Acta Biochimica Polonica

Vol. 54 No. 3/2007, 611–623

on-line at: www.actabp.pl

Regular paper

Amyloid beta enhances cytosolic phospholipase A_2 level and arachidonic acid release *via* nitric oxide in APP-transfected PC12 cells

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Received: 02 February, 2007; revised: 27 April, 2007; accepted: 17 July, 2007
available on-line: 23 August, 2007

Cytosolic phospholipase A_2 ($cPLA_2$) preferentially liberates arachidonic acid (AA), which is known to be elevated in Alzheimer's disease (AD). The aim of this study was to investigate the possible relationship between enhanced nitric oxide (NO) generation observed in AD and $cPLA_2$ protein level, phosphorylation, and AA release in rat pheochromocytoma cell lines (PC12) differing in amyloid beta secretion. PC12 control cells, PC12 cells bearing the Swedish double mutation in amyloid beta precursor protein (APPsw), and PC12 cells transfected with human APP (APPwt) were used. The transfected APPwt and APPsw PC12 cells showed an about 2.8- and 4.8-fold increase of amyloid β (Aβ) secretion comparing to control PC12 cells. An increase of NO synthase activity, cGMP and free radical levels in APPsw and APPwt PC12 cells was observed. $cPLA_2$ protein level was higher in APPsw and APPwt PC12 cells comparing to PC12 cells. Moreover, phosphorylated $cPLA_2$ protein level and [^{3}H]AA release were also higher in APP-transfected PC12 cells than in the control PC12 cells. An NO donor, sodium nitroprusside, stimulated [^{3}H]AA release from prelabeled cells. The highest NO-induced AA release was observed in control PC12 cells, the effect in the other cell lines being statistically insignificant. Inhibition of $cPLA_2$ by $AACOCF_3$ significantly decreased the AA release. Inhibitors of nNOS and γ-secretase reduced AA release in APPsw and APPwt PC12 cells. The basal cytosolic $[Ca^{2+}]_i$ and mitochondrial Ca^{2+} concentration was not changed in all investigated cell lines. Stimulation with thapsigargin increased the cytosolic and mitochondrial Ca^{2+} level, activated NOS and stimulated AA release in APP-transfected PC12 cells. These results indicate that Aβ peptides enhance the protein level and phosphorylation of $cPLA_2$ and AA release by the NO signaling pathway.

Keywords: amyloid beta, nitric oxide synthase, cytosolic phospholipase A_2, arachidonic acid, reactive oxygen species, antioxidative enzymes, calcium, mitochondria

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Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; AD, Alzheimer's disease; APP, amyloid precursor protein; APPsw, Swedish double mutation form of APP; APPwt, wild-type APP; Aβ, amyloid β; $cPLA_2$, cytosolic phospholipase A_2; $[Ca^{2+}]_i$, intracellular free calcium concentration; DAPT, *N*-[*N*-(3,5-trifluorophenacetyl)-L-alanyl]-*S*-phenyglycine t-butyl ester; DCF, 2′,7-dichlorofluorescein; DHE, dihydroethidium; DHR, dihydrorhodamine; DTT, dithiothreitol; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated protein kinase1/2; GR, glutathione reductase; GPx, glutathione peroxidase; HBSS, Hank's balanced salt solution; H_2DCFDA, 2′,7-dichlorofluorescein diacetate; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; MAPK, mitogen-activated protein kinase; NANNG, (4*S*)-*N*-(4-amino-5[aminoethyl]aminopentyl)-*N*′-nitroguanidine, NNLA, *N*-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; p-$cPLA_2$, phospho-$cPLA_2$; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; ROS, reactive oxygen species; SNP, sodium nitroprusside; $sPLA_2$, secretory phospholipase A_2.

INTRODUCTION

Amyloid β (Aβ) accumulation, mitochondrial dysfunction and oxidative stress are early steps in the pathogenesis of Alzheimer's disease (AD) (Holscher, 1998; Yatin *et al*., 1999; Butterfield, 2002; Perry *et al*., 2002; Selkoe, 2001; 2006; Butterfield & Boyd-Kimball, 2004; Pratico & Sung, 2004; Hauptmann *et al*., 2006). Aβ is derived from the amyloid precursor protein (APP) through an initial β-secretase cleavage followed by an intramembraneous cut by γ-seceretase (Buchet & Pikuła, 2000; Selkoe, 2001; Haass, 2004). Certain mutations of the APP gene enhance intracellular Aβ production and lead to intracellular and extracellular Aβ accumulation in transfected cell lines or transgenic animals (Citron *et al*., 1997; Cai *et al*., 1993). In our previous study we showed that the Swedish double mutation in the human APP gene (K670M/N671L) resulted in a significantly higher Aβ production in transfected PC12 cells (APPsw) compared with human wild type APP-transfected PC12 cells (APPwt) (Eckert *et al*., 2001; Keil *et al*., 2004). Further studies have shown that the APPsw mutation induces oxidative stress, enhances nitric oxide (NO) level, induces mitochondrial dysfunction, and finally leads to an enhanced vulnerability to apoptotic cell death through the activation of c-Jun N-terminal kinase, caspase 3 and 9, release of cytochrome *c*, and ATP depletion (Marques *et al*., 2003; Keil, *et al*., 2004).

