Analysis of nitric oxide-induced apoptotic signaling in PC12 rat phaeochromocytoma cells

PhD thesis

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I. Introduction

1. Nitric oxide

Nitric oxide (nitrogen monoxide; NO) is a gaseous neurotransmitter and signaling molecule able to regulate numerous physiological and pathological processes in the cell. NO is synthesized *in vivo* by members of the nitric oxide synthase (NOS) enzyme family. NO passes the cell membrane by simple diffusion and after leaving the cell, it can activate autocrine and paracrine signal transduction.

Intracellular NO may influence the life of cells many ways. On one hand, it is involved in post-translational protein modifications, namely S-nitrosylation (reversible) and nitration (irreversible). S-nitrosylation is the binding of NO to cysteine residues that alters the function of the affected protein; this may be inhibition (e.g. caspase-3; XIAP, X-linked inhibitor of apoptosis protein) or activation (Ras proteins). NO can be removed from cysteine residues by means of an enzymatic reaction called denitrosylation (or trans-nitrosylation when NO is transferred to a cysteine residue of an other protein); several enzymes including thioredoxin and protein disulphide isomerase (PDI) are responsible for this. Denitrosylation may also occur non-enzimatically. In case of nitration, NO binds to tyrosine residues of proteins. On the other hand, NO is able to bind to proteins containing transition metals (haemoglobin, myoglobin, soluble guanylate cyclase, etc.). Moreover, NO may activate the enzyme soluble guanylate cyclase resulting in the production of the second messenger cyclic guanosine monophosphate (cGMP) that, in turn, activates protein kinase G (PKG). Activation of PKG may lead to relaxation of smooth muscles in blood vessels, platelet aggregation or cell proliferation. NO exerts its effect in a cell type- and concentration-dependent manner. Low levels of NO are considered to be protective, they might be involved in learning and memory, while high NO concentrations possess pro-apoptotic capacity. NO often causes apoptosis indirectly; it is weakly reactive by itself but can react with other molecules thereby producing reactive nitrogen species (RNS). For instance, peroxynitrite anion is produced when NO reacts with superoxide. Peroxynitrite is capable of reacting with lipids and DNA, and may lead to apoptosis by causing permeabilization of the mitochondrial membrane. S-nitrosylation of certain proteins under nitrosative stress is also cytotoxic; S-nitrosylation of the PDI and XIAP proteins has already been shown to be involved in neuronal cell loss in case of many neurodegenerative disorders.

Application of the gas NO is problematic, NO donor compounds are used during experiments instead; we applied sodium nitroprusside (SNP) when treating cultured cells. High doses (400 μM) of SNP cause cell death in wild-type PC12 rat phaeochromocytoma

cells and PC12 subclones expressing dominant negative mutant proteins. Similarly to NO, SNP is also capable of activating guanylate cyclase thereby increasing the level of cGMP.

2. Cellular stress / stress signaling / MAPK cascades

Cells react to numerous environmental stimuli; the biological answer depends on the type and intensity of the stimulus, the signaling pathways activated and the cell type as well. Mitogen-activated protein kinase (MAPK) cascades play an important role in the intracellular mediation of environmental signals. These cascades consist of three levels in eukaryotic cells, each level is represented by a protein family.

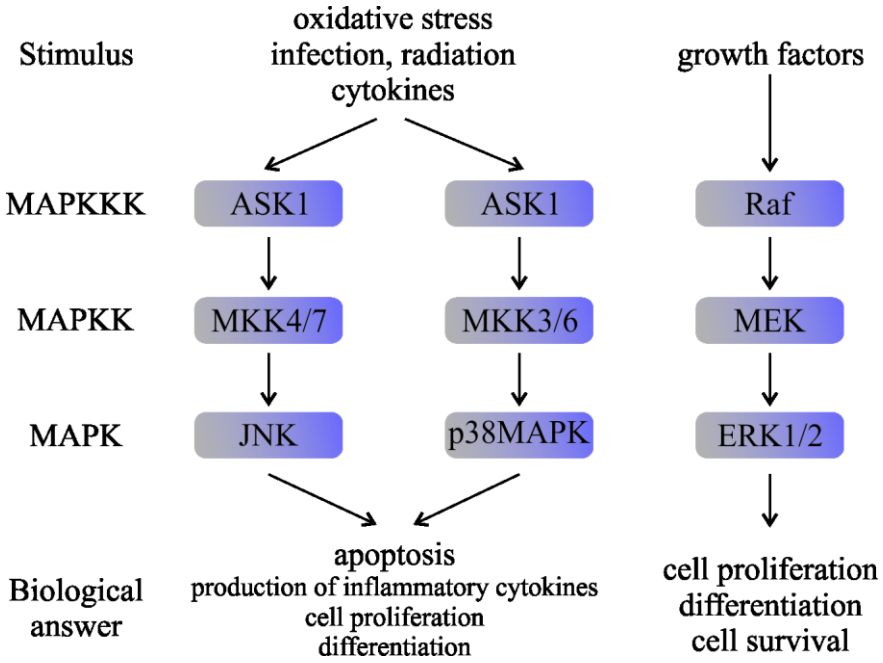

Figure 1: *Summary of MAPK signaling*

At first, proteins belonging to the mitogen-activated protein kinase kinase kinase (MAPKKK) family undergo activation, then they activate members of the mitogen-activated protein kinase kinase (MAPKK) family by means of serine/threonine phosphorylation. The latter family of proteins phosphorylates members of the MAPK family on tyrosine and/or threonine residues; MAPKs are serine/threonine specific protein kinases. Transcription factors may be target proteins of MAPKs; their phosphorylation leads to altered gene expression resulting in the biological answer. MAPKs can be divided into three major groups: c-Jun N terminal kinases (JNK1-3), p38MAPKs (p38α-δ) and extracellular signal regulated kinases (ERK1/2); JNKs and p38MAPKs are often called stress kinases (*Fig. 1*).

2.1. Stress signaling mediated by JNK and p38MAPK pathways

JNK and p38MAPK signaling can be activated by many stimuli among others oxidative and endoplasmic reticulum (ER) stress, viral and bacterial infections, death ligands/inflammatory citokines (e.g. tumor necrosis factor, TNF), UV and ionizing radiation, chemotherapeutic drugs and the increase in the cytoplasmic Ca^{2+} concentration. All these impacts may activate the protein named apoptosis signal-regulating kinase 1 (ASK1) that is a member of the MAPKKK family. ASK1 activation leads to JNK phosphorylation carried out by the kinases MKK4 and MKK7, while p38MAPK is phosphorylated by the MKK3 and MKK6 proteins. Active JNK and p38MAPK proteins not necessarily induce cell death; they can also lead to cell proliferation, differentiation and production of inflammatory citokines. Stress stimuli can induce survival signaling as well; in most cases only sustained JNK and p38MAPK activation induces apoptosis. Disorders of the ASK1-MAPK signaling may be involved in several diseases, such as cardiovascular, neurodegenerative and inflammatory disorders, tumors and other diseases (asthma, diabetes).

