

**The role of Ras protein in the differentiation and apoptosis of PC12 cells
induced by nitrogen monoxide**

Ph.D. thesis

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Introduction

The role, production and metabolism of nitric oxide

Nitric oxide (NO) plays a role in intracellular signal transduction as a second messenger, and as a paracrine mediator due to its membrane permeability. It can be transported from its place of origin by the blood, bound to hemoglobin or albumin.

NO is produced by the nitric oxide synthase (NOS) protein family. These enzymes use L-arginine as a substrate and produce NO and L-citrulline. The members of the enzyme family are the neuronal, the endothelial and the inducible NOS. Endothelial and neuronal NOS produce a relatively small amount of NO, which is below the toxic level. Both of these enzymes are constitutively expressed and activated by Ca^{++} and calmodulin. Inducible NOS is expressed upon stimulation by lipopolysaccharide, cytokines and tumor necrosis factor α (TNF α). As inducible NOS is not activated by calmodulin, the protein is continuously (sometimes for several days) active until the depletion of its substrate or its breakdown. The concentration of NO produced by inducible NOS can reach higher, toxic levels.

The effects of NO under *in vivo* conditions are influenced by different circumstances: first, the distance of potential target proteins from NOS, as NO, being a free radical, is very reactive. Second, the amount of NO produced, because different concentrations are needed for the modification of different proteins. Activation of the cGMP pathway, for example, requires less NO than modification of the p53 protein. Different amino acid residues within one protein may be modified by different concentrations of NO. Third, in a living organism NO may act less efficiently or more transiently than in a cell culture.

Effects of NO

NO activates **soluble guanylate cyclase** (sGC) by binding to its heme component. The resulting cyclic GMP (cGMP) mainly exerts its effects by activating protein kinase G (PKG).

NO binds covalently to certain amino acid residues within proteins. The process is called **nitrosylation**. Binding of the $-\text{NO}$ group to cysteine is called nitrosation or S-nitrosylation, while modification of tyrosine by the $-\text{NO}_2$ group is nitration. Nitration of tryptophan by NO has also been described although its biological significance is not clear. Conformation and activity of proteins are both influenced by NO, the effect can be activating or inhibitory, depending on the position of amino acid within the protein.

NO can bind to metal ions in proteins forming complexes with iron or copper.

Similarly to oxidative stress induced by oxygen free radicals, larger amounts of NO exert general cytotoxic effects called nitrosative stress that may lead finally to cell death.

NO donors

NO donors are often used to model the effects of endogenous NO. They can be easily administered, but their disadvantage is that byproducts other than NO may be formed from them. It can not be excluded that some of their effects are mediated by these byproducts. In the present work sodium nitroprusside (SNP, $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$) was used as NO donor. It generates NO and sodium cyanide, but sodium cyanide formed from toxic doses of SNP does not reach cytotoxic concentrations.

Role of NO in signal transduction of neuronal differentiation

Signal transduction of NGF in PC12 cells

PC12 rat pheochromocytoma cells used in this study show neuronal differentiation upon nerve growth factor (NGF) treatment. Effects of NGF are mediated by tropomyosin-related kinase A (TrkA) and p75 neurotrophin receptor (p75NTR). Binding of NGF is followed by simultaneous activation of several signal transduction pathways: the prolonged activation of the Ras - extracellular signal-regulated kinase (ERK) pathway is required for neurite outgrowth, the phosphatidylinositol 3-kinase (PI3K) - Akt pathway is mainly responsible for cell survival, while the function of the phospholipase C γ (PLC γ) pathway is of less importance.

Activated NGF receptors bind, through adapter proteins, to guanine-nucleotide exchange factors (GEFs) which in turn activate the Ras protein. Activation of the Ras protein is critical: cell lines expressing a dominant inhibitory Ras protein do not differentiate following NGF treatment. The activated Ras protein stimulates, through Raf and mitogen-activated protein kinase kinase (MKK) enzymes, the ERK enzymes. Prolonged ERK phosphorylation and nuclear translocation of the protein is required for neuronal differentiation.

ERK has many target proteins. Among others, p90 ribosomal S6 kinase (p90RSK), which activates the transcription factor called cAMP-responsive element binding protein (CREB). CREB can also be phosphorylated due to the activation of many other signaling pathways (e.g. cAMP-PKA, cGMP-PKG, Ca^{2+} -calmodulin, PI3K-Akt). Neuronal differentiation can be blocked by inhibiting CREB as well.

