of 250 μM of PN for different periods of time (Fig. 1). Consistent with previous studies [8], starting 2 hours post stimulation, PN induced increased levels of caspase-3 activation and apoptotic cell death (Fig. 1A and C). This was accompanied by cleavage of RasGAP into fragments N and N2 (Fig. 1B). In subsequent experiments, the 4 hour post PN stimulation time point was chosen as it allowed PN to efficiently induce its cellular responses.

Exposure of H9C2 cells to increasing doses of PN induced cleavage of caspase-3 activation (Fig. 2A) and RasGAP into fragment N (Fig. 2B). This correlated with Akt activity induction (Fig. 2D). However, there were no conditions where fragment N was produced without a concomitant generation of fragment N2 (Fig. 2B). Moreover, fragment N production did not correlate with cell survival (Fig. 2C) and caspase-3 activation (Fig 2A). These results indicate that PN is not able to induce sequential cleavage of RasGAP and accumulation of fragment N in H9C2 cells that would result in a cell protection response.

Cisplatin induces sequential cleavage of RasGAP in H9C2 cells

The absence of sequential cleavage of RasGAP in PN-treated H9C2 cells could be due to a peculiarity of this cell line. To assess this point, H9C2 cells were treated with increasing doses of cisplatin, which is known to induce sequential cleavage of RasGAP in other cell types [12]. Fig. 3B shows that low concentrations of this genotoxin (< 10 μM) led to fragment N generation in H9C2 cells before fragment N2 was substantially produced and that this correlated with cell survival (Fig. 3C) and mild caspase-3 activation (Fig. 3A). Akt stimulation in these conditions was not increased (Fig. 3D). At higher concentrations of cisplatin (>10 μM), fragment N2 levels markedly increased (Fig. 3B), caspase-3 was strongly activated (Fig. 3A), levels of active Akt decreased (Fig. 3D), and apoptosis occurred (Fig. 3C). These results

demonstrate that partial cleavage of RasGAP into the anti-apoptotic RasGAP fragment N can occur in H9C2 cells as seen in other cells types [12].

PN induces RasGAP cleavage in a non-sequential manner

To assess whether the inability of PN to induce a sequential cleavage of RasGAP could be observed in other cell types than H9C2 cells, HeLa cells were treated with increasing concentrations of this compound. PN induced a similar pattern of caspase-3 activation, RasGAP cleavage, increased Akt activation, and apoptosis in HeLa cells as compared to H9C2 cells (Fig. 4). These results indicate that when PN induces the cleavage of RasGAP and fragment N generation, fragment N is rapidly processed further into smaller fragments. Hence, there are no concentrations of PN that preferentially generate fragment N and that correlate with cell survival, low caspase-3 activity and Akt activation.

Ectopic expression of a caspase-resistant form of fragment N protects cardiomyocytes from PN-induced apoptosis

One possiblity to explain the lack of protection mediated by the endogenously generated fragment N in response to PN is its rapid cleavage. Therefore, if fragment N were not to be degraded, it could counteract PN-induced death. In order to test this possiblity, wild-type fragment N and the uncleavable D157A form were ectopically expressed in H9C2 cells (Fig. 5A) before challenging them with low and high PN concentrations. As hypothesized, wild-type fragment N was degraded in response to PN stimulation while the cleavage resistant mutant was not (Fig. 5B and C). Over-expression of both fragment N forms inhibited caspase-3 activation and apoptosis induced by low PN concentration (50 μM) (Fig. 5B and D), although for apoptosis this did not reach statistical significance in the case of wild-type fragment N (Fig. 5D). At high PN concentration (250 μM), only the D157A fragment N mutant inhibited caspase-3 activation and apoptosis (Fig. 5 B and D), indicating that fragment N, if not degraded, efficiently protects cells against PN-induced death.

Fragment N has been shown to activate the prosurvival Akt kinase [11]. To investigate the prosurvival signals induced by fragment N in cardiomyocytes stimulated or not with PN, we monitored the levels of Akt-Ser^{473} phosphorylation in conditions where the wild-type and uncleavable D157A forms of fragment N were overexpressed. As expected in control H9C2 cells (i.e. cells not stimulated with PN), both forms of fragment N stimulated Akt phosphorylation (Fig. 5B; compare the 0 μM lanes). PN was found, as reported earlier [4, 24], to promote Akt-Ser^{473} phosphorylation (Fig. 5B; first three lanes). However, in the presence of 50 μM of PN, Akt activation was more pronounced in H9C2 cells over-expressing fragment N (Fig. 5B; compare the 50 μM lanes). In the presence of 250 μM of PN, Akt was strongly stimulated and no further increase was brought upon over-expression of fragment N (Fig. 5B; compare the 250 μM lanes).

To clarify the potential role of Akt in protecting cardiomyocytes against PN, H9C2 cells were transfected with a myristoylated constitutively active form of Akt (myr-Akt) and their sensitivity to PN-induced apoptosis was monitored. The myr-Akt protein was, as expected from its constitutive activity, phoshorylated on Ser^{473} (Fig. 5E) and was able to inhibit apoptosis induced by low PN concentrations (50 μM). However at high PN concentrations (250 μM), myr-Akt no longer protected cells (Fig. 5E). These results suggest that Akt activation can mediate the protection induced by fragment N in cardiomyocytes subjected to low concentrations of PN but that there are other pro-survival signals activated by fragment N that allow cardiomyocyte survival at high PN concentrations.