Previous studies from our and others groups indicate that NO is involved in PLA_2 activation through phosphorylation by PKC, ERK1/2 protein kinases as well as cGMP-dependent protein kinase (PKG) (Chalimoniuk *et al*., 2006) and leads to enhanced arachidonic acid (AA) release. Activation of PLA_2 mediates AA release from the *sn*-2 position of phospholipids. Several isoforms of PLA_2 have been identified in neurons, such as cytosolic calcium-dependent PLA_2 ($cPLA_2$), secretory ($sPLA_2$) and Ca^{2+}-independent ($iPLA_2$) (Murakami *et al*., 1997; Farooqui *et al*., 1997; Mollay *et al*., 1998; Sun *et al*., 2004). $cPLA_2$ is activated by increase in $[Ca^{2+}]_i$ (range 100–500 nM) that binds to NH-domain and induces translocation of $cPLA_2$ to membrane. Interestingly, enhanced $cPLA_2$ expression has been described in the brain of AD patients (Stephenson *et al*., 1996).

Excessive NO- and AA release-mediated neurotoxicity seems to be involved in brain aging, amyloid β toxicity and in the pathomechanism of AD (Chalimoniuk & Strosznajder, 1998; Ross *et al*., 1998; Keil *et al*., 2004). Thus, enhanced NO generation may link mitochondrial dysfunction and oxidative stress with the arachidonic acid cascade, but the detailed mechanisms need to be clarified. Accordingly, we investigated the possible relationship between enhanced Aβ secretion, NOS activity, $cPLA_2$ level, and AA release using control, APPwt, and APPsw PC12 cells.

MATERIAL AND METHODS

PC12 cell culture. PC12 (pheochromocytoma) cells were transfected with DNA constructs harboring human mutant APP gene (APPsw, K670M/N671L) or APPwt gene according to Keil *et al*. (2004). Transfected APPwt PC12 cells, PC12 cells bearing the Swedish double mutation in the amyloid precursor protein gene (APPsw), and control PC12 cells (transfected with empty vector) were cultured in 75 cm^2 flasks in DMEM supplemented with 10%-heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum (HS), 0.5% penicillin/streptomycin (50 U/ml), 400 μg/ml G418 and 2 mM glutamine. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were subcultured about once a week. For experiment, confluent cells were subcultured into polyethylenimine-coated 35 mm^2 dishes or 24-well plates. Cells were used for experiments at 75–90% confluence or one day after being plated in the 24-well plate. Prior to treatment, cells were replenished with 2% FBS medium or with serum-free medium.

Determination of $A\beta_{1-40}$. Cells were plated at equal density in 6-well plates. After reaching confluence, 2 ml of conditioned media was collected after 24 h of culture. Media were centrifuged to remove cell fragments, and aliquots were then used to determine the $A\beta_{1-40}$ level. For determination of secreted $A\beta_{1-40}$, a specific sandwich enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies was used. The ELISA was performed according to the instructions given in the Aβ-ELISA kit by Biosources (San Diego, CA, USA). The assay principle is that of a standard sandwich ELISA, which utilizes a site-specific rabbit anti-human $A\beta_{1-16}$ capture, a cleavage site-specific rabbit anti-human $A\beta_{1-40}$ terminal detection antibody, and anti-rabbit IgG peroxidase-cojugated secondary antibody.

PC12 cell treatment. Cells were treated with inhibitors of: $cPLA_2$ (arachidonyl trifluoromethyl ketone, $AACOCF_3$, 1 μM), γ-secretase (*N*-[*N*-(3,5-trifluorophenacetyl)-L-alanyl]-*S*-phenyglycine t-butyl ester, DAPT, 1 μM), Ca^{2+}-dependent NOS (*N*-nitro-L-arginine, NNLA, 10 μM) or neuronal NOS ((4*S*)-*N*-(4-amino-5[aminoethyl]aminopentyl)-*N*′-nitroguanidine, NANNG, 1 μM) for 30 min at 37°C. Moreover, the cells were treated with 1 mM sodium nitroprusside (SNP) in the absence or presence of PLA_2 inhibitor ($AACOCF_3$, 1 μM) for 30 min as described in legend to figures.

Determination of nitric oxide synthase (NOS) activity. NOS activity was determined by measuring

the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as described by Chalimoniuk *et al*. (2006). Briefly, cells were washed with warm PBS and incubated in culture dishes for 20 min at 37°C with 3 μCi/mL [^{3}H]L-arginine (Amersham Biosciences, Piscataway, NJ, USA) in Hank's buffer. Following this incubation period, cells were washed in ice cold PBS and lysed with 0.3 M $HClO_4$. The lysate was neutralized with 1 M Na_2CO_3 and centrifuged at 3000×*g* for 10 min. Then, 400 μl of supernatant was applied to a Dowex AG 50WX (Na^+) column. The [^{3}H]L-citrulline formed was eluted with 1 ml of water and 500 μl of the elution was mixed with 5 ml of scintillation cocktail. The radioactivity was quantified using an LKB 1409 Wallac scintillation counter. To determine the cellular uptake of [^{3}H]L-arginine from each dish, radioactivity from a 50 μl aliquot of supernatant was also quantified**.** NOS activity was expressed as percentage of control (NOS activity in control PC12 cells).

Determination of cellular cGMP level. Cyclic GMP was determined using a commercially available ELISA kit (Amersham Biosciences, Piscataway, NJ, USA) according to the procedure supplied by the manufacturer. This method allows measuring cGMP in the range of 2–512 pmol/well, and it is based on a competition between cGMP present in the sample with peroxidase-labeled cGMP for binding to specific anti-cGMP antibody. Briefly, medium was collected into new tubes and cells were washed with PBS. Then, the medium and cell lysate samples were acetylated. Acetylated samples were incubated with an anti-cGMP antibody for 2 h at 4°C and then with cGMP conjugated to horseradish peroxidase for 60 min at 4°C. 3,3′5′5′-Tetramethylbenzidine was used as a substrate for horseradish peroxidase. To quantify cellular cGMP levels (pmol/10^5 cells) a standard curve was generated using a standard solution of cGMP.