2.2. The ERK pathway

The activation mechanism of the ERK pathway is similar to other MAPK cascades: the monomeric G protein Ras activates Raf (MAPKKK), then Raf phosphorylates MEK (MAPKK) that, in turn, activates ERK1/2 (MAPK). Although ERK signaling can be induced by stress, its best-known activators are mitogenic stimuli. The most possible outcomes of ERK activation are cell proliferation, terminal differentiation and cell survival. The over-activation of the Ras/ERK pathway is known to be involved in many steps of tumor formation (migration, invasion, survival of tumor cells). Upon stress, ERK activation might leads to the induction of genes coding for anti-apoptotic proteins (Bcl-2, B cell lymphoma-2; Bcl-xL, B cell lymphoma-extra large; Mcl-1, myeloid cell leukemia 1) and repression of genes that encode pro-apoptotic proteins (Bim, B-cell lymphoma 2 interacting mediator of cell death; Puma, p53 upregulated modulator of apoptosis; Bmf, Bcl-2 modifying factor). Moreover, ERK can phosphorylate members of the Bcl-2 protein family leading to either stabilization (Mcl-1) or degradation (Bad, Bcl-2-associated death promoter).

3. Apoptosis

Apoptosis is the best-known, most examined, evolutionary conserved type of cell death. It takes part in morphogenesis, maintenance of tissue homeostasis, aging and elimination of damaged and unwanted cells. Its abnormal function leads to diseases (tumor, autoimmune and neurodegenerative disorders, etc.). During apoptosis, the cell membrane

stays intact and cells die without damaging neighbouring cells/tissues or inducing inflammation. Apoptotic cells display several morphological and functional characteristics enabling apoptosis detection using molecular biological techniques. The most common signs of apoptosis are: internucleosomal DNA fragmentation, release of apoptotic bodies, phosphatidylserine externalization and activation of the appropriate signaling pathways/proteins. The two main pathways of apoptotic signaling are the extrinsic (receptor mediated) and intrinsic (mitochondrial) pathways.

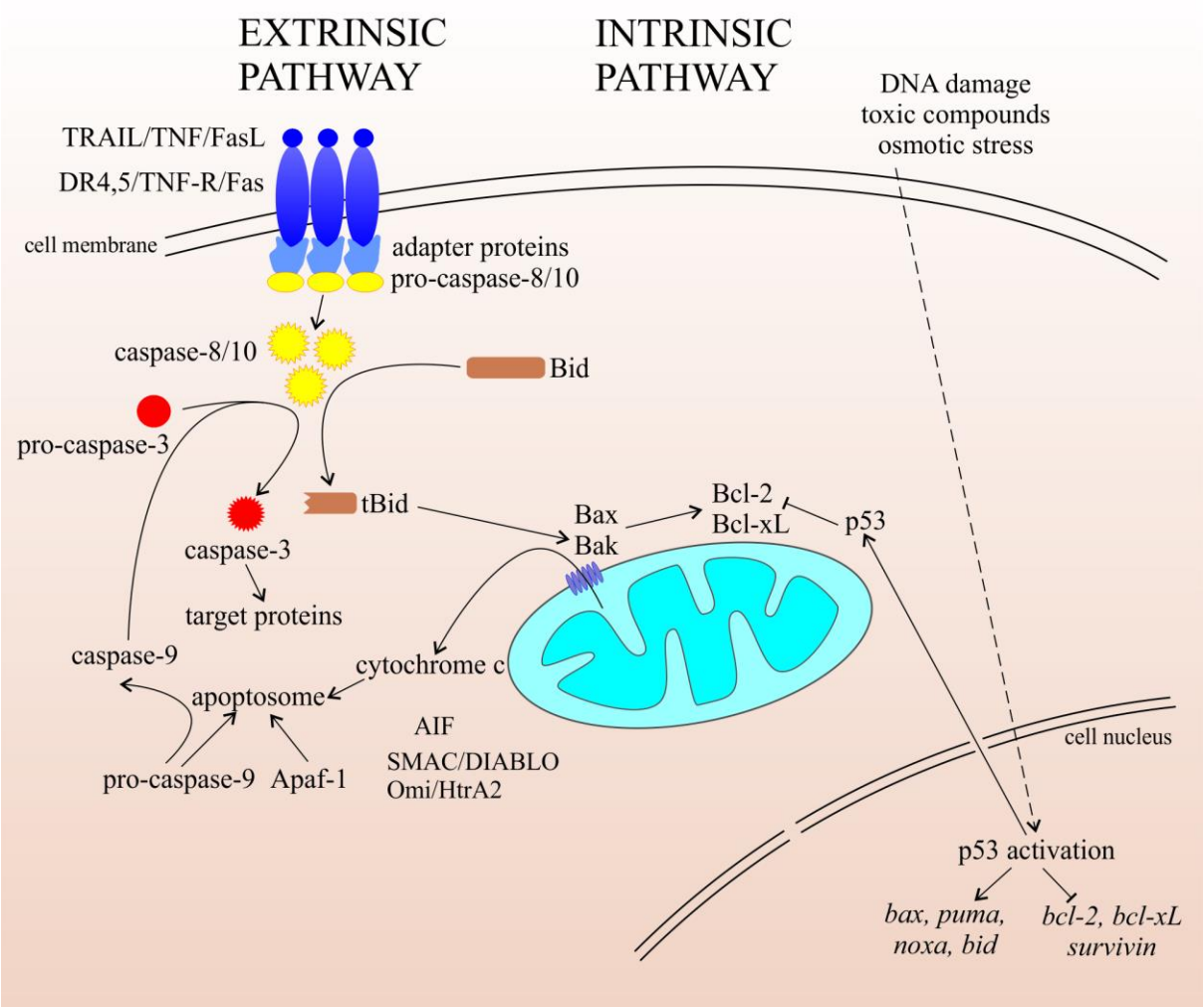

Figure 2: *Summary of the extrinsic and intrinsic apoptotic pathways*

3.1. Extrinsic pathway of apoptosis

Extrinsic apoptosis signaling is activated by ligands that bind to their cell surface receptors (*Fig. 2*); TNF and other members of the TNF family (TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FasL, Fas-ligand, etc.) are such death ligands. Having bound to the appropriate receptor (for TRAIL: DR4 and DR5, death receptor 4 and 5; for

FasL: Fas), a multi-protein complex (DISC, death-inducing signaling complex) is formed on the intracellular domain of the receptor that further transmits the signal. DISC contains adapter proteins (FADD, Fas associated death domain containing protein; TRADD, TNF receptor associated death domain containing protein, etc.) and inactive initiator procaspases (procaspase-8 and -10), the latter ones undergo proteolytic self-cleavage leading to their activaton. Afterwards, the active initiator caspases can take part in the activation of effector procaspases (procaspase-3, -6, -7) via two mechanisms. In type I cells, caspase-8 and -10 are capable of cleaving caspase-3 directly; while in type II cells, extrinsic and intrinsic apoptotic pathways are linked together. In these cells, caspase-8 molecules activated by death receptors cleave a „BH3-only" protein named Bid (BH3 interacting-domain death agonist). The truncated form of the protein (tBid) translocates to mitochondria and activates the Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist/killer) proteins. Signaling mediated by death receptors is not exclusively involved in apoptosis but may take part in development and inflammation, indeed, it can induce cell survival as well.