One possibility that could explain the differential capacity of wild-type fragment N and the D157 form of the fragment to protect cardiomyocyte is that the former can be cleaved into smaller fragments (e.g. fragment N2) that have been shown to sensitize tumor cells to apoptosis induced by various stimuli [13, 25, 26]. We therefore determined whether fragment N2 can sensitize H9C2 cells, which despite having been immortalized are however not tumor cells [23], to PN-induced apoptosis. Figure 5F shows that ectopic expression of fragment N2 in H9C2 cells did not modulate their sensitivity to PN-induced death. This indicates that the lower capacity of fragment N to protect cardiomyocyte from PN-induced apoptosis compared to the

uncleavable fragment N mutant likely results from its degradation rather than the generation of pro-apoptotic fragments such as N2.

Finally, we investigated whether fragment N was able to protect primary cardiomyocytes, the purity of which was assessed by α-actinin staining (Fig. 6A). Ectopic expression of wild-type fragment N and the D157A caspase-resistant form of the fragment both led to Akt activation in primary cardiomyoctes (Fig. 6B; compare the 0 μM lanes). Wild-type fragment N inhibited caspase-3 activation in primary cardiomyocytes stimulated with low, but not high, concentrations of PN. In contrast, the D157A fragment N mutant was able to efficiently reduce caspase-3 activation even at high PN concentrations. This ability of the D157A form of fragment N to inhibit caspase-3 activation was associated with a marked reduction in PN-induced primary cardiomyocyte apoptosis (Fig. 6C-D). Thus, the pathways activated by fragment N are able to efficiently protect cardiac cells from PN-induced cell death.

Discussion

Earlier work has shown that RasGAP is cleaved by caspase-3 sequentially in response to increasing concentrations or doses of stress-inducing agents. This was seen in different cell types exposed to cisplatin, UV light, Fas ligand and doxorubicin [12, 13]. In contrast we provide evidence here that PN does not promote sequential cleavage of RasGAP regardless of whether the cells used are of cardiomyocyte origin or not. No concentration of PN was found that would lead to the partial cleavage of RasGAP into fragment N and that would be associated with cell survival. Fragment N was generated in cells treated with PN but was apparently immediately degraded further into smaller fragments (N1 and N2) that cannot activate the Akt protective pathway [13]. When the wild-type form of fragment N was ectopically expressed in cells, it failed to inhibit PN-induced apoptosis and this was associated with its degradation. In contrast, a caspase-resistant form of fragment N efficiently protected immortalized (Fig. 5) and primary cardiomyocytes (Fig. 6) against PN-induced death. The anti-apoptotic signals generated by fragment N are therefore able to counteract apoptosis induced by PN in cells.

PN has a short half life, ~1.9 sec at pH 7.4 [27], yet it induces potent and adverse effects on cellular processes by modulating signal transduction pathways involving Akt, MAPKs (ERK, c-jun, p38), protein kinase C, and NF-κB (reviewed in [3]). Moreover, PN has the potential to activate multiple caspases including caspase-2, -3, -6, -7, 8 and -9 and thus the cleavage of key cellular proteins such as poly(ADP-ribose) polymerase and lamins [5]. Considering these broad effects on multiple cellular targets, it appears that upon PN stimulation, caspase activation is an all-or-nothing phenomenon. Indeed, PN induces a monophasic caspase-3 activation where fragment N and fragment N2 are concomittantly produced and apoptosis occurs (Fig. 2 and 4). This contrasts with the situation observed with cisplatin that induces a biphasic caspase-3 activation, the first phase (low activation) associated with fragment N production, no formation of fragment N2, and cell survival; the second phase (high activation) associated with fragment N degradation into fragment N2 and apoptosis (Fig. 3).

Interestingly, Akt, the PI3K effector kinase that has been demonstrated to mediate fragment N-induced anti-apoptotic responses [11, 12, 14-16], is strongly activated by PN. The ability of PN to modulate Akt is poorly

understood. In some cell types (pheochromocytoma cells, endothelial cells, macrophages), PN was shown to efficiently inhibit Akt, while in others PN exposure resulted in strong stimulation of the kinase (skin fibroblasts, hepatoma cell lines, neural cells) [3, 28]. It is suggested that it is the nitration capacity of PN that induces Akt inhibition while it is its oxidative properties that mediate Akt activation [3, 28]. Our results indicate that cardiomyocytes and HeLa cells fall in the second category as PN activates Akt in these cells. Nevertheless, activation of Akt by PN in cardiac cells does not impede apoptosis and may therefore represent a futile protection attempt. Yet, expression of a constitutively active form of Akt in cardiomyocytes inhibits apoptosis induced by low concentrations of PN (Fig. 5E). This could therefore be one mechanism used by fragment N to protect cardiomyocyte when the surrounding concentrations of PN are not too elevated. However, fragment N apparently uses additional means to inhibit apoptosis of cardiomyocytes exposed to high concentrations of PN because the active form of Akt does not inhibit apoptosis in these conditions (Fig. 5E). Hence, signaling pathway others than, or in parallel to, Akt activation could participate in the protection mediated by caspase-resistant forms of fragment N in cardiac cells. The NF-κB transcription factor is a downstream target of Akt [29, 30]. However, in the presence of fragment N, Akt-mediated NF-κB activation is inhibited [11, 14, 16]. NF-κB activation leads to cell death of beta cells [31] and the KB epithelial carcinoma cell line [32]. In the heart the role of NF-κB is controversial as it may convey both pro- and anti-apoptotic signals [33]. Nevertheless, recent data are consistent with a detrimental role of NF-κB in heart failure [34]. NF-κB inhibition might be the other signaling event required for fragment N to protect cardiomyocytes as recently shown in pancreatic beta cells [16]. However, inhibition of the NF-κB pathway did not alter cardiac cell apoptosis induced by either low or high concentrations of PN (Fig. S1). This would suggest that fragment N does not use its NF-κB inhibitory capacity to protect cardiomyocytes against PN-induced death. It cannot however be excluded that it is the interplay between Akt activation and NF-κB inhibition generated by fragment N that induces a more potent survival response than when Akt and NF-κB are regulated separately. The protective pathways regulated by fragment N need to be better characterized as they