Determination of free radicals using 2′,7-dichlorofluorescein (DCF). The dichlorofluorescein (DCF) fluorescence assay detects the level of hydrogen peroxide and other free radicals in cells. Free radicals were determined based on reactive oxygen species-mediated conversion of 2′,7-dichlorofluorescein diacetate into fluorescent DCF (Royall & Ischiropoulos, 1993; Adamczyk *et al*., 2006). PC12 cells were loaded with 10 μM 2′,7-dichlorofluorescein diacetate (H_2DCFDA) in DMSO by incubating for 50 min at 37°C. DMSO was used at a final concentration of 0.05% and at this concentration had no effect on free radicals levels. Cells were washed three times in PBS buffer. Fluorescence of DCF was measured using a Perkin Elmer LS 50B spectrofluorometer with excitation and emission wavelengths at 488 and 525 nm, respectively. DCF fluorescence is reported as intensity per mg protein.

Mitochondrial oxidative stress (dihydrorhodamine (DHR) fluorescence). DHR detects the level of reactive oxygen species in mitochondria (Hempel *et al*., 1999). DHR is the non-fluorescent reduced form of rhodamine 123. Due to its lipophilicity it can easily diffuse through cell membranes. Inside the cell, it is oxidized by various ROS to the positively charged fluorescent rhodamine 123 which is incorporated into the mitochondria. DHR mainly detects hydrogen peroxide, especially in the presence of cytochrome *c* oxidase in mitochondria and can thus be used as a marker for mitochondrial ROS production. PC12 cells were incubated with 10 μM dihydrorhodamine in DMEM for 15 min at 37°C. After incubation cells were washed twice with HBSS. Fluorescence of dihydrorhodamine (DHR) was measured using a fluorescence reader (Victor multilabel counter) with excitation and emission wavelengths at 490 and 535 nm, respectively.

Detection of superoxide anions with dihydroethidium (DHE). Superoxide anions can be detected by oxidation of DHE (Rothe & Valet, 1990). Upon oxidation, DHE forms ethidium that intercalates into DNA double strands in the cell. When stacked between the bases, the π-electrons of the dye interact with the π-electrons of the DNA bases, resulting in altered fluorescence properties. PC12 cells were incubated with 40 μM dihydroethidium in Hank's balanced salt solution (HBSS) for 60 min at 37°C. After incubation cells were washed twice with HBSS. Fluorescence of ethidium was measured using a fluorescence reader (Victor multilabel counter) with excitation and emission wavelengths at 535 and 590 nm, respectively.

Cu,Zn-SOD activity. Cu,Zn-SOD activity was measured with the superoxide dismutase assay kit (Calbiochem by VWR International GmbH, Darmstadt, Germany). To remove interfering substances and rule out Mn-SOD activity, the Cu,Zn-SOD activity was assayed after extraction with chloroform and ethanol according to the supplier's manual. SOD activity was calculated based on the Vs/Vc ratio of the autooxidation rates of the chromophore BXT-01050 measured at 37°C in the presence (Vs) and absence (Vc) of sample. The chromophore was measured in a Genesys 5 photometer (Spectronic Instruments, Rochester, USA) at 525 nm. One Cu,Zn-SOD activity unit is defined as the activity that doubles the autooxidation background (Vs/Vc = 2).

Glutathione peroxidase (GPx) activity. GPx (cytosolic GPx) activity was measured using the cellular glutathione peroxidase assay kit (Calbiochem by VWR International GmbH, Darmstadt, Germany) and tert-butylhydroperoxide as substrate. This reaction is based on the enzymatic reduction of hydroperoxide by GPx with consumption of reduced glutathione GSH which is restored from oxidized

glutathione GSSG in a coupled enzymatic reaction by glutathione reductase (GR). This enzyme reduces GSSG to GSH with consumption of NADPH as reducing equivalents. The decrease in absorbance at 340 nm due to NADPH consumption was measured in a Victor multiplate reader using a 355 nm filter with 40 nm bandpass. One unit of GPx was defined as the activity that converts 2 μmol of reduced glutathione per minute at 25°C.

Glutathione reductase (GR) activity. The GR activity was determined using the glutathione reductase assay kit (Calbiochem by VWR International GmbH, Darmstadt, Germany). The enzymatic activity was assayed photometrically by measuring NADPH consumption during the enzymatic reaction. In the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH to yield NADP, resulting in a decrease of absorbance at 340 nm, which was measured in a Victor plate reader. One unit of GR is defined as the activity that reduces 1 μmol of GSSG (corresponding to 1 μmol of NADPH) per minute at 25°C.

Gel electrophoresis and Western blotting for detection of $cPLA_2$ and phosphorylated $cPLA_2$. Cell lysate (40 μg protein) was mixed with an equal volume of sample buffer SB (62.5 mM Tris/HCl, 2% SDS, 100 mM DTT, 20% glycerol and 0.2% bromophenol blue, pH 6.8, (Laemmli, 1970)). The samples were heated for 5 min at 95°C. The proteins were analyzed by electrophoresis on 10% polyacrylamide gel. Then, proteins were electrophoretically transferred from the polyacrylamide gel to nitrocellulose membranes (Bio-Rad) at 4 mA/cm^2 of gel for 2 h. The membrane was blocked in 5% milk powder in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 h at room temp. Then the membrane was incubated with rabbit polyclonal anti-$cPLA_2$ antibody or anti-p-$cPLA_2$ antibody (Cell Signaling, Beverly, MA, USA) (diluted 1:200 or 1:1000 in TBS-T containing 2% (w/v) non-fat milk, respectively) overnight at 4°C. The $cPLA_2$ or p-$cPLA_2$ antibody complex was identified with anti-rabbit IgG antibody conjugated with horseradish peroxidase (ARHRP, diluted 1:5000 in TBS-T containing 5% (w/v) non-fat milk) by incubation for 1 h at room temp. The anti-$cPLA_2$-ARHRP and anti-p-$cPLA_2$-ARHRP were visualized with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and Hyperfilm-Kodak (Sigma, St. Louis, MO, USA). The optical densities of the $cPLA_2$ and phospho-$cPLA_2$ bands on the immunoblot were quantified using a NucleoVision apparatus and GelExpert 4.0 software from NucleoTech (San Manteo, CA, USA).