3.2. Intrinsic pathway of apoptosis

Intrinsic apoptosis signaling is activated by stimuli exerting their effect intracellularly (with the mechanism mediated by tBid being the exception) (*Fig. 2*). Such stimuli are DNA damages that cannot be repaired, toxic agents or osmotic stress. Key players of the intrinsic pathway are members of the Bcl-2 protein family and the p53 transcription factor.

3.2.1. The Bcl-2 protein family

The Bcl-2 protein family includes both anti-apoptotic (inhibit apoptosis) and pro-apoptotic (induce apoptosis) proteins. The ratio of pro- and anti-apoptotic proteins determines the fate of the cell (survival vs. cell death); it leads to several diseases (tumors, autoimmune disorders, etc.), if the balance is disturbed. Anti-apoptotic proteins possess 4 Bcl-2 homology (BH) domains, their best-known representatives are the Bcl-2 and Bcl-xL proteins; both are often over-expressed in tumors. Pro-apoptotic proteins can be further classified according to their structure and function: (1) pro-apoptotic multidomain proteins are called Bax and Bak, they are built up from BH1-3 domains; (2) members of the „BH3-only" subfamily contain no other but the BH3 domain, such proteins are for example Bad, Puma, Noxa and Bid.

Pro-apoptotic multidomain proteins take part in apoptosis by undergoing oligomerization and thereby forming pores in the outer membrane of mitochondria thus leading to mitochondrial outer membrane permeabilization (MOMP). This allows cytochrome c and other proteins (Smac/DIABLO, second mitochondria-derived activator of caspase/direct

IAP-binding protein with low pI; AIF, apoptosis-inducing factor; endonuclease G; Omi/HtrA2, high temperature requirement protein) to be released from the mitochondrial intermembrane space into the cytosol. Endonuclease G and AIF are then translocated into the nucleus, where they contribute to chromatin condensation and DNA fragmentation. The Smac/DIABLO and Omi/HtrA2 proteins bind to and thereby inhibit members of the IAP (inhibitor of apoptosis protein) family thus enhancing caspase activation. Release of cytochrome c into the cytosol is essential for apoptosome formation; besides cytochrome c, this complex contains the apoptotic peptidase-activating factor-1 (Apaf-1) and the inactive procaspase-9 as well. Apoptosome is a platform, where procaspase-9 can execute its proteolytic self-activation thereby becoming able to cleave effector procaspases. Cleavage of other proteins is then fulfilled by effector caspases (caspase-3, -6 and -7). Anti-apoptotic Bcl-2 proteins counteract cytochrome c release by binding Bax/Bak and „BH3-only" proteins involved in Bax/Bak activation.

3.2.2. The p53 protein

Although initially it was considered to be an oncoprotein, p53 (widely known as „the guardian of the genome") is maybe be best-known tumor suppressor protein. First it was thought that its sole function is the regulation of transcription but later it became clear that it possesses transcription-independent activities as well.

Mutations affecting the *p53* gene have been found in several tumors; they are mainly localized to the DNA-binding domain and are of the missense type (the consequence is the exchange of an amino acid). More than 45.000 somatic and germ line mutations have been found in the *p53* gene so far that often lead to the full inactivation of the protein; however, mutant p53 proteins can acquire oncogenic features or are able to inhibit their wild-type counterparts in a dominant negative manner. Besides mutations, normal p53 function can be lost by binding to viral/cellular proteins, epigenetic mechanisms (promoter hypermethylation) and increased degradation as well. Genes regulated by mutant p53 code for proteins involved in survival and proliferation of tumor cells, chemoresistance, genomic instability, inflammation, migration, invasion, angiogenesis and metastasis formation. Germ line mutation/inactivation of the *p53* gene causes the development of the rare Li-Fraumeni syndrome.

The most important functions of the p53 protein are: (1) regulation of the cell cycle, (2) induction of genes encoding proteins involved in DNA repair and (3) regulation of apoptosis. Beyond the aforementioned activites, it regulates autophagy, metabolism of (tumor) cells, vesicular transport, cytoskeleton organization, endocytosis and necrosis.

Full length p53 consists of 393 amino acids (391 in rat). The transactivation domains (TAD1 and 2) and the proline-rich region are located to the N terminus followed by the DNA-binding domain (DBD), the C terminus includes the tetramerization/oligomerization domain (OD) and a regulatory region; the p53 protein possesses nuclear localization and nuclear export signals as well (NLS and NES, respectively). TAD1 and 2 are involved in the regulation of gene expression and provide binding sites for many proteins (e.g. Bcl-2; Bcl-xL; p300/CBP; Mdm2, mouse double minute 2). The proline-rich region is required for the tumor suppressor function of p53 and induction of apoptosis in mitochondria. Most p53 mutations are localized to the DNA-binding domain; there are six „hot-spot" sites most commonly mutated (Arg175His, Gly245Ser, Arg248Trp, Arg249Ser, Arg273His, Arg282Trp). The p53 protein binds to the DNA in tetrameric form, the oligomerization domain is responsible for tetramer formation; NLS and several ubiquitination sites are also found there. Transcription of the *p53* gene produces 8 different mRNA molecules that are translated to 12 isoforms. All the aforementioned domains are present in the full length protein. The other isoforms, however, partially or totally lack them; these isoforms are produced by alternative promoter usage, alternative splicing or alternative initiation of translation. The various p53 isoforms are found in normal tissues but their expression may be altered in tumor cells and due to pathogens (for example H. pylori enhances the expression of certain isoforms in the epithelial cells of the stomach thus contributing to cell survival).

The p53 level is kept low in normal cells because the ubiquitin ligase Mdm2 (Hdm2 in humans) enhances proteasomal degradation of the protein. Upon stress, the p53 level rises but then the p53 protein acting as a transcription factor induces expression of the *mdm2* gene thereby leading to a decline in its own level (negative feedback). Loss of p53 function may arise from overexpression of Mdm2 as well.

The p53 protein is involved in the intrinsic apoptotic signaling by means of two main mechanisms. On one hand, it acts as a transcription factor being able for both activating and inhibiting gene expression: it may induce the *bax, puma, noxa, bid, bad, apaf1, smac/diablo* and repress the *bcl-2, bcl-xL, mcl-1, survivin* genes. On the other hand, it can act in a transcription-independent fashion: it regulates the activity of the Bcl-2 family proteins by binding to them. It is able to bind to the Bcl-2, Bcl-xL and Mcl-1 proteins resulting in Bax/Bak activation; but it is also capable of directly activating Bax and Bak. The p53 protein may stimulate the expression of genes coding for proteins taking part in extrinsic apoptotic signaling, for example Fas, FasL and DR5. The p53 protein may enhance transcription of non-coding RNA genes (micro RNA, long non-coding RNA and long intergenic non-coding RNA molecules) and regulate miRNA maturation.