represent potential targets for the development of cardiac protective therapies.

The loss of adult cardiomyocytes by necrosis or apoptosis is a major factor in the initiation and progression of heart failure as it contributes to the decline in myocardium function [35]. This process is mediated at least in part by PN, which has emerged as a key mediator of cardiomyocyte injury in numerous cardiac pathologies, including myocardial ischemia reperfusion injury, myocarditis, cardiac allograft rejection and anthracycline-induced cardiomyopathy (see [28] for an extended review on this topic). In such conditions, multiple molecular mechanisms have been shown to contribute to peroxynitrite-mediated cytotoxicity, ultimately resulting in the demise of cardiac myocytes, both via the necrotic and apoptotic pathways [10]. Therefore, in light of our current *in vitro* findings, future *in vivo* studies should be designed to assess the importance of fragment N cleavage by PN as a contributing mechanism to cardiomyocyte death in the aforementioned pathological conditions.

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Figures legends

Fig. 1. Apoptosis and RasGAP cleavage kinetics following PN stimulation. One hundred fifty thousand H9C2 cells were seeded in 6-well plates cultured overnight. One day later, the cells were washed once with PBS, placed in phosphate-buffered saline and stimulated with 250 µM PN for 20 minutes. After PN stimulation, the cells were placed in complete culture medium for the indicated periods of time before being lysed. The levels of caspase-3 activation (panel A) and the extent of RasGAP cleavage (panel B) were determined by Western blotting using an antibody recognizing both pro-caspase-3 and the cleaved, active form of the protease, and an antibody recognizing RasGAP fragment N2 sequences, respectively. In both cases, actin levels were also evaluated by reprobing the blots with an actin-specific antibody. The graphs under the blots present the quantitation of the Western blot band intensities normalized to the highest values. (C) Alternatively, the cells were fixed with 2% PFA and apoptosis was determined by scoring cells with pyknotic or fragmented nucleus. In each panel, data correspond to the mean ± 95% CI of three independent experiments.

Fig. 2. PN does not induce sequential cleavage of RasGAP in H9C2 cells. H9C2 cells were treated as in Figure 1 with the indicated PN concentrations for 20 minutes. After PN stimulation, the cells were placed in complete culture medium for an additional 4 hour-period before being lysed. Caspase-3 activation (panel A), RasGAP cleavage (panel B), and apoptosis (panel C) were assessed as described in Figure 1. (D) Akt activation was investigated by Western blot analysis using a phospho-serine^{473} Akt-specific antibody. The blot was reprobed with an actin-specific antibody. Finally, the blot was stripped and probed with an antibody recognizing total Akt. Quantitation of the Western blot band intensities for caspase-3 and RasGAP was performed as in Figure 1. Phospho-Akt band intensities were normalized to the total Akt intensities and the resulting ratios were normalized to the highest values. In each panel, results correspond to the mean ± 95% CI of three independent experiments.

Fig. 3. Cisplatin induces a sequential cleavage of RasGAP in cardiac cells. H9C2 cells were seeded and cultured as in Figure 1. The cells were then incubated with the indicated concentrations of cisplatin for 24 hours. Caspase-3 activation (panel A), RasGAP cleavage (panel B), apoptosis (panel C), and Akt activation (panel D) were assessed as described in Figure 2.

Fig. 4. PN does not induce sequential cleavage of RasGAP in HeLa cells. HeLa cells were treated and analyzed as described for H9C2 cells in Fig. 2.

Fig. 5. Fragment N-mediated protection against PN in H9C2 cells. H9C2 cells (80,000) were infected with an empty lentivirus or with lentiviruses encoding the HA-tagged wild-type form (HA-N) or the caspase-resistant D157A form [HA-N(D157A)] of fragment N. (A) Three days after infection the efficiency of fragment N expression was determined by immunocytochemistry. About 85% of the cells were found to express fragment N. (B) The cells were stimulated for 20 minutes with the indicated concentrations of PN and incubated for a further 7 hour-period, then starved for one hour in serum-free medium to ensure low basal Akt-Ser^{473} phosphorylation. Finally the cells were lysed and expression of HA-tagged fragment N, the phosphorylation of Akt at serine 473, and caspase-3 cleavage were assessed by Western blotting using the indicated antibodies. (C) The expression levels of HA-N and HA-N(D157A) were normalized to actin and further normalized to the expression value of control cells (0 µM PN). (D) Alternatively, three days post-infection, the cells were treated as indicated in panel B. The cells were then were fixed and the percentage of apoptotic cells determined. (E) Overexpression of a constitutive active Akt form protects cardiomyocyte against apoptosis induced by intermediate PN doses. H9C2 cells (150,000) were co-transfected with a GFP-expressing plasmid with either an empty vector or a plasmid encoding the constitutively active myr-Akt construct. The expression levels of activated Akt were detected by Western blotting as indicated in Figure 2. Alternatively the

