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Molecular mechanism of PC12 cell death evoked by sodium nitroprusside, a nitric oxide donor

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Nitric oxide (NO) is a potent extracellular and intracellular physiological messenger. However, NO liberated in excessive amounts can be involved in macromolecular and mitochondrial damage in brain aging and in neurodegenerative disorders. The molecular mechanism of its neurotoxic action is not fully understood. Our previous data indicated involvement of NO in the release of arachidonic acid (AA), a substrate for cyclo- and lipoxygenases (COX and LOX, respectively). In this study we investigated biochemical processes leading to cell death evoked by an NO donor, sodium nitroprusside (SNP). We found that SNP decreased viability of pheochromocytoma (PC12) cells in a concentration- and time-dependent manner. SNP at 0.1 mM caused a significant increase of apoptosis-inducing factor (AIF) protein level in mitochondria. Under these conditions 80% of PC12 cells survived. The enhancement of mitochondrial AIF level might protect most of PC12 cells against death. However, NO released from 0.5 mM SNP induced massive cell death but had no effect on protein level and localization of AIF and cytochrome *c*. Caspase-3 activity and poly(ADP-ribose) polymerase-1 (PARP-1) protein levels were not changed. However, PARP activity significantly decreased in a time-dependent manner. Inhibition of both COX isoforms and of 12/15-LOX significantly lowered the SNP-evoked cell death. We conclude that AIF, cytochrome *c* and caspase-3 are not responsible for the NO-mediated cell death evoked by SNP. The data demonstrate that NO liberated in excess decreases PARP-1 activity. Our results indicate that COX(s) and LOX(s) are involved in PC12 cell death evoked by NO released from its donor, SNP.

Keywords: nitric oxide, apoptosis-inducing factor, PC12, cell death, lipoxygenase, cyclooxygenase

INTRODUCTION

Previous data have shown that nitric oxide (NO) synthesized in excess is a crucial factor leading to cell death in cerebral ischemia (Chalimoniuk & Strosznajder, 1998; Culmsee *et al.*, 2005; Strosznajder *et al.*, 2005a; Yang *et al.*, 2005; Li *et al.*, 2007). Moreover, NO has been implicated in the neurotoxicity of amyloid β in Alzheimer's and Parkinson's diseases (Strosznajder *et al.*, 2000; Keil *et al.*, 2004; Chalimoniuk *et al.*, 2007). Also physiological aging alters the activity of NO synthases and NO-regulated signaling pathway (Chalimoniuk & Strosznajder, 1998; Jesko *et al.*, 2003; Calabrese *et al.*, 2004; Strosznajder *et al.*, 2004).

According to the classical hypothesis (Zhang & Steiner, 1995) the free radical cascade that can be initiated with excessive liberation of NO leads to

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Abbreviations: AA, arachidonic acid; AIF, apoptosis-inducing factor; p-APMSF, 4-amidinophenylmethanesulfonyl fluoride; DTT, dithiothreitol; MTT, 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; βNAD^+, β-nicotinamide adenine dinucleotide; NDGA, nordihydroguaiaretic acid; NF-κB, nuclear factor-κB; PARP-1, Poly(ADP-ribose) polymerase-1; PBS, phosphate-buffered saline; PC12, rat pheochromocytoma cell line; PCD, programmed cell death; SNP, sodium nitroprusside.

DNA damage that activates poly(ADP-ribose) polymerase (PARP-1). An enhancement of this enzyme activity was observed by us in aged brain (Strosznajder *et al.*, 2005b). Massive single- or double strand breaks of DNA are responsible for PARP-1 overactivation. These molecular processes lead to βNAD^+/ATP depletion, lowering of mitochondrial membrane potential and the release of apoptosis-inducing factor (AIF) from mitochondria and to caspase-independent cell death (Chiarugi & Moskovitz, 2002; Strosznajder *et al.*, 2005a; Strosznajder & Gajkowska, 2006; Yu *et al.*, 2002; 2003).