The p53 protein is targeted by numerous post-translational modifications (phosphorylation, acetylation, ubiquitination, sumoylation, neddylation, methylation) that influence both its function and intracellular localization. The multi-mono-ubiquitination carried out by Mdm2 results in the nuclear export of p53, while poly-ubiquitination leads to its proteasomal degradation. 20 serine/threonine and 6 lysine residues have already been identified that undergo stress-induced phosphorylation and acetylation, respectively. DNA damage may cause phosphorylation of the p53 protein. Phosphorylation affecting the serine 15 residue results in p53 stabilization. Serine 46 phosphorylation of p53 (1) enables mitochondrial translocation and then, via binding to Bcl-2, mitochondrial outer membrane permeabilization; (2) causes p53 stabilization allowing transcription of pro-apoptotic genes. Other amino acids within the protein also undergo DNA damage-induced phosphorylation leading to the release of Mdm2 from the transactivation domain of p53; the consequence is the stronger binding of p300/CBP and the enhanced transcriptional potential of p53.

4. The PC12 cell line

Since the establishment of the cell line in 1976, PC12 rat phaeochromocytoma cells are widely used. Due to the relatively short generation time and easy maintenance, this cell line is a well-known model system used for the analysis of signal transduction pathways involved in cell survival, apoptosis and neuronal differentiation. Besides wild-type PC12 cells, PC12 subclones expressing mutant (dominant negative or constitutively active) proteins are also being used in the Department of Medical Biology. Such a subclone is named p143p53PC12; the dominant inhibitory mutant p53 protein expressed by these cells contains alanine instead of valine in position 143 (V143A). As a consequence of this, mutant p53 proteins have lost their ability of sequence-specific DNA binding and have become unable to act as transcription factors. Since p53 regulates transcription in tetrameric form, the presence of a single mutant protein in the tetramer leads to the inactivation of p53. The Val143Ala-mutant p53 protein is known to be less stable than its wild-type counterpart but it may be capable of enhancing the expression of certain genes.

II. Aims

High doses of the NO donor compound SNP cause cell death in PC12 cells. We intended to clarify the followings:

1. Analysis of signaling pathways/proteins activated by the cellular/nitrosative stress evoked by SNP and NO released from it.
2. Investigation of molecular biological mechanisms underlying the higher SNP sensitivity of p143p53PC12 cells expressing a mutant p53 protein.

Apoptosis induced by high SNP concentrations can be inhibited by dbcGMP or low dose (100 μM) SNP pre-treatments in murine macrophage cells. By adjusting the experimental conditions to our PC12 model system, we aimed to investigate the followings:

1. Possible prevention of SNP-induced apoptosis by 100 μM SNP pre-treatment.
2. Decreasing the pro-apoptotic potential of certain other stimuli (serum starvation, cisplatin, tunicamycin, anisomycin) by low-dose SNP pre-treatment.
3. Analysis of signaling pathways providing partial protection against apoptosis upon 100 μM SNP pre-treatment.

III. Materials and methods

1. Cell lines

Wild-type PC12 rat phaeochromocytoma and p143p53PC12 cells were used; the latter one is a PC12 subclone expressing a dominant negative mutant version of the p53 protein. Cells were cultured under optimal conditions (37°C, 5% CO_2), the culturing medium (DMEM, Dulbecco's Modified Eagle's Medium) was supplemented by 5% fetal bovine and 10% horse sera. The medium p143p53PC12 cells were cultured in contained 200 μg/ml geneticin as well.

2. Analysis of cell viability

ATP assay allows the determination of live cell number in cultures by measuring cellular ATP content. $2x10^3$ cells were plated into the wells of 96-well plates, cell culturing was carried out as described above. Cells underwent SNP treatments: they either were treated for 72 hours with different concentrations of SNP (20, 50, 100, 200, 400 and 2000 μM) or received 400 μM SNP treatment for 2, 4, 6, 8, 18 or 24 hours to determine the time-course of SNP cytotoxicity. Cellular ATP content was measured according to the instructions of the manufacturer.

3. Analysis of DNA fragmentation

10^7 cells were plated onto 100-mm plates prior to treatments. The next day cells were collected by scraping and then subjected to DNA isolation. Cells were lysed in the appropriate buffer,

then cellular debris was removed by centrifugation. Proteins were removed from the resulting supernatant by phenol/chloroform extraction. DNA molecules were precipitated in the mixture of 3M sodium acetate and 96% ethanol, salts were removed by 70% ethanol, while RNA contamination was eliminated by RNase digestion. DNA samples were electrophoresed in ethidium bromide-containing agarose gels, photos of the gel were taken using the Kodak IS440 CF gel documentation system.

4. Western blot analysis

$6x10^6$ cells were plated onto 100-mm plates. After the appropriate treatments cells were harvested and lysed in a buffer containing protease and phosphatase inhibitors. When analysing caspase activation, protein isolation was performed using a special buffer (Chaps cell extract buffer). Protein isolation was followed by heat denaturation, then samples of equal volumes were fractionated by means of SDS polyacrylamide gel electrophoresis (SDS PAGE). After electrophoresis proteins were transferred from the gels onto PVDF membranes and proteins of interest were detected by using the indirect method. Membranes were incubated in 5% solution of milk powder to block non-specific binding sites; blocking was followed by the incubation of the membrane in the solution of the appropriate primary antibody; then horse radish peroxidase (HRP)-conjugated secondary antibodies were applied. The signal was detected with the Kodak IS440 CF gel documentation system. The following antibodies were used: anti-P-p38MAPK, anti-p38MAPK, anti-P-JNK, anti-JNK, anti-P-Akt, anti-P-eIF2α, anti-eIF2α, anti-P-p53Ser15, anti-P-p53Ser46, anti-p53, anti-CHOP, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-caspase-8, anti-cleaved caspase-9, anti-cleaved caspase-3, anti-ERK1/2, anti-cytochrome c, anti-PKR, anti-actin, anti-β-actin, HRP-conjugated secondary anti-rabbit and anti-mouse antibodies.

5. Analysis of cytochrome c release

$6x10^6$ cells were cultured in 100-mm plates. After treatments were completed, cells were harvested and then collected by centrifugation. The resulting pellet was re-suspended in a buffer supplemented by the same protease and phosphatase inhibitors used during protein isolation prior to Western blotting. Suspensions of cells were homogenized and then centrifuged. The supernatant was centrifuged again, then cytoplasmic proteins were isolated from the resulting postmitochondrial supernatant. Re-suspension of the pellet was followed by centrifugation and then used as mitochondrial fraction. Isolation of cytoplasmic and mitochondrial proteins was performed as described above.

6. Examination of nuclear morphology

10^5 cells were plated onto glass coverslips into wells of 6-well cell culture plates; in advance, coverslips have been covered with poly-L-lysine to allow attachment of cells. Half of the samples received 24-hour low-dose (100 μM) SNP pre-treatment the next day; then old media were removed and cells underwent serum starvation, SNP, cisplatin, tunicamycin or anisomycin treatments. After completing the treatments, cells were fixed and nuclear DNA was stained by a fluorescent dye

(Hoechst 33342). Specimens were then mounted and cells with apoptotic nuclear morphology were examined in a fluorescence microscope (Olympus BX-61).