Assessment of AA release from PC12 cells. AA release was quantified essentially as described previously (Samanta *et al*., 1998; Xu *et al*., 2002) with some minor modifications. Briefly, 0.1 μCi of [^{3}H]AA (NEN, specific radioactivity 55 Ci/mol) was suspended in DMEM medium containing 0.5% BSA and added directly to PC12, APPsw or APPwt cells cultured in a 35 mm dish. After incubation for 4 h at 37°C, unincorporated labeled AA was removed by three successive washings with buffer A containing 145 mM NaCl, 5.5 mM KCl, 1.1 mM $MgCl_2$, 1.1 mM $CaC1_2$, 5.5 mM glucose, 20 mM Hepes, pH 7.4, and BSA (0.5 mg/ml). Cells were preincubated in buffer A for 30 min at room temp. Then the cells were treated with inhibitors of $cPLA_2$ ($AACOCF_3$, 1 μM), γ-secretase (DAPT, 1 μM), calcium-dependent NOS (NNLA, 10 μM) or with a specific neuronal NOS inhibitor (NANNG, 1 μM) for 30 min at 37°C. In some experiments, cells were subsequently incubated with sodium nitroprusside (SNP, 1 mM) without or with 1 μM $AACOCF_3$ for 30 min at 37°C. After incubation, cell media were transferred to scintillation tubes and radioactivity in the supernatant was determined using an LKB 1409 Wallac scintillation counter.

Cytosolic calcium measurements. Cells were plated onto polylysine-coated glass cover slips or multi-well plates one day before experiments. Measurement of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was carried out using the fluorescent indicator fura-2 in combination with a monochromator-based imaging system (T.I.L.L. Photonics, Gräfelfing, Germany) attached to an inverted microscope (Axiovert 100, Zeiss, Jena, Germany). PC12 cells were loaded with 5 μM fura-2-AM (Molecular Probes, Leiden, The Netherlands) supplemented with 0.05% Pluronic F127 for 45 min at 37°C in a standard solution containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.5 mM glucose and 10 mM Hepes (adjusted to pH 7.4 with NaOH). The cells were washed once with HBSS and, to ensure complete AM ester hydrolysis, kept for 30 min at room temp. in the dark. For $[Ca^{2+}]_i$ measurements, fluorescence was excited at 340 and 380 nm. After correction for the individual background fluorescence, the fluorescence ratio F340/F380 was calculated.

Mitochondrial calcium measurements. For qualitative estimation of mitochondrial calcium $[Ca^{2+}]_m$, the cells were loaded with 3 μM dihydrorhod-2/AM (rhod-2/AM reduced with $NaBH_4$) in the presence of 0.02% Pluronic® in Hank's balanced salt solution (HBSS, pH 7.4) for 45 min at 37°C. The cells were washed once with HBSS and, to ensure complete AM ester hydrolysis, were kept for 20–30 min at room temp. in the dark. After a second wash step, the fluorescence was determined with a fluorescence reader (Victor® multilabel counter) at 550/574 nm.

Statistic analysis. The results were expressed as mean values ±S.E.M. Differences between means were analyzed using one-way or two-way (type of cells and treatment) ANOVA followed by the New-

Table 1. $AB_{1–40}$ secretion by APP-transfected and control PC12 cells under basal conditions

	PC12 cells	APPwtPC12 cells	APPswPC12 cells
$A\beta_{1–40}$ [pg/ml]	1.30±0.35	3.69±0.28**	6.19±0.85**##

Cells were plated at equal density in 6-well plates. After reaching confluence, 2 ml of conditioned media was collected for 24 h. $A\beta_{1–40}$ level was determined by ELISA kit. Values are means ±S.E.M. from 4 experiments. **$P<0.01$ *versus* PC12 cells, ##$P<0.01$ *versus* APPwt PC12 cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

man-Keuls test when appropriate. $P<0.05$ was considered statistically significant. The statistical analyses were performed by using Statistic version 4.0.

RESULTS

Control PC12 cells, PC12 cells bearing the Swedish double mutation of human APP gene (APPsw) or those transfected with human APP (APPwt) have been described previously (Marques *et al*., 2003; Keil *et al*., 2004). The cells expressing human wild-type APP and mutant APP showed 2.8- and 4.8-fold higher Aβ production comparing to PC12 cells transfected with empty vector (Table 1). Moreover, the data of Eckert *et al*. (2001) demonstrated similar properties of non-transfected PC12 cells and those transfected with empty vector.

We observed an increase of NOS activity and cGMP level in APPsw and APPwt PC12 cells compared to control PC12 cells (Fig. 1A, B and C). The NOS activity in APPsw PC12 cells was 1.4-fold higher than in control PC12 cells (Fig. 1A). The activity of NOS increased 1.3-fold in APPwt PC12 cells compared to control cells, but was significantly lower than in APPsw PC12 cells (Fig. 1A). The increased NOS activity was accompanied by enhanced intracellular and extracellular cGMP levels in APPsw and APPwt cells (Fig. 1B and C). The highest intracellular and extracellular cGMP levels were observed in APPsw PC12 cells (Fig. 1B and C). Thus, elevated Aβ generation increased NOS activity and cGMP concentration.