Under physiological conditions AIF plays a role in oxidative phosphorylation and in antioxidant defense (Modjtahedi *et al.*, 2006) and its absence is lethal during early stages of embryonic life. The lowering of AIF protein level can lead to neurodegeneration (Modjtahedi *et al.*, 2006). However, after oxidative or genotoxic insults AIF is translocated from mitochondria to the nucleus and induces apoptosis (Daugas *et al.*, 2000; Yu *et al.*, 2002, 2006; Cohausz *et al.*, 2008). Recent data of Moubarak *et al.* (2007) indicate that AIF is also essential in programmed necrosis. The role of AIF in cell death appears to be highly cell type- and stimulus-specific (Modjtahedi *et al.*, 2006). Recent results have demonstrated a potential role of AIF in brain aging, Alzheimer's disease and cerebral ischemia (Reix *et al.*, 2007) and pointed to a potential role of AIF as a therapeutic target (Lorenzo & Susin, 2007).

Overactivation of PARP-1 and the appearance of its product poly(ADP-ribose) is suggested to be involved in AIF release from mitochondria under several pathological conditions (Yue *et al.*, 2006; Cohausz *et al.*, 2008). The NO donor sodium nitroprusside (SNP) has been widely used to study NO-dependent biochemical processes and cell death (Inoue *et al.*, 2003; Kühn & Lotz, 2003; Nie *et al.*, 2006; Gui *et al.*, 2007; Kawasaki *et al.*, 2007). Our previous data indicates that NO is involved in the regulation of cytosolic phospholipase A_2, its phosphorylation and activity, arachidonic acid (AA) release and in consequence in up-regulation of expression and activity of cyclooxygenases (COX) and lipoxygenases (LOX) (Chalimoniuk *et al.*, 2006; 2007).

The aim of this study was to determine the level and localization of AIF during PC12 cell death evoked by NO liberated from its donor, sodium nitroprusside. Moreover, the role of NO-regulated COX and LOX isoforms in this process was investigated.

MATERIALS AND METHODS

Cell culture. PC12 cells were kindly provided by Professor Walter E. Müller from the Department of Pharmacology Biocenter (University of Frankfurt, Germany). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 5% horse serum (HS), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2.

Cell treatment protocols. PC12 cells were treated with SNP at 0.1 or 0.5 mM for different times up to 24 h. In some experiments, cells treated with 0.5 mM SNP were cultured for 24 h with 0.5 mM SNP and following inhibitors: NS-398 at 1 μM; indomethacin at 25 μM; baicalein at 2.5 μM, 5 μM or 10 μM or with NDGA at 0.5 μM.

MTT reduction assay. Mitochondrial function and cellular viability were evaluated using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). PC12 cells were seeded into 24-well culture plates coated with 0.1% polyethyleneimine (PEI) in 25 mM borate buffer and allowed to attach. Medium with 2% FCS, 50 units/ml penicillin and 50 μg/ml streptomycin, containing SNP (0.1 mM or 0.5 mM) was added to the cells for a given period of time. MTT was added to all wells and the cells were incubated at 37°C for 2 h. Then cells were lysed and spectrophotometric measurement at 595 nm was performed.

Isolation of cytosolic, mitochonrial and nuclear fractions. Cells were washed and scraped into ice-cold PBS and pelleted at 900 × *g* for 3 min at 4°C. The pellet was resuspended in hypotonic buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl with Complete™ protease inhibitors cocktail; Roche). The cell membranes were disrupted by 10 passes through a 26 gauge needle and pelleted at 500 × *g* for 10 min at 4°C. The pellet (P1) was used as a crude nuclear fraction for Western blot analysis. The supernatant (S1) was used for isolation of mitochondria and the cytosolic fraction by centrifugation at 15 000 × *g* for 10 min at 4°C. The pellet (P2) (crude mitochondria) was resuspended in 25 mM Tris, pH 7.4, with protease inhibitors. Supernatant (S2) was used as a cytosolic fraction.

Caspase 3 activity. Caspase activity was determined using a colorimetric assay kit from Sigma (St. Louis, USA). Cells were cultured at 3×10^6 cells/well, harvested with lysis buffer (caspase colorimetric assay kit; Sigma), incubated for 20 min at 4°C, disrupted by 10 passes through a 26 gauge needle and centrifuged at 14 000 × *g* for 15 min. The activity of caspase-3 was measured in 10 μl of supernatant using 20 μM synthetic caspase-3 substrate Ac-DEVD-AMC in reaction buffer in a final volume of 200 μl; the incubation was carried out at 37°C for 4 h. The concentration of AMC, a product of cleavage of the caspase-3 substrate Ac-DEVD-AMC was measured at the excitation and emission wavelengths of 360 nm

and 460 nm, respectively. Caspase-3 activity was estimated as nmol AMC/min per mg protein.