7. Immunocytochemistry

$5x10^3$ cells were plated onto plastic coverslips into wells of 96-well plates. Completion of treatments was followed by fixation and permeabilization of cells; non-specific binding sites were blocked with 5% solutions of either milk powder or bovine serum albumin. Samples were then incubated in solutions containing the appropriate primary antibodies. The next day fluorescently labelled secondary antibodies were applied; cell nuclei were visualized by staining with the fluorescent dye Hoechst 33342. Samples were washed with a buffer containing Triton X-100 between each step. After mounting, localization of proteins was analysed using a laser scanning confocal microscope (Olympus FluoView 1000).

8. TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase-dUTP nick end labeling) assay utilizes that huge amounts of DNA fragments having a free OH group at their 3' ends are generated due to DNA fragmentation during apoptosis. The enzyme terminal transferase can attach fluorescently labelled nucleotides to such OH groups, this can be detected by fluorescence or confocal microscopy. $2x10^4$ cells were plated onto plastic coverslips into wells of 96-well plates. The next day, half of the samples received low concentration (100 μM) SNP pre-treatment that lasted for 24 hours. Having completed the pre-treatments, cells were subjected to certain pro-apoptotic stimuli (serum withdrawal, 400 μM SNP, cisplatin, tunicamycin, anisomycin) followed by fixation and permeabilization. After the reaction catalysed by terminal transferase and the staining of DNA (Hoechst 33342), specimens were examined by laser scanning confocal microscope (Olympus FluoView 1000).

IV. Results

1. The p143p53PC12 cell line is more sensitive to high-dose SNP treatment than wild-type PC12 cells

Cellular viability was determined by performing ATP assay; live cell number can be deduced from cellular ATP content after various treatments. 100 μM and lower concentrations of SNP were not cytotoxic but the 400 μM SNP treatment (lasting for 72 hours) lowered the amount of ATP to approximately 10% in both cells lines; this SNP dose was later used for apoptosis induction. p143p53PC12 cells reacted more sensitively to 20, 50, 100 and 200 μM SNP treatments, with a significant difference between the two cell lines. The vast majority of cells underwent cell death when SNP was applied at 400 and 2000 μM concentrations. Long-term (18- and 24-hour) 400 μM SNP treatment was significantly more toxic to cells expressing the dominant negative p53 protein.

2. SNP treatment leads to more robust stress kinase activation in cells expressing a dominant negative p53 protein

Stress stimuli often lead to the activation of the p38MAPK and JNK proteins. After the 18-hour 400 μM SNP treatment phosphorylation of p38MAPK and JNK could be detected in both cell lines, stress kinase activation was more pronounced in p143p53PC12 cells. High amounts of both phosphorylated stress kinases were detected in wild-type PC12 cells following the 8-hour exposure to SNP; treatments lasting longer than 8 hours were less effective in activating the p38MAPK and JNK proteins. On the contrary, cells expressing the mutant version of the p53 protein displayed permanent p38MAPK and JNK activation. The CHOP transcription factor may be activated by certain stress stimuli thereby contributing to the enhanced expression of genes encoding proteins required during apoptosis (for example Bim, DR5). CHOP induction was the most powerful upon 200 μM SNP treatment in both cells lines, although 400 μM SNP was proved to be more toxic than this concentration. In line with stress kinase activation, rise in the CHOP level was more pronounced and prolonged in p143p53PC12 cells.

3. p143p53PC12 cells react with enhanced protein kinase R cleavage and eIF2α phosphorylation to 400 μM SNP treatment

Viral infection and other stimuli may result in the activation (caspase-mediated proteolytic cleavage) of protein kinase R (PKR). Active PKR can phosphorylate its target proteins, for instance the α subunit of the eukaryotic initiation factor 2 (eIF2α). Phosphorylation of eIF2α can be carried out by other kinases as well but the common consequence is the inhibition (of the initiation) of translation. High doses of SNP (200 and 400 μM) led to the proteolytic cleavage of PKR in both cell lines. PKR activation evoked by 400 μM SNP was the strongest following the 8-hour treatment, while more prolonged treatments (18 and 24 hours) caused less powerful PKR cleavage. The most powerful phosphorylation of eIF2α could be detected when cells were exposed to 400 μM SNP for 18 hours; while there was no remarkable difference regarding PKR activation between the two cell lines, eIF2α phosphorylation was much stronger in p143p53PC12 cells. Only cells with the mutant p53 protein displayed sustained and increasing eIF2α phosphorylation.

4. 400 μM SNP evokes strong p53 induction and phosphorylation in p143p53PC12 cells

The p53 protein, upon undergoing stress-induced activation, can function both in the cell nucleus (as a transcription factor) and in the cytoplasm. Pro-apoptotic stimuli often cause the phosphorylation of serine residues in the 15. and the 46. positions leading to the

stabilization of the protein, thus enhancing transcription of genes involved in apoptosis. When exposed to 200 and 400 μM SNP, both cell lines displayed p53 phosphorylation on both the residues mentioned above. In p143p53PC12 cells, phosphorylation of the p53 protein started earlier, was stronger and lasted longer than in wild-type PC12 cells. Normal cells exhibit low amount of the p53 protein; stress induces a vigorous rise in the cellular p53 level. Increasing amounts of the p53 protein could be detected in both cell lines upon long-lasting (18- and 24-hour) exposure to 400 μM SNP.

5. Toxic dose SNP treatment reduces the level of the Bcl-2 protein

The decline in the level of the anti-apoptotic Bcl-2 protein is a common feature of apoptosis. High-dose and prolonged SNP treatment effectively reduced the Bcl-2 level in both cell lines; this was observable in cells expressing the mutant p53 protein following short-term SNP treatments as well.

6. p143p53PC12 cells display enhanced cytochrome c release and caspase activation upon 400 μM SNP treatment

Activation of the intrinsic apoptotic pathway is accompanied by the release of proteins from the intermembrane space of mitochondria that are of great importance during cell death. Such a protein is cytochrome c, its release into the cytoplasm is essential for the activation of the initiator caspase-9 and the effector caspase-3 proteins. Compared to wild-type PC12 cells, the increase in the cytosolic cytochrome c concentration was more pronounced and could be detected earlier in the p143p53PC12 cell line. There was a difference in the concentration of the anti-apoptotic Bcl-2 and Bcl-xL proteins between the two cell lines; cells with the mutant p53 protein displayed lower amounts of both proteins. The cleaved form of caspase-9 was present in PC12 cells exposed to high SNP doses (200 and 400 μM); lower concentrations of SNP were also capable of causing caspase-9 activation in p143p53PC12 cells. Cleavage of caspase-3 occurred upon toxic-dose (400 μM) SNP treatment only. In both cell lines, activation of both caspases was the strongest if receiving 8-hour 400 μM SNP treatment; however, activation of both caspases was stronger and more prolonged in p143p53PC12 cells.

7. Nuclear translocation of the p53 protein cannot be observed following 400 μM SNP treatment

Immunocytochemistry was performed in order to analyse the intracellular localization of the p53 protein after 400 μM SNP treatment. Although following 2 hours of 400 μM SNP treatment cells showed p53 phosphorylation and cytochrome c release from mitochondria,

nuclear translocation of the p53 protein was not detectable in microscopic images after 2- or 8-hour treatments; there was no difference between the two cell lines in this sense.