The Ras-ERK pathway also mediates the induction of neuronal NOS in NGF-treated PC12 cells. NO then induces the p21WAF1 (wild-type p53-activated fragment 1), a cyclin dependent kinase inhibitor, through the p53 protein. The amount of cyclin D1 also increases, and it plays a role in differentiation: upon NGF treatment the number of cells entering the S-phase declines. This antimitogenic effect of NGF is indicated by decreased expression of proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase. The antiproliferative effect of NGF is abolished in PC12 cells expressing a dominant inhibitory RasH protein.

As a consequence of growth factor receptor activation, PI3K phosphorylates phosphatidyl-inositol-bisphosphate (PIP₂) to phosphatidyl-inositol-trisphosphate (PIP₃), resulting in the activation of Akt protein (also known as protein kinase B). A number of target proteins of Akt are mainly involved in survival signaling. Phosphatase and tensin homologue (PTEN) inhibits the effects of PI3K by dephosphorylating PIP₃. The PI3K-Akt pathway is directly not involved in neuronal differentiation.

The role of NO in NGF signaling

Although NO does not cause differentiation of PC12 cells by itself, it plays a role in NGF-induced neuronal differentiation. This is supported by the fact that NOS-inhibitors inhibit this process. The sGC-cGMP pathway activated by NO is not required for the neuronal differentiation of PC12 cells. It is thus likely that the effects are mediated by protein nitrosylation.

Several proteins involved in the signal transduction of neuronal differentiation are targets of nitrosylation: their nitrosylation can be either stimulatory or inhibitory.

Antiapoptotic effects of NO

In small concentrations NO has cytoprotective functions, but in higher concentrations (this concentration varies in different cell types) it induces cell death. Apoptosis caused by the withdrawal of growth factors or serum, by oxidative stress and by death ligands can be overcome by NO. Its antiapoptotic effect is mediated by the cGMP pathway in several cell types: the PI3K-Akt pathway is activated through PKG, the phosphorylation of CREB and expression of the antiapoptotic Bcl2 protein are increased. In other cell types the role of the sGC-cGMP pathway is questionable, thus the antiapoptotic effects may be results of nitrosylation events. The protective role of Ras nitrosylation has also been suggested.

The antiapoptotic effect can be mediated not only through activating survival signaling, but also through the inhibition of proapoptotic proteins. For example, nitrated cytochrome c fails to activate the apoptosome. Activation of caspases is inhibited by S-nitrosylation of their catalytic domain.

Cell death induced by NO

At a high concentration NO induces nitrosative stress and even cell death. This concentration varies considerably among cell types: 3-4 mM SNP required to kill heart muscle and dental pulp cells is ten times more than the 200-400 μ M SNP leading to cell death of PC12 cells. Not only the toxic dose, but also the types of cell death evoked by NO vary: cells dying by necrosis, caspase-dependent or caspase-independent apoptosis can be seen in the culture. The dominant type of cell death depends on cell culture conditions, the glycolytic capacity of the cell type and other circumstances. In general, NO induces apoptosis in smaller concentrations, and necrosis at very toxic concentrations.

NO-induced necrosis

Necrosis caused by high concentrations of NO is due to the inhibition of ATP synthesis. The most important event is the irreversible inhibition of complexes of the respiratory chain caused by protein nitrosylation. Under these conditions the cell is able to produce ATP only via glycolysis; if this is not enough, it dies by necrosis.

NO-induced caspase-independent programmed cell death

Poly-ADP-ribose polymerase (PARP) is also activated by NO. PARP plays a role in different types of cell death. Its effect is mediated mainly by apoptosis inducing factor (AIF). This protein is present in mitochondria of resting cells, but translocates to the nucleus after being released from its original location. It promotes cell death via endonuclease activation, leading to chromatin condensation.

Proapoptotic effects of NO

The Akt kinase has a central role in the survival of PC12 cells and many other cell types. Its inactivation by itself can lead to different types of cell death. Signaling through the PI3K-Akt pathway is influenced by NO on several signaling proteins: at lower concentrations NO inhibits PTEN via nitrosylation, at higher concentrations Akt is also inhibited.