Measurement of PARP-1 activity. ARP activity was determined using ^{14}C-labeled βNAD$^+$ as a substrate. The incubation mixture in a final volume of 100 μl contained 200 μM (adenine-^{14}C)βNAD$^+$ (4×10^5 d.p.m.), 100 mM Tris/HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 50 μM p-APMSF and 200 μg of protein. The mixture was incubated for 1 min at 37°C and the reaction was stopped with 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitates were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. The radioactivity was measured in Bray scintillation fluid using Wallac 1409 scintillation counter from LKB.

Western blot. After determination of the total protein content according to Lowry *et al.* (1951), the cytosolic, mitochondrial or nuclear fraction was mixed with 5 × sample buffer according to Laemmli (1970) and denatured for 5 min at 95°C. Protein (40 μg) was loaded onto each lane of 10% acrylamide gels and resolved by SDS/PAGE. The proteins were transferred onto PVDF membranes at 100 V. Membranes were incubated in 5% dry milk in TBS (Tris-buffered saline) with Tween-20 (TBS-T) for 1 h and exposed overnight to the following antibodies: anti-AIF (1:100, from Santa Cruz, USA), anti-PARP-1 (1 : 500, from Sigma, USA), anti-cytochrome *c* (1 : 500, from BD Bioscience Pharmingen), or anti-actin (1 : 400, from MP Biomedicals). After treatment for 1 h with appropriate horseradish peroxidase-coupled secondary antibodies (anti-rabbit from Sigma St. Louis, USA, or anti-mouse from Amersham Biosciences, UK), the protein bands were visualized with ECL reagents (Amersham Biosciences). After detection, the membranes were treated with stripping buffer (50 mM glycine, pH 2.5, 1% SDS) for detection of another protein.

Statistical analysis. Statistical analyses between two groups were conducted using a two-tailed, unpaired Student's *t*-test. Analyses among multigroup data were conducted using one-way analysis of variance (ANOVA), followed by Newman-Keuls *post hoc* test. The data are given as the mean ± S.E.M. P values < 0.05 were considered statistically significant.

RESULTS

Time- and concentration-dependent effect of NO liberated from SNP on PC12 cell viability

PC12 cells in culture were treated with two concentrations of the NO donor SNP (0.1 or 0.5 mM) for 6, 12 or 24 h. Cell viability was determined using the MTT assay. Treatment of cells with 0.1 mM SNP for 12 or 24 h caused about 20% PC12 cell death (Fig. 1A). In the higher (0.5 mM) concentration of SNP, the cell viability strongly decreased. After 6 to 24 h about 30–40% of cells survived (Fig 1B).

AIF and cytochrome *c* protein levels in mitochondria of PC12 cells treated with 0.1 and 0.5 mM SNP

To characterize the molecular events leading to SNP-induced cell death, the level of AIF protein was determined by Western blot analysis in mitochondrial and nuclear fractions. AIF immunoreactivity in mitochondria of cells treated with 0.1 mM SNP increased with time. This effect was significant after 4 h of SNP treatment and the highest value was reached after 12 h of incubation with 0.1 mM SNP (Fig. 2A). A similar but statistically insignificant result was observed in the case of cytochrome *c* immunoreactivity. The level of this protein was slightly increased with time in mitochondria after 0.1 or 0.5 mM SNP treatment (Fig. 3A, B). At higher (0.5 mM) concentration, SNP had no effect on AIF