8. Low-dose SNP pre-treatment weakens the pro-apoptotic potential of 400 μM SNP in PC12 cells

Internucleosomal DNA fragmentation is a well-known hallmark of apoptosis; the 24-hour exposure to 400 μM SNP caused DNA fragmentation in PC12 cells. Intensity of SNP-induced DNA fragmentation could be lowered by applying low-dose (100 μM) SNP or 0,5 mM dbcGMP pre-treatments, both of which lasting for 24 hours. (100 μM SNP is not cytotoxic in PC12 cells.) PC12 cells displayed powerful JNK and p38MAPK phosphorylation upon 24-hour 400 μM SNP treatment. Activation of both stress kinases could be partially inhibited by the 100 μM SNP and 0,5 mM dbcGMP pre-treatments. Stimuli inducing apoptosis, like 400 μM SNP, are known to reduce Akt phosphorylation. Following 100 μM SNP or 0,5 mM dbcGMP pre-treatments, the decline in the level of Akt phosphorylation was less prominent.

9. 100 μM SNP pre-treatment is able to lower the intensity of internucleosomal DNA fragmentation evoked by serum starvation, cisplatin, tunicamycin or anisomycin

Due to the removal of growth factors, cells reside in the G_0 phase of the cell cycle after serum withdrawal; cells undergo apoptosis in case of prolonged serum starvation. Cisplatin is a chemotherapeutic drug able to bind to purine bases thereby causing cross-linking between the two strands of DNA; this leads to the apoptosis of tumor cells. The antibiotic tunicamycin evokes ER stress by inhibiting N-linked glycosylation; anisomycin causes cell death via the inhibition of translation. All the above mentioned conditions possess pro-apoptotic potential and are thus able to cause internucleosomal DNA fragmentation in PC12 cells. DNA fragmentation evoked by serum withdrawal, cisplatin or tunicamycin could be inhibited if cells received 100 μM SNP pre-treatment; however, the rate of anisomycin-induced DNA fragmentation was only slightly lowered by the pre-treatment.

10. Pre-treatment with 100 μM SNP partly abolishes the pro-apoptotic potential of certain treatments

When exposed to 400 μM SNP, the majority of PC12 cells underwent apoptosis; high number of cell nuclei bearing signs of apoptotic morphology (highly condensed and fragmented chromatin) and containing DNA fragmented at many sites were detectable. All these alterations could be partially eliminated if cells were pre-treated with 100 μM SNP.

Similarly, the pre-treatment was effective in minimising the extent of chromatin condensation and DNA fragmentation evoked by serum starvation and cisplatin; there was no significant difference when comparing tunicamycin-treated cells with or without the pre-treatment. Nevertheless, cells receiving 100 μM SNP prior to anisomycin treatment displayed increased number of cell nuclei showing apoptotic morphology than those not having the pre-treatment.

11. Low-dose SNP pre-treatment effectively reduces the rate of protein phosphorylation and caspase activation caused by certain apoptosis-inducing conditions

400 μM SNP treatment is followed by the activation of stress kinases, the PKR, eIF2α, p53, CHOP proteins and caspases, as well as the decline in the levels of the anti-apoptotic Bcl-2 and Bcl-xL proteins. Pre-treating cells with 100 μM SNP diminished the activation of the aforementioned proteins and the reduction of Bcl-2/Bcl-xL protein levels. The CHOP transcription factor is to be mentioned as the only exception: its induction was further strenghtened by the pre-treatment. Likewise, phosphorylation of the p38MAPK and the eIF2α proteins as well as PKR and caspase cleavage evoked by serum starvation, cisplatin or tunicamycin could all be lowered by the low-dose SNP pre-treatment. On the contrary, the intensity of cisplatin-induced p53 phosphorylation was not weakened by the pre-treatment; in addition, levels of the pro-apoptotic Bax and the anti-apoptotic Bcl-2/Bcl-xL proteins were hardly affected by the treatment/pre-treatment combinations (except for 400 μM SNP). Unlike the other toxic conditions, anisomycin treatment combined with the pre-treatment enhanced JNK and p53 phosphorylation, increased levels of the p53 and the CHOP proteins and led to stronger caspase activation.

12. Pre-treatment with 100 μM SNP reduces the concentration of TRAIL in cell culture medium

High-dose SNP treatment causes cell death via the activation of the intrinsic apoptotic pathway; however, 400 μM SNP caused activation of caspase-8 that is mainly involved in extrinsic apoptosis signaling. Upon binding to the appropriate receptor, the TRAIL protein can activate extrinsic apoptotic signaling; besides this, it may activate intrinsic signaling as well, via inducing the cleavage of the Bid protein. Moreover, TRAIL molecules may be present in exosomes/microvesicles released from cells. Upon 400 μM SNP treatment, increase in the amount of the 34 kDa form of the TRAIL protein could be detected in cell culture medium of PC12 cells. This was not detectable if high-dose SNP treatment was preceded by the 100 μM SNP pre-treatment.

V. Discussion

1. Nitrosative stress

Although low concentrations of NO may facilitate cell survival (by S-nitrosylating caspases, inducing the expression of the *bcl-2* gene, enhancing the phosphorylation of the CREB protein and activating the phosphatidylinositol-3-kinase (PI3K)/Akt pathway), high NO doses are cytotoxic. However, NO does not damage cells directly: it can react with free radicals thus producing reactive nitrogen species (RNS), such as peroxynitrite. The powerful rise in the cellular levels of various reactive oxygen and nitrogen species leads to oxidative or nitrosative stress, respectively. Peroxynitrite causes lipid peroxidation and protein nitration by irreversibly binding to tyrosine residues, thereby mostly leading to the inactivation of the affected protein. Peroxynitrite may bind to numerous proteins in the cell, among others haemoglobin, myoglobin, cytochrome c and NOS isoforms. Peroxynitrite is also known for its ability to cause DNA damage: bases (mainly guanine) as well as the sugar-phosphate backbone can be affected; besides this, it can cause single-strand breaks, may inactivate enzymes involved in DNA repair and the p53 protein as well.

Cellular NO may exert its effect via covalently binding to proteins. The irreversible nitration of tyrosine residues (also called 3-nitrotyrosination) is mediated by peroxynitrite and is mostly harmful for cells. S-nitrosylation is the reversible binding of NO to cysteine residues. If NO levels are low, S-nitrosylation is a physiological process regulating protein function. On the contrary, nitrosative stress arising from high NO concentrations is cytotoxic due to abnormal S-nitrosylation and nitration of proteins; this may contribute to the development of neurodegenerative disorders. Prolonged and increased NO production may lead to the S-nitrosylation of such proteins that are not S-nitrosylated under low/physiological NO concentrations, for example glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH), PDI and XIAP.