The stress kinases (c-Jun N-terminal kinase /JNK/ and p38 mitogen activated protein kinase /p38MAPK/), members of the MAPK family, are activated by various stress events, including high doses of NO. They phosphorylate diverse target proteins promoting the stress response. Their transient activation may contribute to cell survival, prolonged activation leads to apoptosis.

NO used in pharmacological concentrations induces endoplasmic reticulum stress. Accumulation of unfolded proteins provokes the unfolded protein response (UPR). General translation activity decreases as a part of stress response. In the presence of the phosphorylated α -subunit of eukaryotic translation initiation factor 2 (eIF2 α) initiation of cap-dependent translation is inhibited. The phosphorylation of eIF2 α is mediated by several kinases. Prolonged phosphorylation of eIF2 α may lead to apoptosis caused by general inhibition of cap-dependent translation.

The p53 protein is also expressed in unstressed cells. Its amount is negligible in unstressed cells, as the p53 protein is degraded by the ubiquitin-proteasome system. Due to different stress stimuli it can be stabilized, thus its amount increases. Different posttranslational modifications take part in its stabilization (among others phosphorylation of the protein). The p53 protein can induce cell cycle arrest or apoptosis. The activated p53 protein stimulates the mitochondrial (intrinsic) pathway of apoptosis, acting in the nucleus (as a transcription factor), or directly on the mitochondria. The exact mechanism of its direct mitochondrial effect is not clear: it may activate the proapoptotic Bax and/or Bak proteins, or inhibit the antiapoptotic Bcl2 or Bcl-xL proteins. During apoptosis induced by p53 several proteins, like cytochrome c, are released to cytosol. The apoptosome is formed, and procaspase-9, an initiator caspase, is activated. Cleaved caspase-9 induces apoptosis through cleavage of effector procaspases, among others procaspase-3.

Several examples for the effects of NO under pathological conditions

A growing body of evidence shows that NO produced in toxic concentrations, plays a role in the development of neurodegenerative diseases. Excessive amounts of NO lead to the accumulation of unfolded proteins, which in turn may contribute to the development of Alzheimer- and Parkinson-disease. Several pathoneurological conditions are accompanied by an inflammatory reaction, including activation of microglia and astrocytes. In these cells iNOS is induced and may produce a considerable amount of NO, killing the neighbouring nerve cells.

Aims of the study

NO takes part in the signal transduction events of nerve cells as a signaling molecule in small concentrations, and by causing cellular stress in toxic concentrations. The aim of this work was to analyze the modulating effect of small SNP doses on NGF signaling and the cytotoxic effects of toxic SNP doses in PC12 cells, as a model system. Primarily the role of RasH protein in NO-signaling was investigated using a subclone of PC12 cells expressing a dominant inhibitory RasH protein.

In the neuronal differentiation PC12 cell model:

- Ras dependent or independent manner of the antiproliferative effect of SNP,
- the effect of combined SNP- and NGF-treatment on neuronal differentiation in cells with deficient Ras-function,
- influence of SNP and NGF on the activation of neuritogenic signal transduction pathways were studied.

In the SNP-induced cellular stress PC12 model:

- the effect of Ras inhibition on the survival pathways (Akt- and ERK pathways),
- the effect of Ras inhibition on the activation of stress signaling pathways,
- the Ras-dependence of p53 activation and induction,
- the effect of Ras inhibition on caspase activation were studied.

Materials and methods

Cell culture

The wild-type PC12 cell line derived from a rat pheochromocytoma and its subclones were used in this study. The M-M17-26 cell line is a subclone of PC12 cells expressing a dominant inhibitory RasH protein (N17 mutant). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose supplemented with 5% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum.

Proliferation assay

5x10³ cells per well were seeded in twenty-four-well plates. Next day the cultures were treated with SNP. The culture media were changed after three days. After six days, cells were trypsinized, collected and cell numbers were determined by hemocytometric quantitation. Statistical significance was analyzed using Student's t-test (p<0.05).