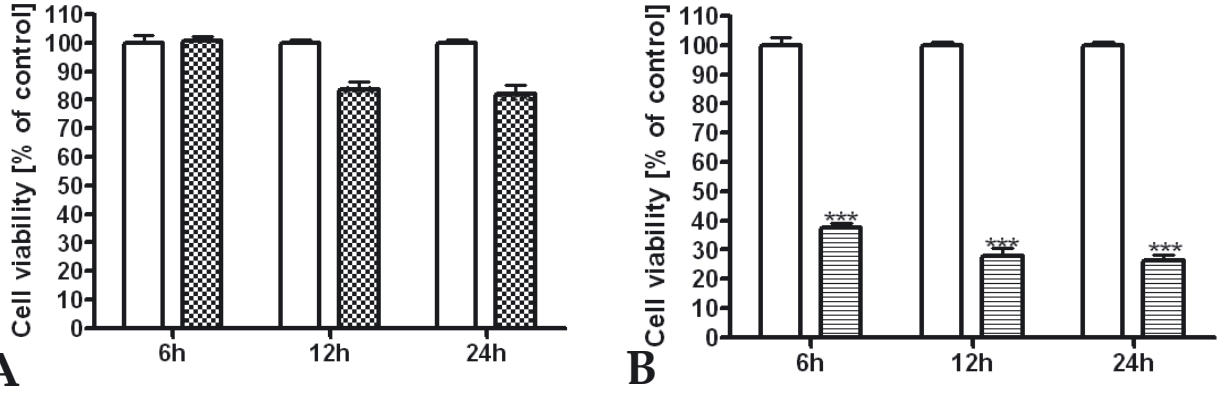

Figure 1. Time-dependent changes of PC12 cell viability evoked by SNP treatment.
PC12 cells were cultured in medium without (open bars) or with (textured bars) 0.1 mM (A) or 0.5 mM SNP (B) for 6, 12 or 24 h. MTT test was then used for determination of their viability. Results are the mean ± S.E.M. (n = 3–4). Each experiment was carried out six times. Statistical significance compared to control group. ***P < 0.001.

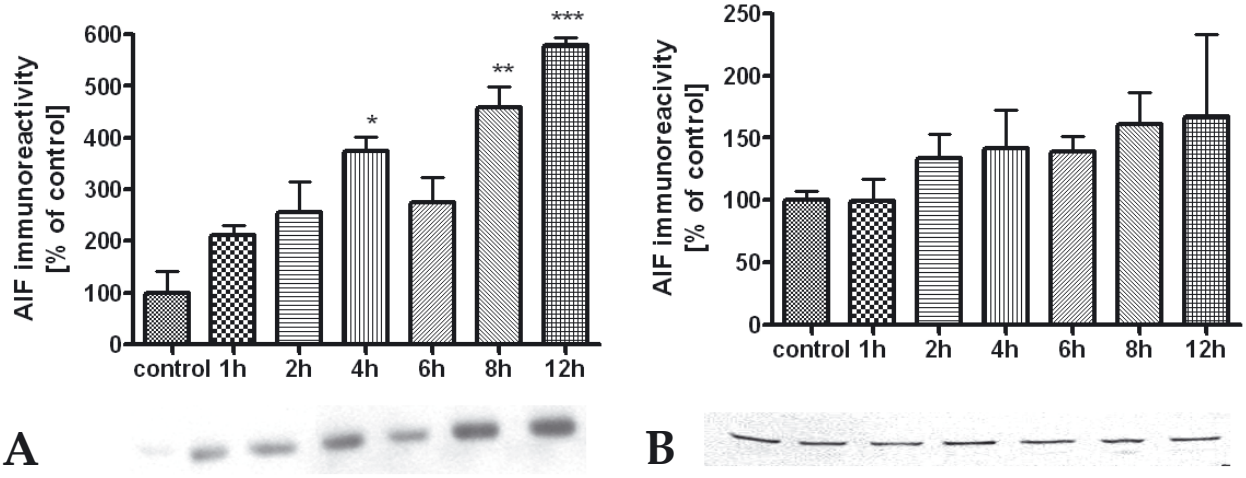

Figure 2. Western blot analysis of AIF protein level in mitochondrial fraction of PC12 cells treated with SNP. The cells were cultured in medium without or with 0.1 mM (A) or 0.5 mM SNP (B) for different times up to 12 h. Results are means ± S.E.M. from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