In agreement with our previous findings that $cPLA_2$ activity and AA release can be enhanced by *in vitro* added NO donors like sodium nitroprusside

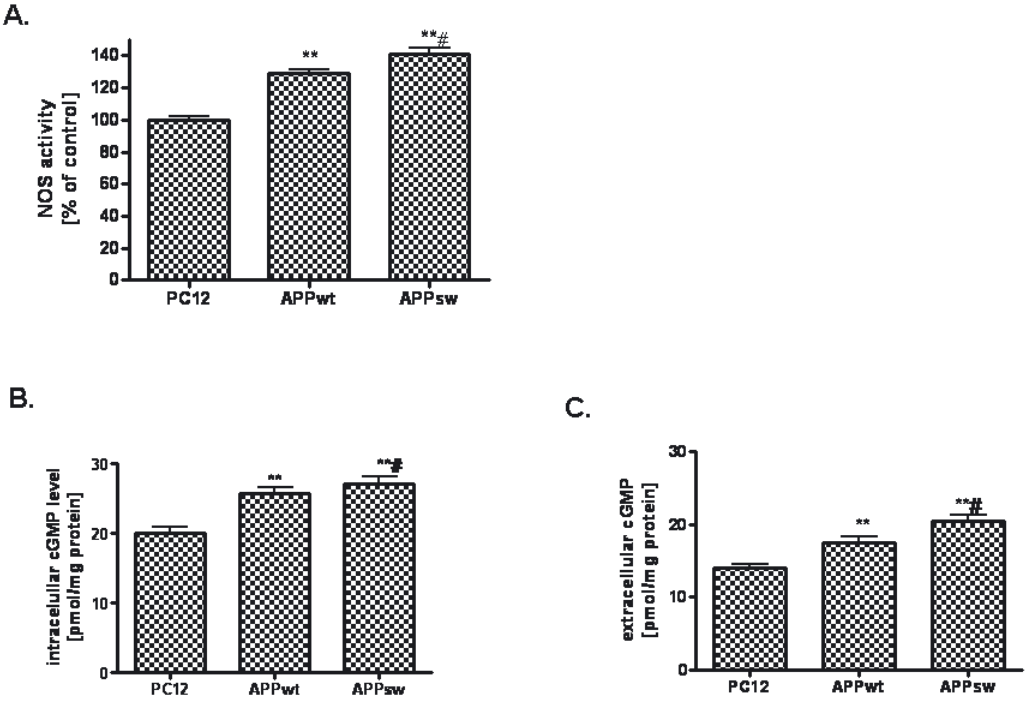


Figure 1. NOS activity and cGMP levels in APP-transfected and control PC12 cells.
NOS activity (A) was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as described in Material and Methods**.** The cGMP levels (B and C) were determined by ELISA. Values are means ±S.E.M. from 5 experiments. **$P<0.001$ *versus* control PC12 cells, #$P<0.05$ *versus* untreated APPwt cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

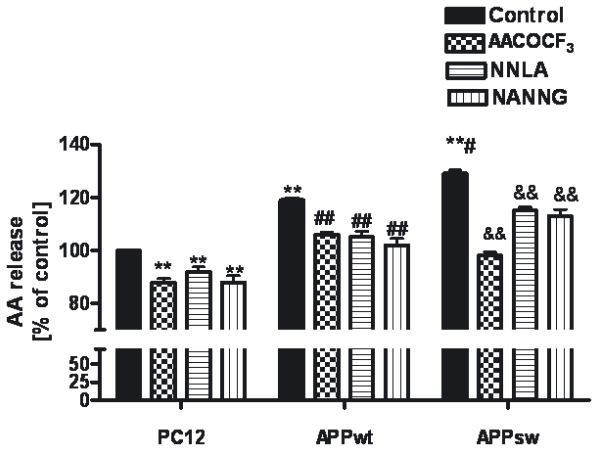
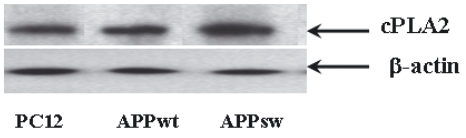


Figure 2. Effect of $AACOCF_3$, NNLA and NANNG on AA release in APP-transfected and control PC12 cells.
[^{3}H]AA-labeled cells were treated with inhibitor of $cPLA_2$ ($AACOCF_3$, 1 μM), calcium-dependent NOS inhibitor (NNLA, 10 μM), or with specific neuronal NOS inhibitor, (NANNG, 1 μM) for 30 min at 37°C. Values are means ±S.E.M. from 5 experiments. $^{**}P<0.01$ *versus* control (non-treated) PC12 cells, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* non-treated APPwt PC12 cells, $^{\&\&}P<0.01$ *versus* non-treated APPsw PC12 cells by two-way ANOVA followed by Newman-Keuls post-hoc test.