Western blotting

5x10⁶ cells per plate were seeded in 100-mm plates, and treated on the next day with different doses of SNP and/or NGF for different times. After treatment cells were harvested and total protein extracts were prepared using RIPA buffer. For caspase Western blots, cells were lysed in Chaps buffer. 30-50 µg protein extracts were electrophoresed in 12-15% polyacrylamide gels containing 0.1 % SDS and transferred to polyvinylidene difluoride membranes. Membranes were treated with the following antibodies: anti-ERK1 and anti-p53, anti-p21, anti-PCNA, anti-cleaved-caspase-9, anti-cleaved-caspase-3, anti-P-ERK, anti-P-AktSer473, anti-Akt, anti-P-JNK, anti-JNK, anti-P-p38MAPK, anti-p38MAPK, anti-P-eIF2α, anti-eIF2α and anti-P-p53Ser15 primary antibodies followed by administration of horse radish peroxidase conjugated secondary antibodies (anti-rabbit and anti-mouse). Antibody binding was detected using ECL reagent and visualized by the Kodak Image Station 440 gel documentation system.

Neuronal differentiation assay

10³ cells per well were seeded in twenty-four-well plates. Next day the cultures were treated with SNP and/or NGF. The culture media were changed after three days. After 2, 4 and 6 days of treatment, 200 cells per well were counted. Statistical significance was analyzed using Student's t-test (p<0.05).

Cell viability assay

To determine cell viability the WST-1 assay was used. The reagent WST-1 is a formazan salt modified by the mitochondrial dehydrogenases of living cells. 2 x 10⁴ cells per well were plated in twenty-four-well plates. Next day the cells were treated with different concentrations of SNP. After three days of treatment cells were incubated with 10 % WST-1 reagent for 4 hours and light absorption at 450 nm of the yellowish product against control was determined in an ELISA reader. Results were analyzed for statistical significance (p<0.05) using Student's t-test.

Analysis of nuclear morphology

1-2x10⁵ cells were seeded in six-well dishes containing poly-L-lysine-pretreated coverslips. Cultures were treated with different concentrations of SNP or for different time periods. At the end of the treatment cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100. DNA was stained with 0.5% Hoechst 33258 solution, and mounted using Vectashield H-1000. Cell nuclei showing apoptotic chromatin condensation or fragmentation were identified using a fluorescent microscope, and the fraction of apoptotic cells was

determined by counting 100 cells. Results were analyzed for statistical significance ($p < 0.05$) using Student's t-test.

Analysis of DNA fragmentation

Cells were plated in 100-mm plates at a density of 5×10^6 cells per plate. Next day the cells were treated with different concentrations of SNP. At the end of the incubation period cells were scraped in their culture medium centrifuged for 5 minutes with 2000 rpm speed, and lysed in a buffer containing 0.5 % Triton-X 100, 5 mM Tris pH 7.4, 5 mM EDTA. After centrifugation DNA was isolated from the supernatant using the phenol-chloroform protein extraction method, then after RNase treatment samples were electrophoresed in a 1.8 % agarose gel and stained with ethidium bromide or SYBR Gold. The characteristic DNA ladder was photographed using a Kodak Image Station 440 gel documentation system.

Results

Effects of SNP used in nontoxic concentrations

SNP inhibits proliferation in a Ras-independent manner

In order to test the antiproliferative effect of NO wild-type PC12 cells and M-M17-26 cells highly expressing a dominant inhibitory RasH protein were treated with 5 or 50 μM SNP for 6 days. The antiproliferative effect of SNP was dose-dependent in both wild-type PC12 and M-M17-26 cell lines.

As it is known, that the antiproliferative effect of NGF is mediated by the Ras-ERK-p53-p21WAF1 pathway, we tested, whether these proteins are also involved in mediating the antiproliferative effects of SNP. The p53 protein was induced in SNP-treated wild-type PC12 and M-M17-26 cells dose-dependently. Simultaneously, expression of the S-phase marker PCNA was inhibited by SNP in both cell lines.

Combined treatment of M-M17-26 cells with NGF and SNP does not overcome the blockade of neuronal differentiation

Treatment of PC12 cells with 0.5, 5 or 50 μM SNP alone did not cause neurite formation. 50 ng/ml NGF treatment induced normal neuronal differentiation. Combined treatment with NGF and 5 μM SNP made neurite outgrowth faster, as the ratio of differentiated cells after 2 days was higher compared to cells treated only with NGF. In M-M17-26 cells neither different doses of SNP nor 50 ng/ml NGF treatments resulted in

differentiation. We tried to induce differentiation in these cells by combined SNP- and NGF treatments, but neither of the used treatments succeeded. Small concentrations of NO and cell cycle arrest is thus not able to overcome the effect of inhibition of the Ras/ERK pathway on differentiation.