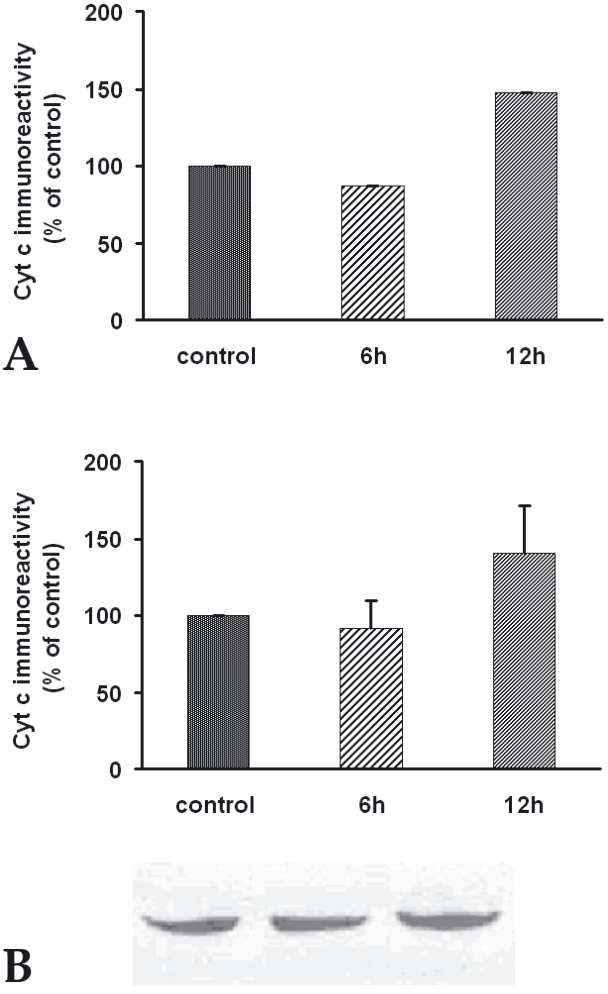

Figure 3. Western blot analysis of cytochrome *c* protein level in mitochondrial fraction after SNP treatment. PC12 cells were cultured in medium without or with 0.1 mM (A) or 0.5 mM (B) SNP for 6 or 12 h. Results are means ± S.E.M. from 3 independent experiments.

protein level in mitochondrial fraction (Fig. 2B) or on its translocation into nucleus (not shown).

Effect of NO on caspase-3 activity and on PARP-1 protein level and activity

Because caspase-3 is an executioner of apoptosis induced by a variety of stimuli, we examined whether NO generated by SNP activated caspase-3 in PC12 cell. Our data demonstrated that caspase-3 activity was not changed by death signaling induced by NO liberated from 0.5 mM SNP (Fig. 4A).

Moreover, Western blot analysis showed lack of changes in the level of PARP-1 protein, one of the best substrates for caspase-3 (Fig. 4B). However, SNP strongly decreased PARP-1 activity (Fig. 4C). The inhibition of PARP by 0.5 mM SNP was already observed after 5 min of treatment and was time-dependent (Fig. 4C).

Effect of COX and LOX inhibitors on NO-induced PC12 cell death

Our recent results indicated that NO activated arachidonic acid release and its metabolism by COX and LOX. In the following experiments we examined the effects of COX and LOX inhibitors on SNP-induced PC12 cell death (Fig. 5). Treatment with 0.5 mM SNP caused > 70% cell death. Incubation with NS-398, a COX-2-specific inhibitor slightly reduced cell death triggered by 0.5 mM SNP treatment. A nonselective inhibitor of both COX isoforms, indomethacin, significantly enhanced cell viability, about 50% of cells survived. An enhancement of cell viability was also observed in the presence of baicalein, an inhibitor specific for 12/15-LOX, and after incubation with nordihydroguaiaretic acid (NDGA;