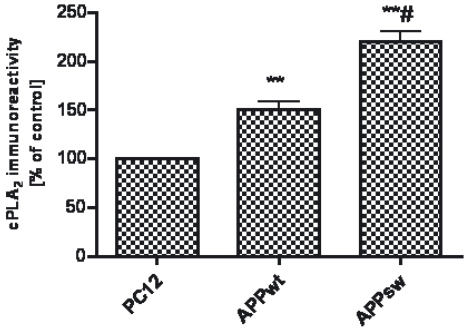


Figure 3. $cPLA_2$ immuoreactivity in APP-transfected and control PC12 cells.
Lysed cells (40 μg of protein) were subjected to 10% polyacrylamide gel SDS/PAGE and analyzed for $cPLA_2$ expression by immunoblotting using rabbit polyclonal anti-$cPLA_2$ antibody (diluted 1:200) as described in Material and Methods. The photographic inserts show representative Western blot analysis from 6 separate experiments. The results of densitometric analysis are means ±S.E.M. from 6 experiments. $^{**}P<0.01$ *versus* control PC12 cells, $^{\#}P<0.05$ *versus* APPwt PC12 cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

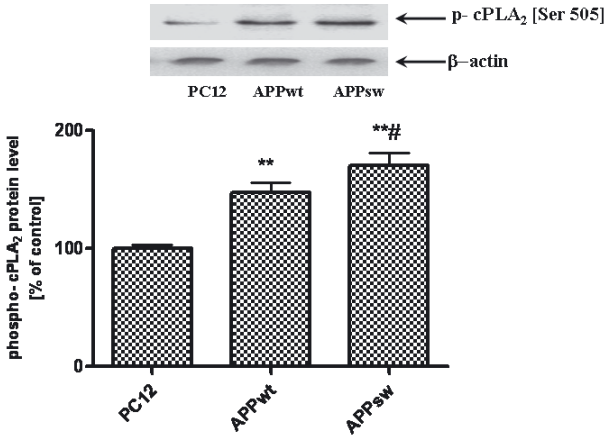


Figure 4. $cPLA_2$ phosphorylated protein levels determined by Western blot analysis using anti-p-$cPLA_2$ antibody in APP-transfected and control PC12 cells.
Lysed cells (40 μg of protein) were subjected to 10% polyacrylamide gel SDS/PAGE and analyzed for phospho-$cPLA_2$ protein level by immunoblotting using rabbit polyclonal anti-cPLA2 phospho-serine 505 antibody (diluted 1:1000) as described in Material and Methods. The photographic inserts show representative Western blot analysis from 6 separate experiments. The results of densitometric analysis are means ±S.E.M. from 6 experiments. $^{**}P<0.01$ *versus* control PC12 cell, $^{\#}P<0.05$ *versus* APPwt PC12 cells.

(SNP) to brain cortex synaptoneurosomes (Chalimoniuk *et al*., 2006), AA release was increased 1.3-fold in APPsw and 1.2-fold in APPwt cells compared to control cells, indicating that the rather small elevation of NO in both cell lines is sufficient to stimulate $cPLA_2$ (Fig. 2). An inhibitor of $cPLA_2$, $AACOCF_3$, decreased the AA release significantly, by about 30%,

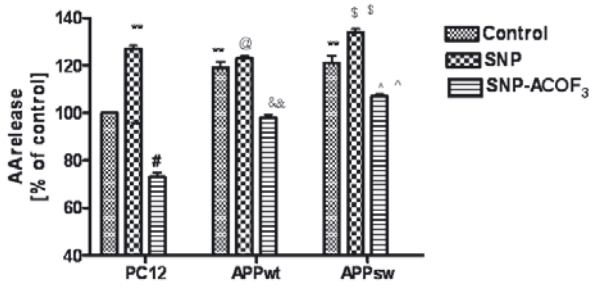


Figure 5. Effect of SNP on AA release in APP-transfected and control PC12 cells.
[^{3}H]AA–prelabeled cells were treated with 1 mM SNP without or with inhibitor of $cPLA_2$ ($AACOCF_3$, 1 μM) for 30 min at 37°C. After incubation, the radioactivity was measured in medium as described in Material and Methods. Values are means ±S.E.M. from 8 experiments. $^{**}P<0.01$ *versus* untreated PC12 cells, $^{\#}P<0.05$ *versus* PC12 cells treated with SNP, $^{@}P<0.05$ *versus* untreated APPwt PC12 cells, $^{\&\&}P<0.01$ *versus* APPwt PC12 cells treated with SNP, $^{\$\$}P<0.01$ *versus* untreated APPsw PC12 cells, $^{\wedge\wedge}P<0.01$ *versus* APPsw PC12 cells treated with SNP by two-way ANOVA followed by Newman-Keuls post-hoc test.

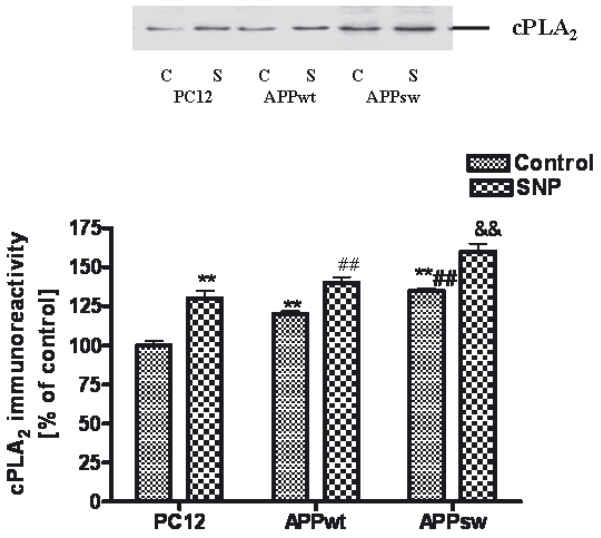


Figure 6. Effect of SNP on $cPLA_2$ immunoreactivity in APP-transfected and control PC12 cells.
Cells were treated with 1 mM SNP for 30 min at 37°C. Then, 40 μg of proteins was subjected to 10% polyacrylamide gel SDS/PAGE and analyzed for $cPLA_2$ expression by immunoblotting using rabbit polyclonal anti-$cPLA_2$ antibody (diluted 1:200) as described in Material and Methods. The photographic inserts show representative Western blot analysis from 6 separate experiments. The results of densitometric analysis are means ±S.E.M. from 6 experiments. $^{**}P<0.01$ *versus* untreated PC12 cells, $^{\#\#}P<0.01$ *versus* untreated APPwt PC12 cells, $^{\&\&}P<0.01$ *versus* untreated APPsw PC12 cells by two-way ANOVA followed by Newman-Keuls post-hoc test.