SNP does not potentiate the stimulating effect of NGF on ERK phosphorylation

As expected, NGF treatment caused a strong ERK phosphorylation in PC12 cells. ERK phosphorylation caused by SNP was negligible in wild-type PC12 cells compared to the effect of NGF. SNP and NGF did not synergize in promoting ERK phosphorylation. Activation of the ERK protein was strongly inhibited by the dominant inhibitory RasH protein in M-M17-26 cells treated with SNP or NGF. Combined treatment with SNP and NGF in this cell line also did not cause ERK phosphorylation comparable with that of PC12 cells.

SNP does not influence NGF mediated Akt phosphorylation

The Akt protein undergoes activating phosphorylation through a Ras-independent signaling pathway in PC12 cells. Phosphorylation of Akt on Ser473 increased in both cell lines following NGF treatment. Non-toxic doses of SNP slightly stimulated Akt phosphorylation in both cell lines, but did not synergize with NGF in combined treatments.

Phosphorylation of CREB is Ras-dependent following both SNP and NGF treatment

Phosphorylation of CREB on Ser133 was studied, as this phosphorylation site plays a central role in NGF signaling. As expected, strong CREB phosphorylation was detected following NGF treatment in wild-type PC12 cells. SNP treatment also caused a dose-dependent increase in CREB phosphorylation in PC12 cells, but this did not synergize with NGF. In M-M17-26 cells neither SNP nor NGF or combined treatment increased the phosphorylation of CREB.

Cytotoxic effects of SNP

The effect of SNP on cell viability

According to the results of the WST-1 assay cell viability was decreased in both cell lines dose-dependently, leading to complete cell death at 400 μ M SNP concentrations. M-M17-26 cells were found to be slightly more sensitive to SNP, than wild-type PC12 cells.

SNP induces apoptotic changes in the chromatin

Chromatin condensation is a hallmark of apoptosis or apoptosis-like cell death. Cell nuclei showing condensed chromatin were detectable in both cell lines following treatment with SNP (at least with 100 μ M and at least for 4 hours), more prominently in M-M17-26 cells.

Internucleosomal DNA fragmentation is considered to be a marker of apoptosis. It was detectable in wild-type PC12 cells treated with 200 μ M SNP, while in M-M17-26 cells already after 100 μ M SNP treatment.

The tests used here underline the higher sensitivity of M-M17-26 cell line to the cytotoxic effect of SNP, and that apoptosis is the dominant cell death type in SNP-treated PC12 cell lines.

SNP treatment results in biphasic ERK phosphorylation

Activation of the Ras-ERK pathway may provide survival signals in PC12 cells. SNP treatment resulted in dose-dependent increase in ERK-phosphorylation in PC12 cells. The strongly toxic, 400 μ M SNP treatment caused a biphasic increase of ERK-phosphorylation with an early peak at 2-4 hours, and a late peak at 18 hours of treatment. ERK phosphorylation was strongly inhibited in the M-M17-26 cell line. The early increase in ERK phosphorylation was completely absent, while the late phosphorylation was detectable, although weaker than in wild-type PC12 cells. Reduced ERK phosphorylation may contribute to the increased sensitivity of this cell line to SNP.

SNP inhibits Akt phosphorylation only in strongly toxic concentration

Phosphorylation of Akt on Ser473 was inhibited only by the highest SNP concentration (400 μ M) used for prolonged treatment (at least 18 hours) in PC12 cells. This inhibition was also found in M-M17-26 cells. Different activity of an Akt-mediated survival pathway can not be responsible for the different sensitivity of the two cell lines to SNP.

SNP activates stress kinase mediated signaling pathways

As a part of general cellular stress response dose-dependent and prolonged JNK and p38MAPK activation was found upon SNP treatment. As a sign of endoplasmic reticulum stress, phosphorylation of eIF2 α was also detectable, with similar time course as that of stress kinases. The activation of neither of the examined kinases was stronger in the M-M17-26 cell

line than in PC12 cells. Therefore, although signaling pathways activated via these kinases contribute to the cellular stress response in both cell lines, they cannot explain the higher sensitivity of M-M17-26 cells to SNP.