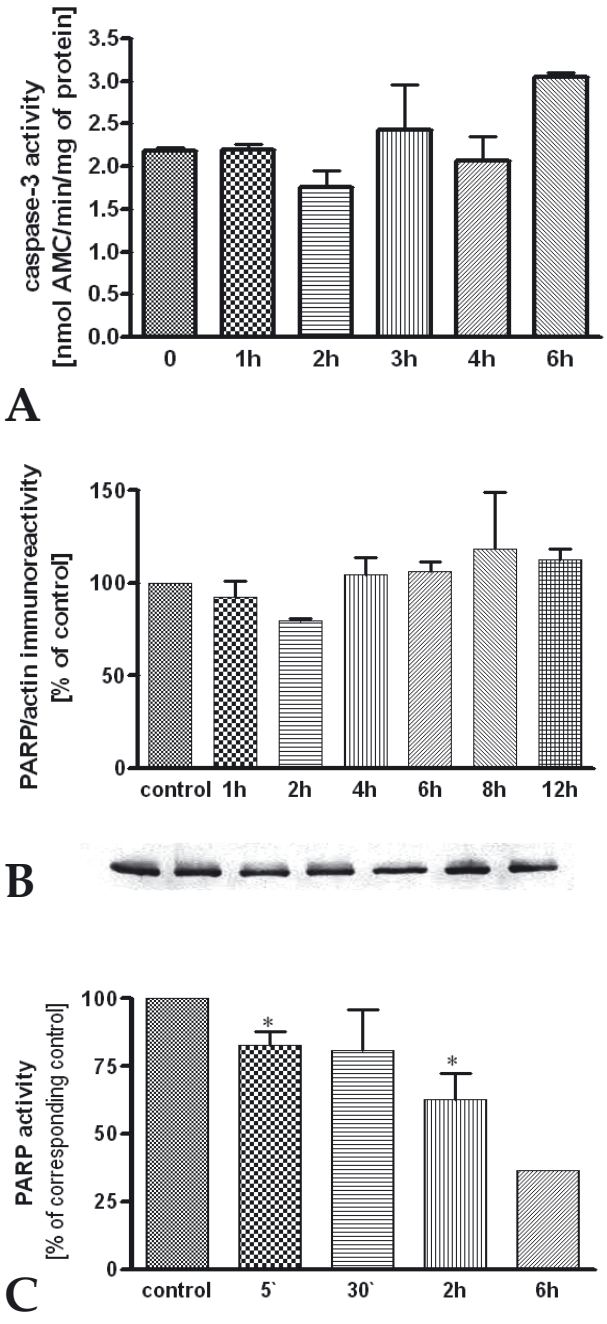

Figure 4. The effect of 0.5 mM SNP on caspase-3 and PARP-1.

Time-dependent effect of 0.5 mM SNP on caspase-3 activity in cytosolic fraction of PC12 cells (A), PARP-1 protein (B) and activity (C) in nuclear fraction of PC12 cells. The data are means ± S.E.M. from 3–4 independent experiments with the exception of PARP-1 activity after 6 h (2 experiments). $^{*}P < 0.05$.

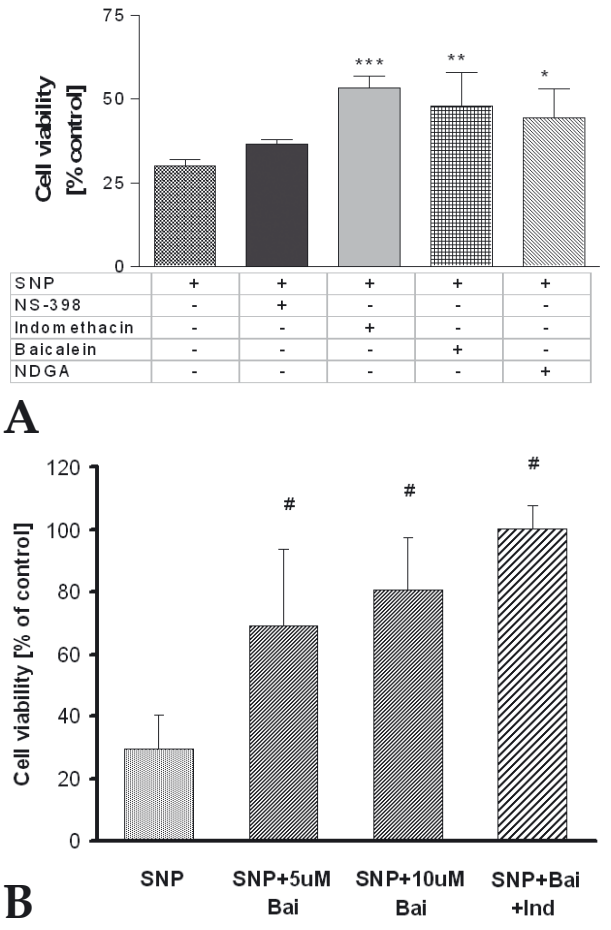

Figure 5. Effect of COX and LOX inhibitors on SNP-induced PC12 cell death.

The cells were cultured for 24 h in the presence of inhibitors of COX (1 μM NS-398, 25 μM indomethacin) or LOX (2.5 μM, 5 μM or 10 μM baicalein, 0.5 μM NDGA) and 0.5 mM SNP (A). The cells were cultured for 24 h in the presence of 5 or 10 μM baicalein and with 25 μM indomethacin and 0.5 mM SNP (B). The control cells were not treated with SNP nor with inhibitors. Results are the mean ± S.E.M. from 3 independent experiments with the exception of 0.5 mM SNP + 5 μM and 10 μM baicalein which is mean value from 2 experiments. Each experiment was carried out six times. Statistical significance compared to the respective control. $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$, $^{\#}P < 0.05$ *vs* SNP.

inhibitor of all LOX isoforms) (Fig. 5). Baicalein at 5–10 μM significantly enhanced cell viability and its protective effect was much stronger in the presence of indomethacin. The viability of cells treated with SNP returned to the control (non SNP-treated) value in the presence of both inhibitors.