transfected were treated with the indicated concentrations of PN for four hours and apoptosis was then scored in GFP-positive cells. (F) Fragment N2 has no effect on PN-induced apoptosis. H9C2 cells (150'000) were co-transfected with a GFP expressing plasmid (GFP.dn3) in combination with S·tag- or S·tag-N2-expressing plasmids. S·tag-N2 expression levels were detected by immune-blotting using HRP-conjugated S-protein. Alternatively the transfected cells were treated with the indicated concentrations of PN for four hours and then apoptosis was scored in GFP-expressing cells. Results in panels C-F correspond to the mean ± 95% CI of three independent experiments performed in monoplicate (panels C-D) or in duplicate (panels E-F). Statistical significance was assessed by one-way ANOVAs.

Fig. 6. A caspase-resistant fragment N protects primary cardiomyocytes from PN-induced apoptosis. Seventy-five thousands primary cardiomyocytes were seeded in 24-well plates and cultured for three days. (A) The cells were then infected wih an empty lentivirus or with lentiviruses encoding the HA-tagged wild-type form (HA-N) or the caspase-resistant D157A form [HA-N(D157A)] of fragment N. The purity of the cardiomyocyte preparation was monitored by immuno-fluorescence using an alpha actinin-specific antibody, and the efficiency of fragment N expression was determined by immunocytochemistry. (B) The cells were stimulated for 40 minutes with the indicated concentrations of PN and then treated and analyzed as indicated in Figure 5B. Alternatively, the infected cardiomyocytes were left untreated or stimulated for 40 minutes with 50, 100 or 250 µM PN. Eight hours later, apoptosis was determined by scoring the percentage of cells with pyknotic or fragmented nuclei (panel C) or by measuring the extent of DNA-coated histones released from nuclei (panel D). Results correspond to the mean ± 95% CI of three independent experiments performed in triplicates. The indicated p values were derived from paired t-tests. Asterisks indicate statistically significant differences after Bonferonni corrections.