Phosphorylation and stabilization of p53 protein due to SNP treatment

The Ser15 residue of the p53 protein is a phosphorylation site for both JNK and p38MAPK. Nuclear export and proteasomal breakdown of p53 is inhibited by its phosphorylation on Ser15. SNP treatment leads to dose-dependent phosphorylation with a similar time course than activation of stress kinases. It is more prominent in wild-type PC12 cells than in cells expressing the dominant inhibitory Ras, therefore it may contribute to the stabilization of the p53 protein, so this does not explain the increased sensitivity of M-M17-26 cells to SNP.

Activation of caspases following SNP treatment

Dose-dependent cleavage of procaspase-9 was detected in SNP-treated wild-type PC12 cells. Activation of caspase-9 in the M-M17-26 cell line was much more intense, than in PC12 cells. Cleavage of procaspase-3 was similar to caspase-9 activation in both cell lines. The stronger activation of caspases can be responsible for the higher sensitivity of M-M17-26 cells to SNP.

Discussion

The antiproliferative effect of SNP is Ras-independent

NGF exerts its antiproliferative effect in PC12 cells through activation of Ras. If the antimitogenic effect of NGF is inhibited, PC12 cells fail to differentiate. Epidermal growth factor (EGF) combined with an antiproliferative agent (e.g. dbcAMP, SNP) induces differentiation in PC12 cells. We therefore tested whether the block of NGF-induced neuritogenesis could be overcome by SNP.

The antiproliferative response of wild-type PC12 cells and the M-M17-26 subclone to SNP treatment was compared. No difference between the two cell lines in p53 induction and decrease of PCNA expression was found. According to these results the Ras protein does not play a role in mediating the antimitogenic effect of SNP. The effects of NO are dose-

dependent, but concentrations of SNP higher than 50 μM used in the differentiation experiments already have toxic effects (5 or 50 μM SNP does not induce cell death).

Combined treatment with NGF and SNP does not lead to neuronal differentiation in M-M17-26 cells

Although combination treatment with 5 μM SNP and NGF makes the differentiation of wild-type PC12 cells somewhat faster, it does not change the final percentage of differentiated cells.

M-M17-26 cells proliferate in the presence of NGF comparably to untreated cells, and their proliferation can be inhibited by small doses of SNP. They fail to grow neurites upon combined treatment with SNP and NGF, thus the inhibition of Ras protein cannot be overcome by NO. A possible explanation is that NO is unable to stimulate the GTP-binding of endogenous Ras proteins in the presence of dominant inhibitory Ras. M-M17-26 cells are able to differentiate in the absence of Ras activation in response to the combined effect of NGF and certain second messenger analogues. Thus a second possible explanation for the failure of differentiation is, that NO does not stimulate a signal transduction mechanism which bypasses the Ras blockade. The results of combined SNP and NGF treatment also show, that cell cycle arrest, although is necessary for neurite outgrowth, requires additional neuritogenic signals to induce it.

SNP treatment is not able to overcome the blockade of NGF-induced ERK and CREB phosphorylation imposed by a dominant negative RasH protein

ERK phosphorylation stimulated by non-toxic doses of SNP is much weaker than that induced by NGF treatment and they do not synergize. ERK is not phosphorylated in M-M17-26 cells treated with low doses of SNP, and SNP combined with NGF is not able to overcome the block in the activation of the Ras-ERK pathway.

Another important member of this signal transduction pathway is the CREB transcription factor, which can be activated by other, ERK-independent signals as well. CREB phosphorylation was found to be Ras-dependent not only for NGF but also for SNP treatment: both agents stimulate CREB phosphorylation through activating the Ras-ERK pathway. Since CREB binds to the promoters of one third of the genes induced during neuronal differentiation, it is not surprising that failure of CREB activation results in failure of differentiation.

Summarizing the results of the differentiation experiments, NO is partially able to substitute the inhibited Ras-function (antiproliferative effect), but does not sufficiently activate pathways required for neurite outgrowth (Figure 1).