DISCUSSION

The data presented in this paper suggest that the enhanced level of AIF protein in mitochondria under mild oxidative stress evoked by NO liberated from a donor, 0.1 mM SNP is probably involved in PC12 cell survival (Figs. 1A, 2A). The protein level of AIF was not changed in PC12 cells treated with 0.5 mM SNP (Fig. 2B). This NO donor at 0.5 mM inhibited significantly PARP-1 activity (Fig. 4C), probably disturbing the role of this enzyme in DNA repair. Under these conditions massive cell death was

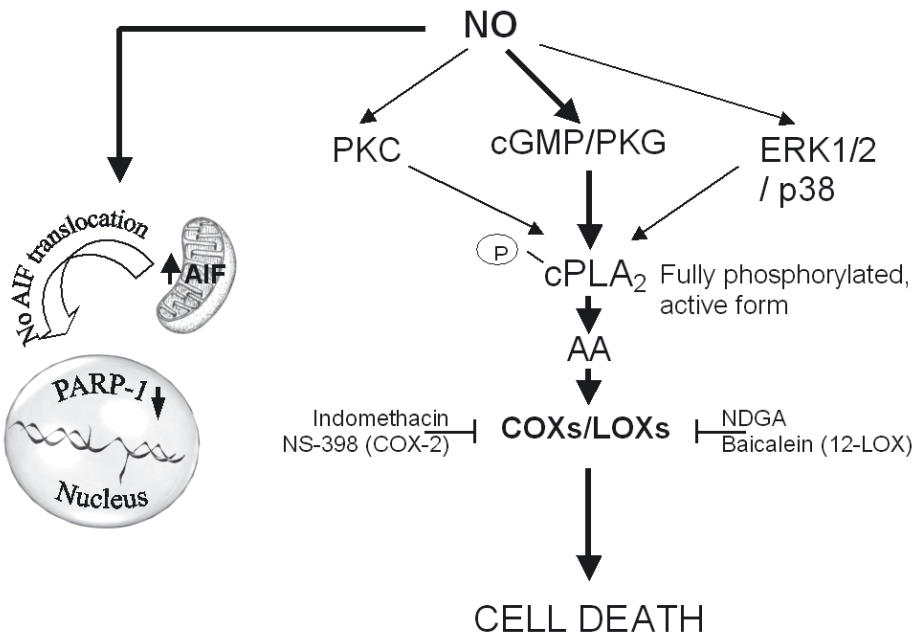

Figure 6. Proposed mechanism of SNP-evoked PC12 cell death.

observed (Fig. 1B). Our study also shows that inhibitors of COXs and LOXs protect PC12 cells against the NO-evoked death (Fig. 5).

NO plays key roles in brain aging, cerebral ischemia and amyloid β and α-synuclein toxicity in Alzheimer's and Parkinson's diseases (Chalimoniuk & Strosznajder, 1998; Jesko *et al.*, 2003; Strosznajder *et al.*, 2003; 2005; Culmsee *et al.* 2005; Adamczyk *et al.*, 2006). Our previous data indicated that in cerebral ischemia, NO is one of the key factors leading to death of some populations of hippocampal neurons (Strosznajder *et al.*, 1994; Chalimoniuk & Strosznajder, 1998). There is significant evidence that the most important isoform in this condition is nNOS (Strosznajder *et al.*, 2005a). Aging differently affected the isoforms of NOS, enhancing nNOS and decreasing eNOS activity (Jesko *et al.*, 2003; Strosznajder *et al.*, 2004). Moreover, the toxicity of Aβ is connected with enhancement of NO liberation that leads to activation of $cPLA_2$ and to the release of AA, the substrate of COXs and LOXs (Keil *et al.*, 2004; Chalimoniuk *et al.*, 2006; 2007; Kim *et al.*, 2006). Our data demonstrate that inhibitors of COX and LOX protect PC12 cells against the cytotoxic action of NO liberated from SNP. Results of Notoya *et al.* (2000) and Lim *et al.* (2003) show that chondrocyte and PC12 cell death triggered by NO released from its donor SNP is mediated by up-regulation of COX-2. The enhanced production of PGE_2 could sensitize cells to the cytotoxic action of NO in an auto- or paracrine way (Notoya *et al.*, 2000). Because both MEK-1/2 and p38 kinase inhibitors abolish the SNP-induced PGE_2 production and prevent the enhancement of COX-2 expression in chondrocytes (Shalom-Barak *et al.*, 1998; Notoya *et al.*, 2000), it is suggested that these proteins act upstream to COX-2 in the cell death pathway (Fig. 6). Our previous data showed that ERK-1/2 and PKC together with cGMP-regulated PKG were involved in $cPLA_2$ phosphorylation and in the release of AA, the substrate of COXs and LOXs (Chalimoniuk *et al.* 2006). Both families of enzymes have an oxidase activity and produce superoxide radical that may participate in oxidative stress.