Figure1

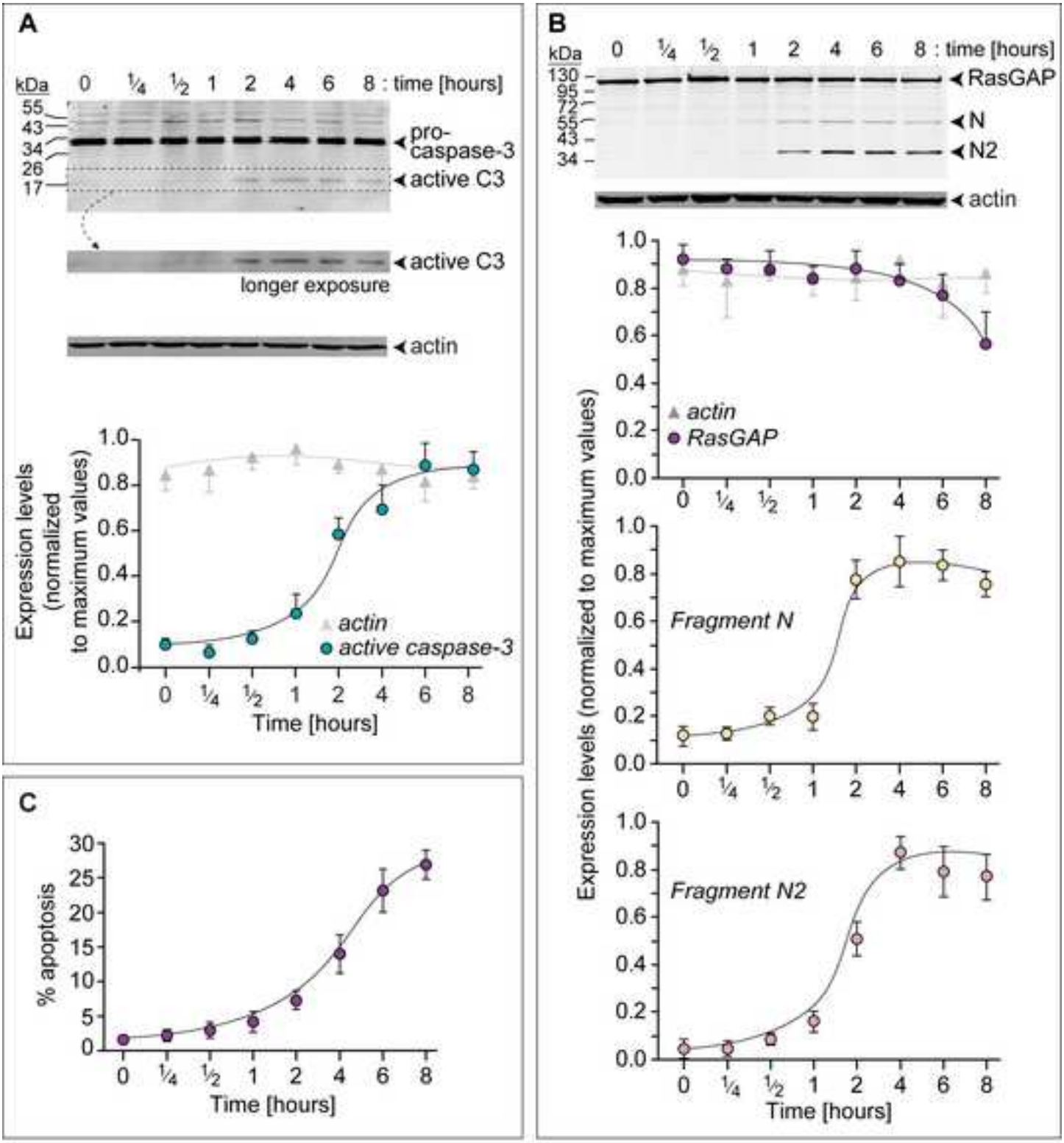


Figure 1

Figure2