2. Mutant p53 proteins and their potential role in apoptosis

A mutant p53 protein is synthesized in p143p53PC12 cells that contain valine instead of alanine in position 143. This mutation affects the DNA binding domain of the protein but does not belong to the „hot spot" sites most commonly mutated in cancer. The p53 protein binds to DNA in tetrameric form; if the complex contains V143A mutant p53 as well, wild-type p53 is not capable of sequence-specific DNA binding either. Wild-type and V143A mutant p53 proteins possess different DNA binding affinity and specificity, DNA binding and transcriptional activity of the mutant protein is known to be temperature-dependent. Various

mutant p53 proteins not necessarily lose their DNA binding capacity; but they bind in a structure-specific manner (there is no such DNA sequence known that is generally/specifically recognized by mutant p53 proteins). Unlike wild-type p53 (that acts as a tumor suppressor), mutant p53 proteins often facilitate cell survival and cell proliferation, because normal and mutant proteins regulate the expression of different genes. Genes regulated by mutant p53 may encode multidrug transporters or growth factor receptors; all these proteins may be overexpressed/too active in cancer. Although, due to the mutation(s) in the DNA binding domain, mutant p53 proteins are unable to regulate the expression of the same genes as wild-type p53 proteins do, they are still capable of inducing apoptosis via transcription-independent mechanisms.

3. NO stimulates intrinsic apoptotic signaling in PC12 cells

SNP was used as NO donor to investigate signaling pathways contributing to the nitrosative stress-induced apoptosis of PC12 cells. When applied at high concentration (400 μM), SNP caused apoptosis in both wild-type and p143p53PC12 cells. NO mainly evokes cell death via the generation of peroxynitrite that is able to cause DNA damage. DNA damage leads to the phosphorylation of the p53 protein; phosphorylation of the serine 15 residue results in the stabilization of the protein, while serine 46 phosphorylation enhances mitochondrial translocation and apoptosis. Following 400 μM SNP treatment both of the above mentioned amino acids underwent phosphorylation; besides this, the level of the p53 protein was raised as well. On one hand, the p53 protein is known to induce the expression of genes encoding pro-apoptotic members of the Bcl-2 family (for example Bax, Puma, Noxa); on the other hand, it may contribute to apoptosis in a transcription-independent manner. In the latter case, p53 resembles a „BH3-only" protein (activates Bax/Bak and inhibits Bcl-2/Bcl-xL) thereby contributing to the permeabilization of the mitochondrial outer membrane followed by the release of cytochrome c and other pro-apoptotic proteins from the mitochondrial intermembrane space. Cytochrome c release is followed by the activation of caspase-9 and -3 resulting in the destruction of the cell by means of apoptosis. After treating cells with 400 μM SNP, release of cytochrome c along with activation of caspase-9 and -3 could be detected. These results indicate that SNP, and NO released from it, cause apoptosis via the stimulation of the intrinsic apoptotic pathway.

4. The p143p53PC12 cell line is more sensitive to the toxic effects of NO

400 μM SNP caused cell death in both wild-type and p143p53PC12 cells; however, cells expressing the mutant p53 protein reacted to the cytotoxic effects of SNP with higher

sensitivity. We examined the activation status and the level of numerous proteins, and all changes were more pronounced and/or more persistent in p143p53PC12 cells. Cells with the V143A mutant p53 protein displayed earlier p53 phosphorylation and an increase in the level of the p53 protein; moreover, cytochrome c release from mitochondria occurred more rapidly in this cell line. In line with these results, differences between the two cell lines could be observed regarding the time course and strength of caspase cleavage: activation of both caspase-9 and -3 started earlier, was more robust and long-lasting in p143p53PC12 cells. Thus we can conclude that the p143p53PC12 cell line displays higher sensitivity towards SNP because, compared to wild-type PC12 cells, the activation of the intrinsic apoptotic pathway is much stronger in them.

Phosphorylation of the serine 46 residue within the p53 protein enables mitochondrial translocation of p53, the stress kinase p38MAPK may be responsible for this phosphorylation event. According to other data, serine 15 phosphorylation may also enhance the mitochondrial translocation of p53. Strong activation of p38MAPK and serine 46 phosphorylation of the p53 protein occurred earlier in p143p53PC12 cells than in the wild-type cell line. The reason may be that in cells with the V143A mutant p53 protein the mitochondrial translocation of p53 occurred earlier; this might be one of the mechanisms underlying the higher SNP sensitivity of the p143p53PC12 cell line.

The p53 protein is not only able to induce gene expression, it may repress genes as well; it is involved in the inhibition of *bcl-2* gene expression indirectly, by binding to the TATA binding protein (TBP). The binding of p53 to TBP requires the N terminal transactivation or the C terminal oligomerization domain of the protein; the DNA-binding domain carrying the point mutation within the V143A mutant p53 protein does not contribute to this. Compared to wild-type PC12 cells, the expression level of the Bcl-2 protein was lower in the p143p53PC12 cell line; when increasing the duration of the 400 μM SNP treatment, the Bcl-2 level was further decreased. It may be possible that V143A mutant p53 proteins are able to repress the *bcl-2* gene, maybe via indirect mechanisms, thereby enhancing the NO sensitivity of the p143p53PC12 cell line.

The ER stress evoked by NO can lead to the induction of the CHOP transcription factor also able to repress the *bcl-2* gene; this might contribute to the high NO sensitivity of cells with the mutant p53 protein. This notion is supported by the observation that the CHOP protein was strongly induced by 400 μM SNP treatment in p143p53PC12 cells; CHOP induction was far less powerful in wild-type PC12 cells.

5. 100 μM SNP pre-treatment lowers the pro-apoptotic potential of certain treatments

SNP, NO released from it and peroxynitrite produced from NO are all able to evoke apoptosis in cell lines other than PC12 as well; although the NO sensitivity of various cells may be different. SNP applied at the 400 μM concentration is sufficient in causing apoptosis of PC12 cells, while in RAW264 murine macrophage cells the cell-killing dose is 4 mM. Upon 4 mM SNP treatment, p38MAPK is phosphorylated, the Bax protein undergoes conformational change and translocates to mitochondria thereby contributing to the release of cytochrome c from the mitochondrial intermembrane space. All the aforementioned events can be inhibited if cells are exposed to 100 μM SNP or 0,5 mM dbcGMP prior to the high-dose SNP treatment.

400 μM SNP induced apoptosis in PC12 cells, accompanied by internucleosomal DNA fragmentation and changes in the activity/level of numerous proteins. 100 μM SNP or 0,5 mM dbcGMP pre-treatments reduced the rate of SNP-induced internucleosomal DNA fragmentation, stress kinase activation and the decrease in the Akt phosphorylation in PC12 cells. Based on these results, some signal transduction pathway/mechanism (not identified yet) able to enhance cell survival/inhibit cell death may be activated by the low-dose NO pre-treatment.