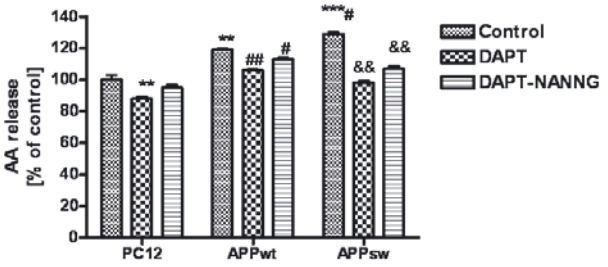


Figure 7. Effect of DAPT and NANNG on AA release by APP-transfected and control PC12 cells.
[^{3}H]AA-prelabeled cells were treated with inhibitor of γ-secretase (DAPT, 1 μM) or with DATP together with specific neuronal NOS inhibitor (NANNG, 1 μM) for 30 min at 37°C. Values are means ±S.E.M. from 5 experiments. $^{**}P<0.01$, $^{***}P<0.001$ *versus* control non-treated PC12 cells, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* non-treated APPwt PC12 cells, $^{\&\&}P<0.01$ *versus* non-treated APPsw PC12 cells by two-way ANOVA followed by Newman-Keuls post-hoc test.

15% and 15% in APPsw, APPwt and control PC12 cells, respectively (Fig. 2).

N-Nitro-L-arginine (NNLA), an inhibitor of Ca^{2+}-dependent NOS, and the specific neuronal NOS inhibitor NANNG also significantly lowered the AA release in all investigated cells (Fig. 2).

Enhanced AA release was paralleled by higher immunoreactivity of $cPLA_2$ protein in APPsw and APPwt PC12 cells compared with control PC12 cells. APPsw PC12 cells showed a 1.50- and 2.25-fold increased immunoreactivity of $cPLA_2$ compared to APPwt and control PC12 cells, respectively (Fig. 3). The activity of PLA_2 is regulated by phosphorylation. For determination of phosphorylated $cPLA_2$ protein level we used Ser 505 phospho-$cPLA_2$ antibody. Phosphorylated $cPLA_2$ protein levels were about 1.7- and 1.47-fold higher in APPsw and APPwt PC12 cells *versus* control PC12 cells, respectively (Fig. 4).

After incubation with the NO donor, SNP (1 mM), [^{3}H]AA release was significantly increased in all investigated cells (APPwt, APPsw, control PC12 cells) (Fig. 5). However, the highest SNP-induced the AA release was noted in control PC12 cells, where SNP increased AA release about 1.3-fold relative to non-treated PC12 cells. In APPsw PC12 cells and APPwt cells, AA release after SNP treatment was significantly increased but to a lesser extend than in control PC12 cells probably due to the already enhanced AA liberation by elevated endogenous NO concentration (Fig. 5). The basal but also the SNP-elevated AA release was significantly inhibited by the $cPLA_2$ inhibitor $AACOCF_3$ (Fig. 5).

Similar to its effect on $cPLA_2$ activity, SNP also enhanced $cPLA_2$ protein level and again this effect was higher in control PC12 than in APPwt or APPsw PC12 cells (Fig. 6). We have previously shown that the γ-secretase inhibitor DAPT reduced efficiently Aβ overproduction and mitochondrial dysfunction by normalizing NO and ATP levels as well as mitochondrial membrane potential in APPsw PC12 cells (Keil *et al*., 2004). In agreement with those data, DAPT reduced the enhanced AA release in APPsw PC12 cells (Fig. 7), confirming the relationship between enhanced Aβ levels on the one hand and elevated NO and $cPLA_2$ levels on the other.

As $cPLA_2$ activity is regulated by the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, we investigated possible changes of $[Ca^{2+}]_i$ in control and transfected PC12 cells. The basal cytosolic and mitochondrial Ca^{2+} levels are the same in all investigated cell lines (Fig. 8 A and C). Under these basal conditions $cPLA_2$ is mainly activated by NO-regulated cGMP-dependent protein kinase and other kinases. However, treatment with thapsigargin led to a significant enhancement of cytosolic as well as mitochondrial Ca^{2+} level in APPsw PC12 cells (Fig. 8B and D). These data suggest that after an ER stress the Ca^{2+} homeostasis is easily disturbed in APP-transfected cells.

We also found the highest levels of NOS activity and increased AA release following thapsigargin stimulation in APPsw PC12 cells, suggesting that NOS and AA release are, at least in part, regu-