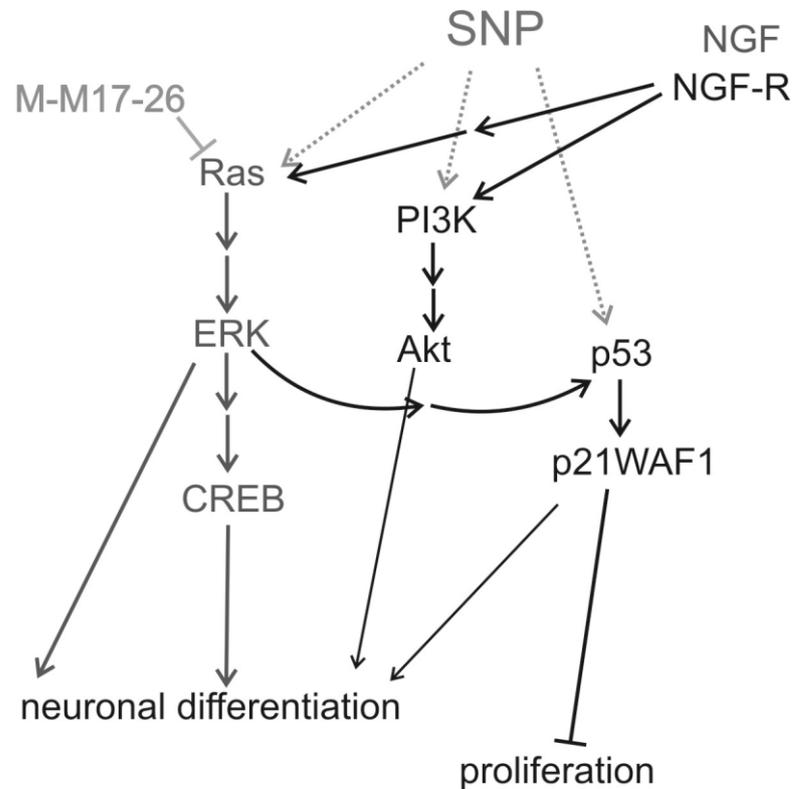


Figure 1.: Combined effects of SNP and NGF treatment in PC12 and M-M17-26 cells.
 →: activation/induction, →→: activation/induction in several steps, ⊥: inhibition

SNP induces cell death through activating different signal transduction pathways

SNP may induce several types of cell death in PC12 cells. An individual cell in a culture will be killed by the pathway activated the strongest/fastest. In PC12 cell cultures treated with SNP this principle is reflected by the heterogeneity of nuclear morphology. Chromatin condensed to a lesser extent may be a sign for an early phase of apoptosis, for apoptosis-like cell death or caspase-independent apoptosis. The appearance of nuclei with condensed chromatin upon SNP treatment preceded the activation of caspases. Beside the activation of mitochondrial pathway of apoptosis, caspase-independent cell death mechanisms may also operate in SNP-treated cells.

Treatment with toxic concentrations of SNP induces biphasic ERK phosphorylation through the Ras signaling pathway

The activated Ras protein stimulates several signal transduction pathways, including proapoptotic pathways. Since the PC12 cell line is less sensitive to SNP than M-M17-26 cells, the activation of such proapoptotic Ras-effectors is unlikely. Activation of the PI3K-Akt pathway occurs Ras-independently in PC12 cells: there is no difference between the two cell lines in the phosphorylation of Akt on Ser473. Early activation of ERK proteins by SNP is completely Ras-dependent, thus may be mediated by a nitrosylated Ras protein or by the activation of an upstream regulator of Ras. The late phase of ERK phosphorylation (after 18 hours of SNP treatment) is partially Ras-independent, as it also appears in M-M17-26 cells. It may be caused by direct activation of ERK enzymes by peroxynitrite. Alternatively, late ERK activation may be caused by stimulation of MEKK1, a MAPKKK not regulated by Ras. This protein primarily stimulates JNK, but may also activate ERK through the phosphorylation of MKK1.

Treatment with toxic concentrations of SNP activates stress signaling independently of Ras-function

The kinetics of p38MAPK and JNK phosphorylation are similar to the phosphorylation of Ser15 of the p53 protein; either of the stress kinases may activate the p53 protein. Phosphorylation of Ser15 may lead to the stabilization of p53 protein, but by itself gives no explanation for the different sensitivity of the two cell lines to SNP.