In order to examine whether the 100 μM SNP pre-treatment is able to inhibit apoptosis in general, PC12 cells have been exposed to certain apoptosis-inducing stimuli (namely serum starvation, cisplatin, tunicamycin or anisomycin). Similar to SNP, all these conditions are known to activate the intrinsic signaling pathway of apoptosis. When applying the 100 μM SNP pre-treatment, less powerful DNA fragmentation could be detected upon serum withdrawal, cisplatin or tunicamycin treatments than without the SNP pre-treatment. Other techniques used to detect apoptosis (analysis of nuclear morphology and TUNEL assay) showed the same result: pre-treatment lowered the rate of apoptosis evoked by SNP, serum starvation and cisplatin; it did not significantly affect cell death induced by tunicamycin. Results of Western blotting experiments could not explain the mechanism by which low-dose SNP pre-treatment provides partial protection against apoptosis. 100 μM SNP pre-treatment was effective in reducing p38MAPK phosphorylation caused by 400 μM SNP, cisplatin or tunicamycin. This could lead to the dimished activation of caspase-9 and -3 thereby reducing the rate of apoptosis. However, the exact mechanism partially inhibiting cell death upon low-dose SNP pre-treatment is still unknown.

Activation of p38MAPK can be evoked by various stress stimuli (oxidative stress, DNA damage, etc.). Pre-treatment with low concentration of SNP may inhibit MAPKKKs and MAPKKs mediating p38MAPK phosphorylation; another possible explanation could be

the involvement/activation of phosphatases responsible for the dephosphorylation of the p38MAPK protein.

6. The SNP pre-treatment further strengthens anisomycin-induced cell death

Unless the other cytotoxic compounds, anisomycin treatment preceded by 100 μM SNP pre-treatment was more toxic than anisomycin alone, according to results of nuclear morphology analysis and TUNEL assay. This was supported by results of Western blotting analysis as well; although the 100 μM SNP pre-treatment was effective in diminishing anisomycin-induced p38MAPK phosphorylation, the SNP pre-treatment prior to anisomycin exposure resulted in the enhanced activation of certain proteins (e.g. JNK, PKR, p53, caspases). It may be possible that anisomycin applied at the 1 μg/ml concentration inhibits translation and thereby induces apoptosis in such an effective manner that cannot be prevented by the SNP pre-treatment.

7. The potential role of the TRAIL protein in the apoptosis of PC12 cells

Although all pro-apoptotic stimuli we applied stimulate the intrinsic apoptotic signaling, activation of caspase-8, typically involved in the extrinsic pathway, could also be detected in each case. Caspase-8 may be activated by death ligands through the stimulation of the extrinsic apoptosis signaling; we analysed the amount of the TRAIL protein in the cell culture medium to confirm this notion. The presence of the transmembrane form of TRAIL in the culturing medium indicates that cells release apoptotic bodies or extracellular vesicles. The latter category includes two main types named microvesicles and exosomes; they differ in both the size and the way of formation. Their primary function is intercellular communication but they may be linked to certain diseases as well. They are involved in the transportation of proteins, lipids and nucleic acids (mRNA, miRNA and non-coding RNA molecules).

K562 cells expressing TRAIL produce such exosomes that induce apoptosis in tumor cells. It is possible that extracellular vesicles containing TRAIL in their membrane are released from PC12 cells; via binding to the corresponding cell surface receptor, TRAIL may activate extrinsic apoptotic signalling in an autocrine/paracrine manner. Although this hypothesis requires further confirmation, it may be supported by results showing that the pre-treatment preceding 400 μM SNP treatment reduced the level of TRAIL in the cell culture medium and partly inhibited the activation of caspase-8 as well.

8. Possible significance of the work/project

Diseases affecting the central nervous system are often characterized by the production of abnormally folded proteins; their accumulation evokes ER stress. This can liberate the ER from the load exerted by abnormal proteins through the inhibition of translation and the „correction" of defective protein conformation. Protein conformation can be repaired by chaperone proteins like PDI; the other function of PDI is the rearrangement of disulphide bonds, if they are not formed between the appropriate cysteine residues. If protein homeostasis cannot be restored, the cell undergoes apoptosis. Various isoforms of the PDI family have been identified in neurodegenerative disorders (Alzheimer's, Parkinson's, Creutzfeldt-Jakob diseases; amyotrophic lateral sclerosis, ALS). Plaques consisting of the amyloid-β peptide and aggregation of the tau protein are typical of Alzheimer's disease. In the dopaminergic neurons of patients with Parkinson's disease so called Lewy bodies are found that contain abnormally folded α-synuclein proteins; huge complexes of the huntingtin protein in nerve cells are associated with Huntington's disease. Neurons of patients suffering from ALS are characterized by protein aggregates built up from superoxide dismutase (SOD), neurofilament and other proteins. Oxidative and nitrosative stress affecting neurons are factors possibly contributing to the formation of the abovementioned protein aggregates. Nitrosative stress can cause the abnormal S-nitrosylation of certain proteins; this may lead to abnormal protein folding, mitochondrial function and transcription may be disturbed, neurons and synapses can be damaged; all these events might result in neurodegenerative disorders. S-nitrosylation of the ubiquitin-ligase parkin might contribute to the development of Parkinson's disease, because this reduces the activity of parkin and the effectiveness of proteasomal protein degradation resulting in the formation of Lewy bodies and neuronal cell loss. Abnormal S-nitrosylation inhibits PDI as well, contributing to the aforementioned protein aggregation. Certain diseases are not only caused by S-nitrosylation; enhanced protein nitration, mainly mediated by peroxynitrite, may also be harmful. Nitration of tyrosine (or far less commonly tryptophane) residues results in the inactivation of the affected protein and the formation of protein aggregates. Nitration of α-synuclein can be detected in Parkinson's disease, while nitrated tau proteins and amyloid-β peptides are signs of Alzheimer's disease. Moreover, tyrosine nitration may contribute to ALS, cardiovascular and inflammatory diseases and diabetes as well.

NO plays a dual role in tumor development. On one hand, it may inhibit it, depending on the duration/strength of exposure, cell type and NO sensitivity of the cell. On the other hand, NO can evoke DNA damage and thereby mutations, it may enhance the expression of

vascular endothelial growth factor (VEGF) thus leading to angiogenesis and can potentiate tumor cell migration and invasion via activation of guanylate cyclase and MAPK signaling.

The PC12 cell line is a widely accepted neuronal model system, since these cells undergo neuronal differentiation following nerve growth factor (NGF) treatment. Thus, SNP-induced apoptosis of PC12 cells can mimic nitrosative stress conditions in nerve cells. Protection against high-dose SNP induced cytotoxicity by low concentrations of NO may establish the possibility to lower the rate of neuronal cell loss evoked by nitrosative stress.

VI. Summary

Nitrosative stress evoked by 400 μM SNP:

- causes apoptosis in PC12 cells.
- leads to higher apoptosis rate in the p143p53PC12 subclone expressing dominant inhibitory mutant p53 protein than in wild-type PC12 cells.
- induces stronger activation of the intrinsic apoptotic pathway in the p143p53PC12 cell line.
- raises the amount of the TRAIL protein in the culture medium of PC12 cells.

100 μM SNP pre-treatment of wild-type PC12 cells:

- partially inhibits cell death evoked by 400 μM SNP.
- reduces the rate of apoptosis induced by serum starvation, cisplatin or tunicamycin; probably through the inhibition of the intrinsic apoptosis signaling.
- enhances the cytotoxicity of anisomycin.

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Publications

The thesis is based on the following publications:

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