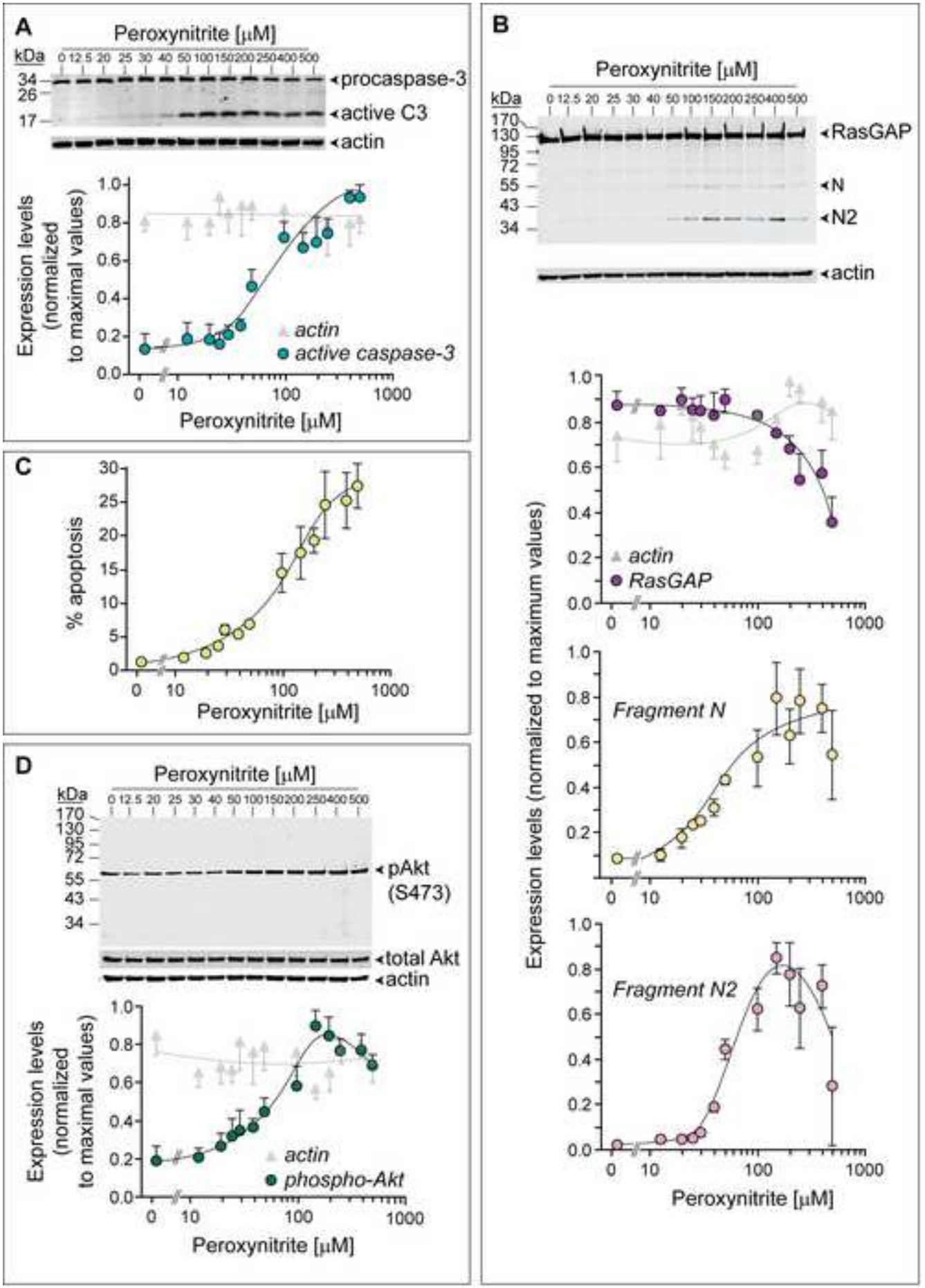
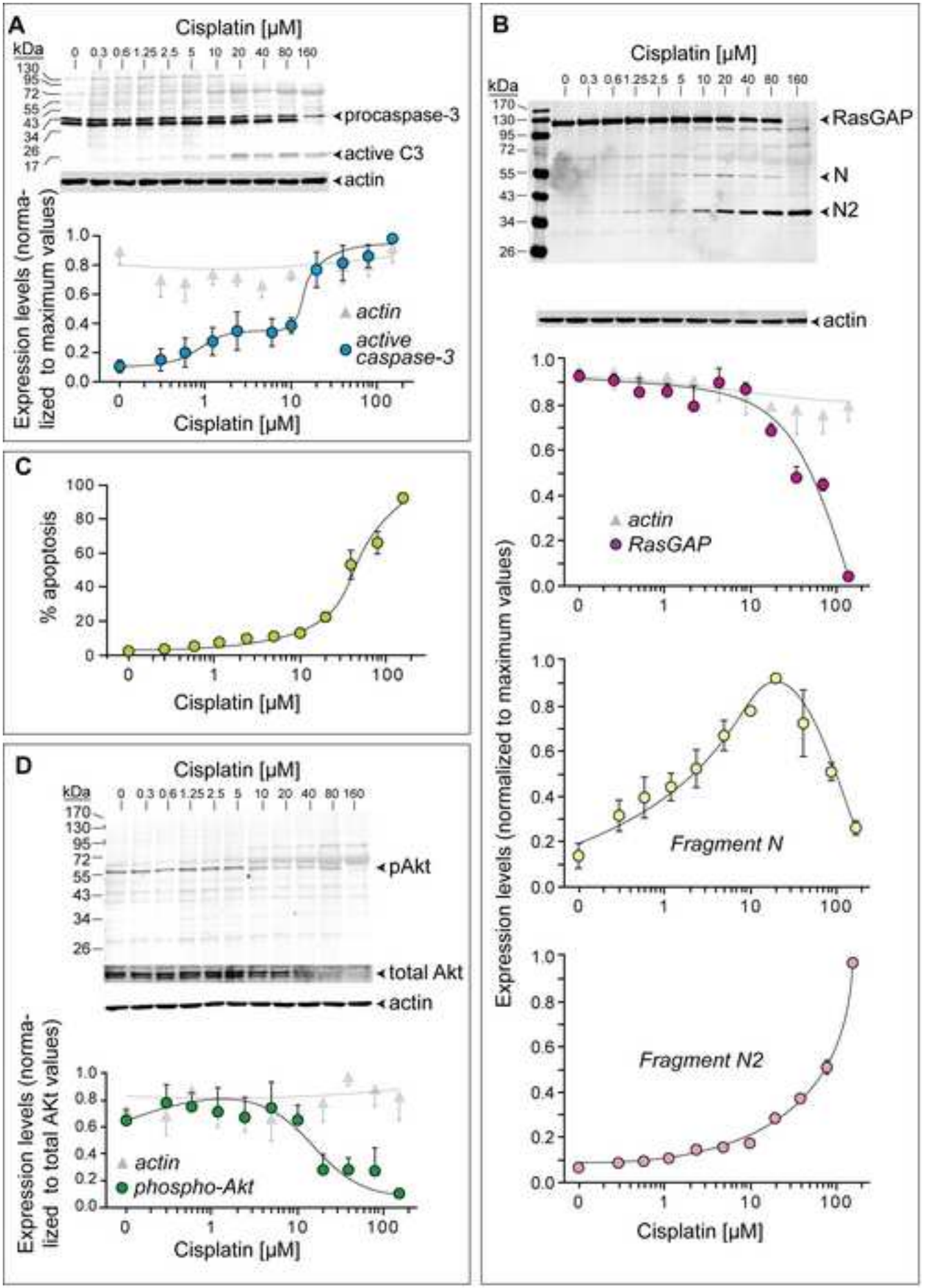