The free radical-mediated damage of DNA causes activation of PARP-1 and depletion of βNAD^+ and may lead to a decrease of mitochondrial membrane potential and AIF release from mitochondria (Chiarugi & Moskowitz, 2002; Yu *et al.*, 2002; 2003; Strosznajder *et al.* 2005b).

Our study was done to better understand the mechanism of cell death evoked by NO. The NO donor SNP has been widely applied in the research of NO biology and pharmacology (Kühn & Lotz, 2003; Nie *et al.*, 2006; Kawasaki *et al.*, 2007).

Our data demonstrated that the preservation and increase of mitochondrial AIF pool could exert a neuroprotective influence against cytotoxicity evoked by NO liberated from 0.1 mM SNP. When NO was released in excessive amounts from 0.5 mM SNP, about 30–40% of PC12 cells survived.

Our data indicate that NO-evoked PC12 cell death is not mediated by AIF translocation and PARP-1 activation and degradation, in contrast to the recent results of Kawasaki *et al.* (2007). The study showed that NO liberated from SNP induced AIF translocation in glial cells. However, results of Kühn and Lotz (2003) obtained in chondrocytes

indicated that NO-dependent cell loss was not connected with AIF release. The question arose what kind of mediator was responsible for the NO-dependent PC12 cell death. We demonstrated that indomethacin, a nonselective COXs inhibitor, significantly enhanced cell viability reduced by the NO donor, SNP. These data suggested that COX isoform(s) are involved in the mechanism of NO-evoked cell death. This is in agreement with the results obtained by Notoya *et al.* (2000). Moreover, using a 12/15-LOX inhibitor, baicalein and NDGA, a nonselective inhibitor of all LOXs, we found that 12/15-lipoxygenases and probably other isoforms (e.g. 5-LOX) might also participate in PC12 cell death evoked by the NO donor SNP. The data of Kawasaki *et al.* (2007) suggest that the MAP kinase pathway plays a key role in SNP-induced apoptosis-like cell death. In relation to NO-induced cytotoxicity in astrocytes it was shown that the NO donor SNP induced mitochondrial dysfunction in these cells (Phuangphong *et al.*, 2004). Another study carried out by Suk *et al.* (2001; 2002) demonstrated caspase-dependent apoptosis evoked by NO in astrocytes. However, Yung *et al.* (2004) presented an NO-induced, caspase-independent, p53- and Bax-mediated apoptotic death in astrocytes. On the other hand, Nagano *et al.* (2005) indicated that NO induced caspase-dependent apoptosis in microglial cells in culture. According to Kawasaki *et al.* (2007) all those studies suggest that there is a cell-specific mechanism for NO-induced death. The effect of NO on cell survival or death depends probably not only on the type of cells but also on the NO concentration and on the duration of its action. Our previous study demonstrated a significant role of ERK, PKC and PKG in NO-dependent regulation of cytosolic PLA_2 and AA release. On the basis of these data it is possible to postulate that these kinases are involved in NO-evoked PC12 cell death upstream of COXs and LOXs. Another NO donor, SNAP, exerted a similar effect on $cPLA_2$ and AA release.

In sumary, our results show that PC12 cell death evoked by SNP/NO involves cyclooxygenase(s) and lipoxygenase(s) but not AIF, cytochrome *c* or caspase-3. Combined administration of COX and LOX inhibitors may offer a very efficient cytoprotective approach. Moreover, our data show for the first time that NO in high concentration significantly decreases PARP-1 activity *in vivo* and in this way may affect the efficiency of this enzyme in DNA repair.

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