Treatment with SNP and the presence of the dominant inhibitory RasH protein alters the expression pattern of p53 isoforms

The distinct isoforms of the p53 protein differently regulate processes of proliferation, differentiation and apoptosis. These isoforms influence the signal transduction effects of each other as well. The amount of the “classical”, 53 kDa α isoform increases in SNP-treated wild-type PC12 and M-M17-26 cells in a similar manner. All the 46-48 kDa isoforms are able to induce apoptosis and are induced following SNP-treatment in both cell lines. The 35 kDa $\Delta 133p53\alpha$ isoform acts as a dominant inhibitory protein of p53 α , thereby inhibiting apoptosis induced by p53. The band detectable only in untreated M-M17-26 cells disappears following SNP treatment, so this may enhance p53-induced apoptosis.

The increased sensitivity of the M-M17-26 cell line to the apoptotic effect of SNP is mediated by increased caspase 9 and 3 cleavage

Activation of caspases is inhibited by signaling pathways promoting cell survival. Among others a not yet identified member of the PI3K-Akt pathway and the ERK enzyme may phosphorylate procaspase-9. Both of these phosphorylations inactivate the protein. SNP-stimulated dephosphorylation of the Akt protein happens similarly in both cell lines: it cannot account for the difference in caspase activation. Reduced ERK activation in M-M17-26 cells may lead to increased caspase-9 activity and higher SNP-sensitivity of this cell line (Figure 2).

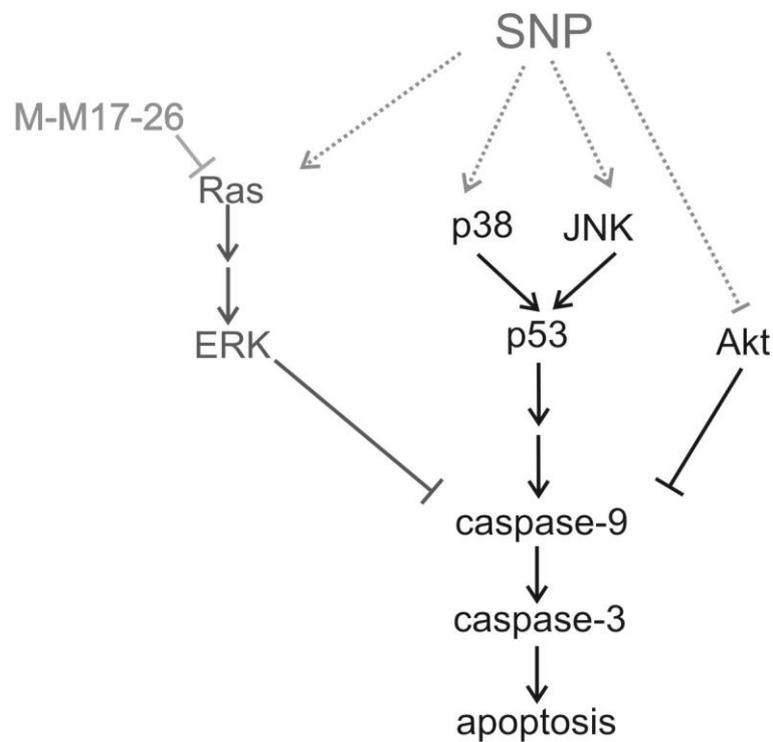


Figure 2. Apoptotic effects of SNP in PC12 and M-M17-26 cells. →: activation/induction, →→: activation/induction in several steps, ⊥: inhibition

Summary

Non-toxic concentrations of SNP:

- The antiproliferative effect of SNP is Ras-independent.
- Combined treatment with NGF and SNP does not lead to neuronal differentiation in M-M17-26 cells.
- Treatment with SNP is not able to overcome the inhibition of NGF-induced ERK and CREB phosphorylation by a dominant negative RasH protein.

SNP treatment using toxic concentrations activates different signal transduction pathways:

- It induces biphasic ERK phosphorylation using the Ras signaling pathway.
- It activates stress signaling independently of Ras function.
- Expression of p53 is influenced by treatment with SNP and expression of a dominant inhibitory RasH protein.
- The increased sensitivity of the M-M17-26 cell line to the apoptotic effect of SNP is mediated by increased activation of caspase-9 and -3.

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Publications

Peer-reviewed articles in the topic of the thesis

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