Figure 2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95
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55
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34
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17
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active C3
actin
Expression levels (normalized to maximum values)
actin
active caspase-3
Cisplatin [μM]
B
Cisplatin [μM]
kDa
170
130
95
72
55
43
34
26
RasGAP
N
N2
actin
actin
RasGAP
Fragment N
Fragment N2
Expression levels (normalized to maximum values)
Cisplatin [μM]
C
% apoptosis
Cisplatin [μM]
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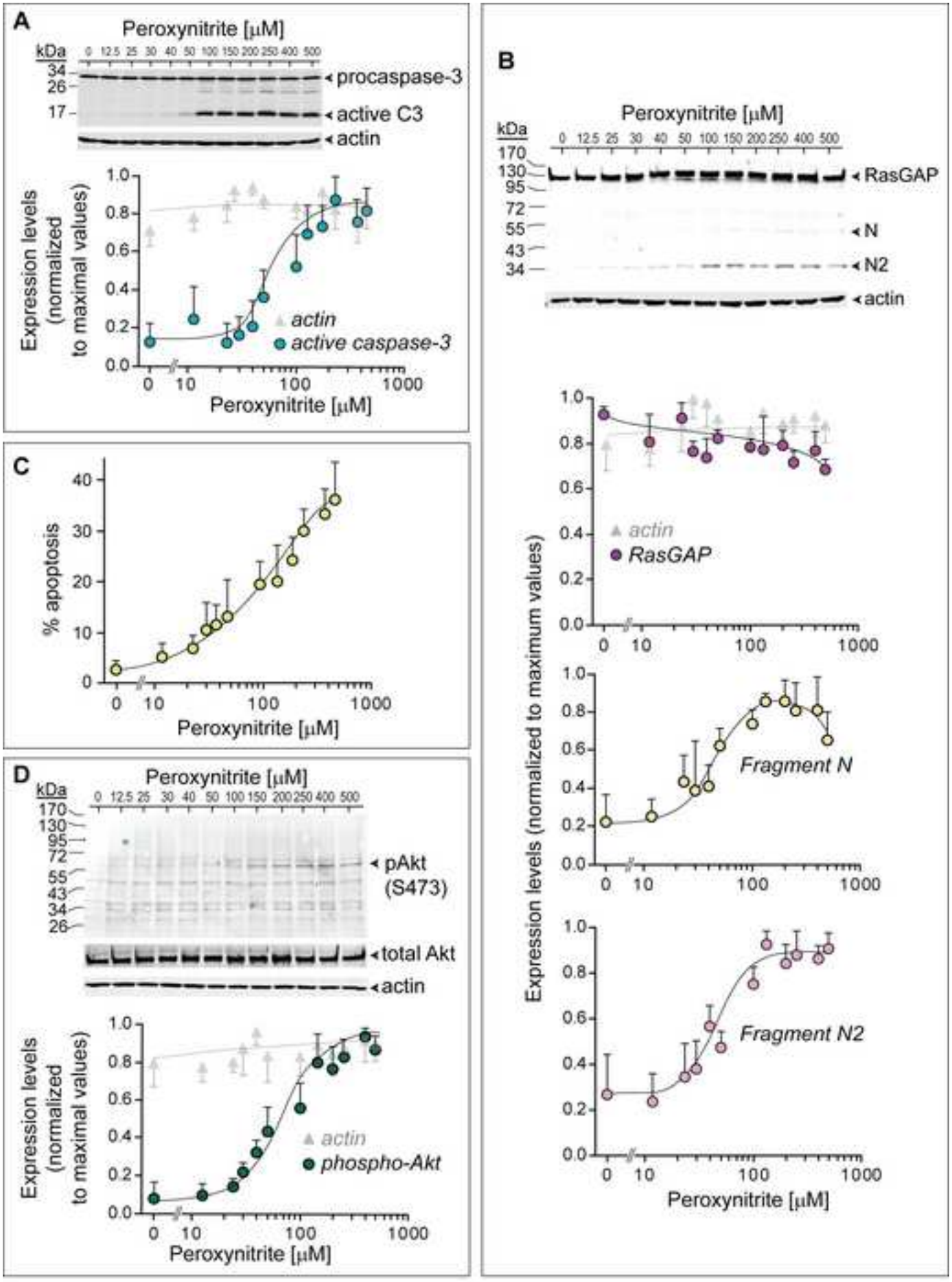
actin
phospho-Akt
Cisplatin [μM]

Figure 3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kDa
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34
26
17
procaspase-3
active C3
actin
Expression levels (normalized to maximal values)
actin
active caspase-3
Peroxynitrite [μM]
B
Peroxynitrite [μM]
kDa
170
130
95
72
55
43
34
RasGAP
N
N2
actin
actin
RasGAP
Fragment N
Fragment N2
Expression levels (normalized to maximum values)
Peroxynitrite [μM]
C
% apoptosis
Peroxynitrite [μM]
D
Peroxynitrite [μM]
kDa
170
130
95
72
55
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actin
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phospho-Akt
Peroxynitrite [μM]

Figure 4

Figure5

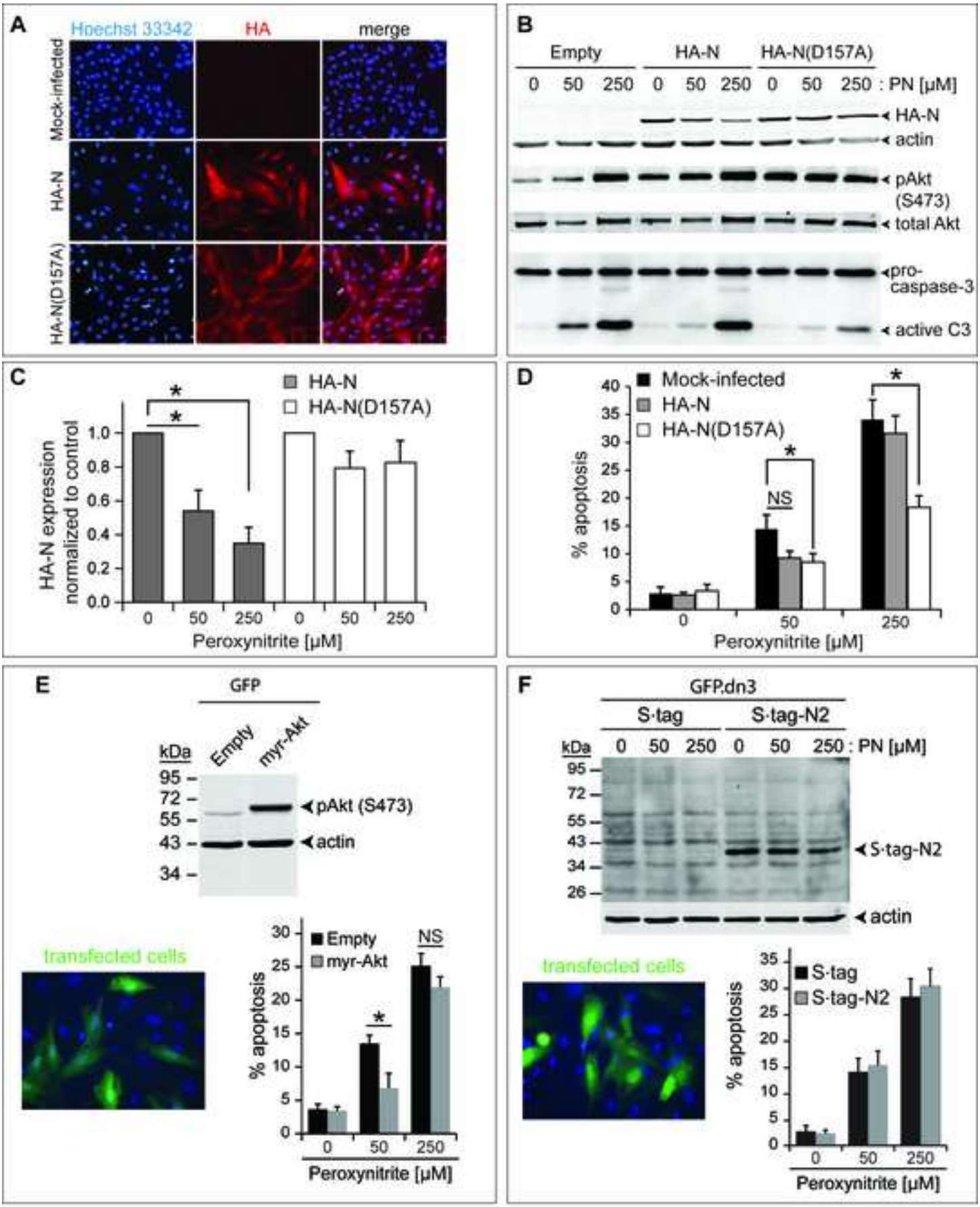

Figure 5

Figure6

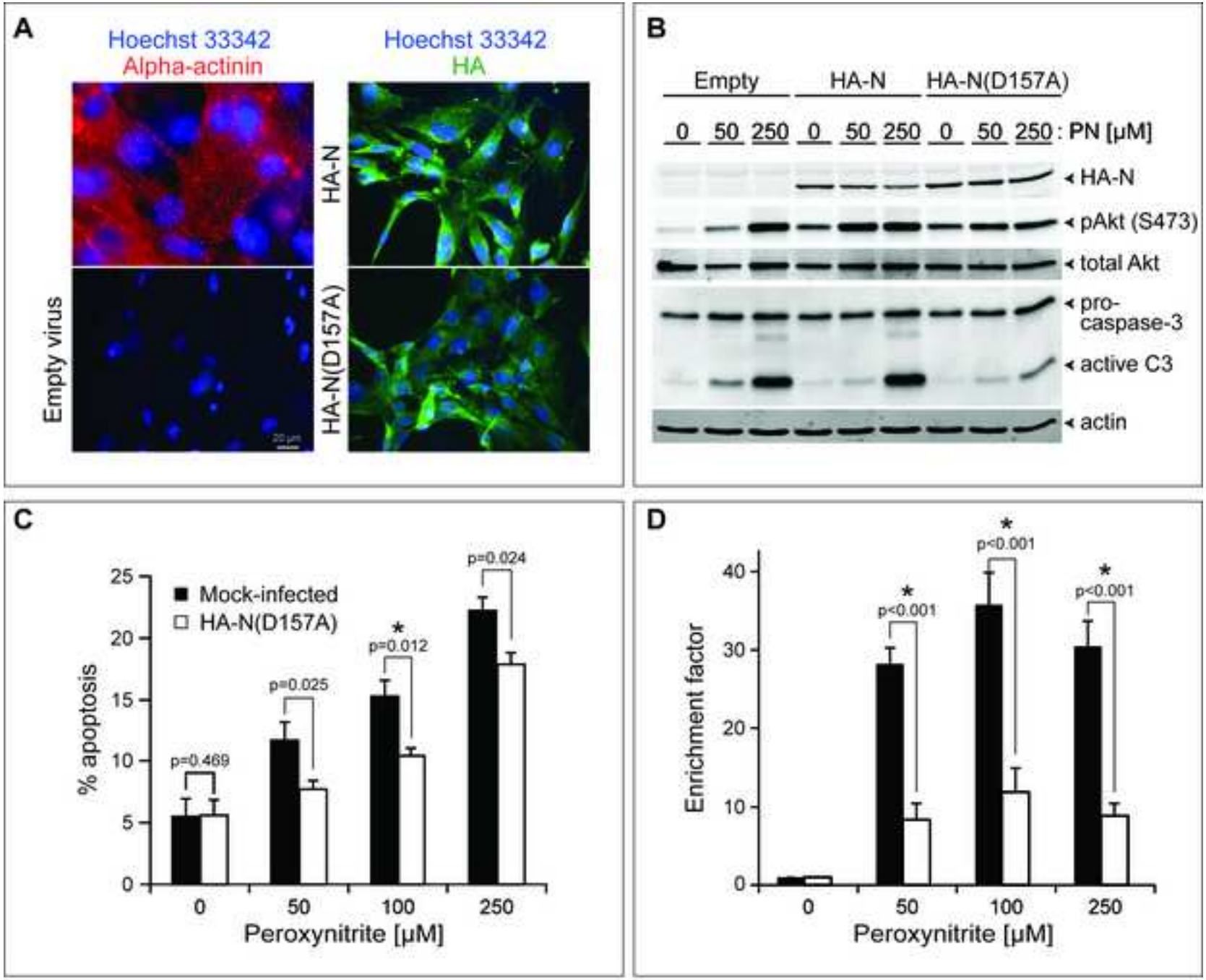

Figure 6