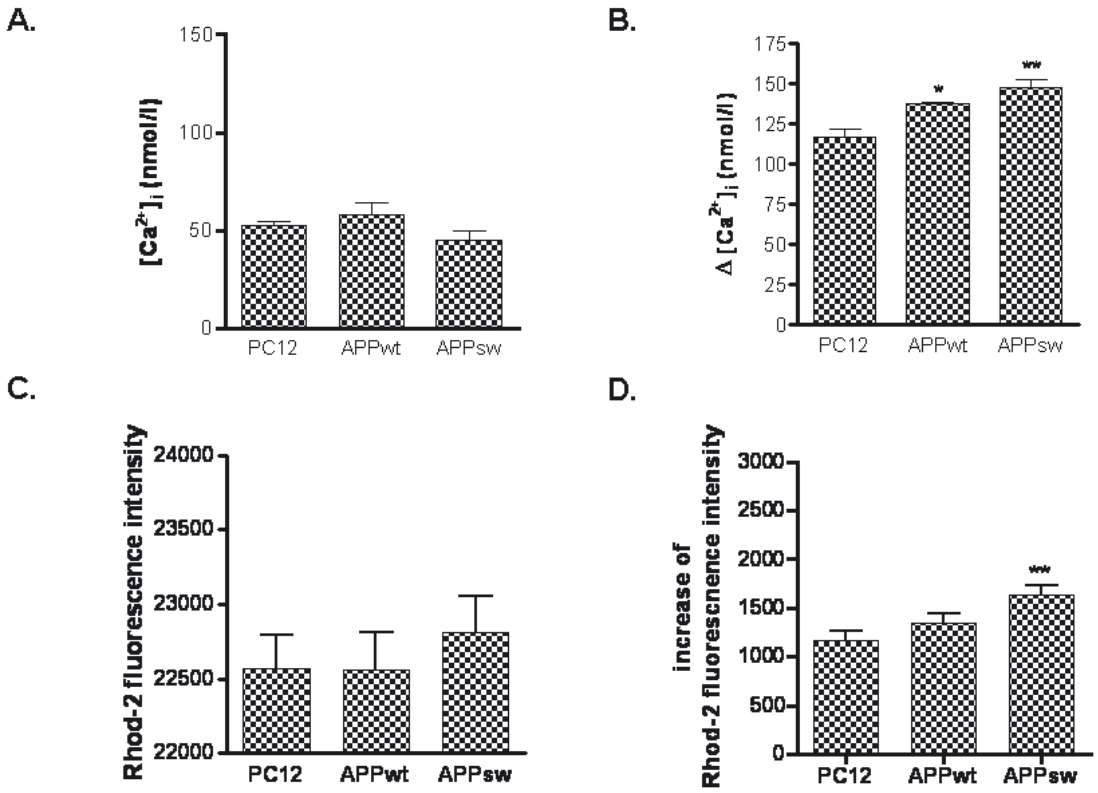
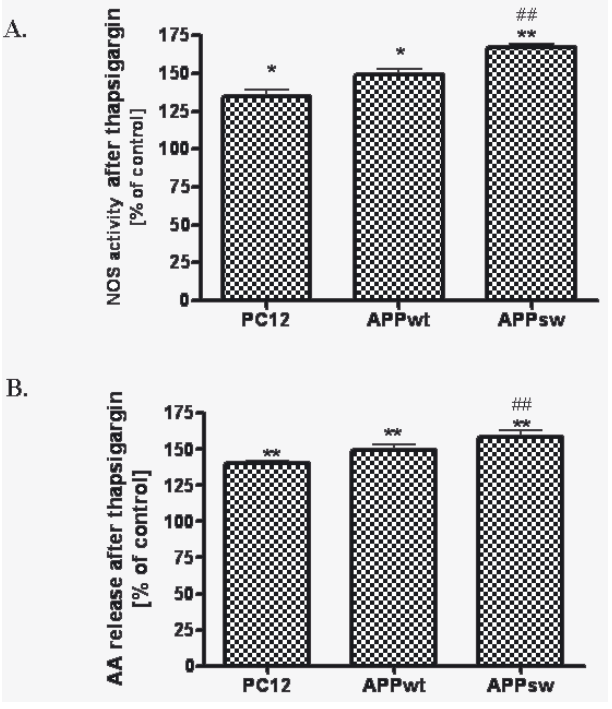


Figure 8. Cytosolic and mitochondrial calcium levels under basal conditions (A, C) and after stimulation with thapsigargin (B, D).
For measurement of cytosolic $[Ca^{2+}]_i$, the cells were loaded with 5 μM fura-2-AM supplemented with 0.05% Pluronic F127 in Hank's balanced salt solution pH 7.4 for 45 min at 37°C as described in Material and Methods. For measurement of mitochondrial calcium $[Ca^{2+}]_m$, the cells were loaded with 3 μM dihydrorhod-2/AM (rhod-2/AM reduced with $NaBH_4$) in the presence of 0.02% Pluronic® in Hank's balanced salt solution pH 7.4 for 45 min at 37°C. The cells were exposed to thapsigargin (10 ng/ml) for 30 min at 37°C. Values are means ±S.E.M. from 6–13 experiments.$^{**}P<0.01$, $^{*}P<0.05$ *versus* control PC12 cells by one-way-ANOVA followed by Newman-Keuls post-hoc test.



lated by an increase in $[Ca^{2+}]_i$ after thapsigargin-induced release from the ER (Fig. 9).

Taken together, our observations of enhanced NOS activity and AA release but unchanged cytosolic and mitochondrial $[Ca^{2+}]$ in APP-transfected cells do not support the assumption of a major role of Ca^{2+} in modulating the NOS and $cPLA_2$ activity in these cells (Fig. 8).

Figure 9. The effect of thapsigargin on NOS activity and AA release in APP-transfected and control PC12 cells.
Cells were exposed to thapsigargin (10 ng/ml) for 30 min at 37°C. NOS activity assay was performed by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, AA release was estimated by measuring the radioactive [^{3}H]AA release into medium from [^{3}H]AA-prelabeled cells as described in Material and Methods. Values are means ±S.E.M. from 5 experiments. $^{*}P<0.05$, $^{**}P<0.01$ *versus* non-treated PC12 cells, $^{\#}P<0.05$ *versus* non-treated APPwt PC12 cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

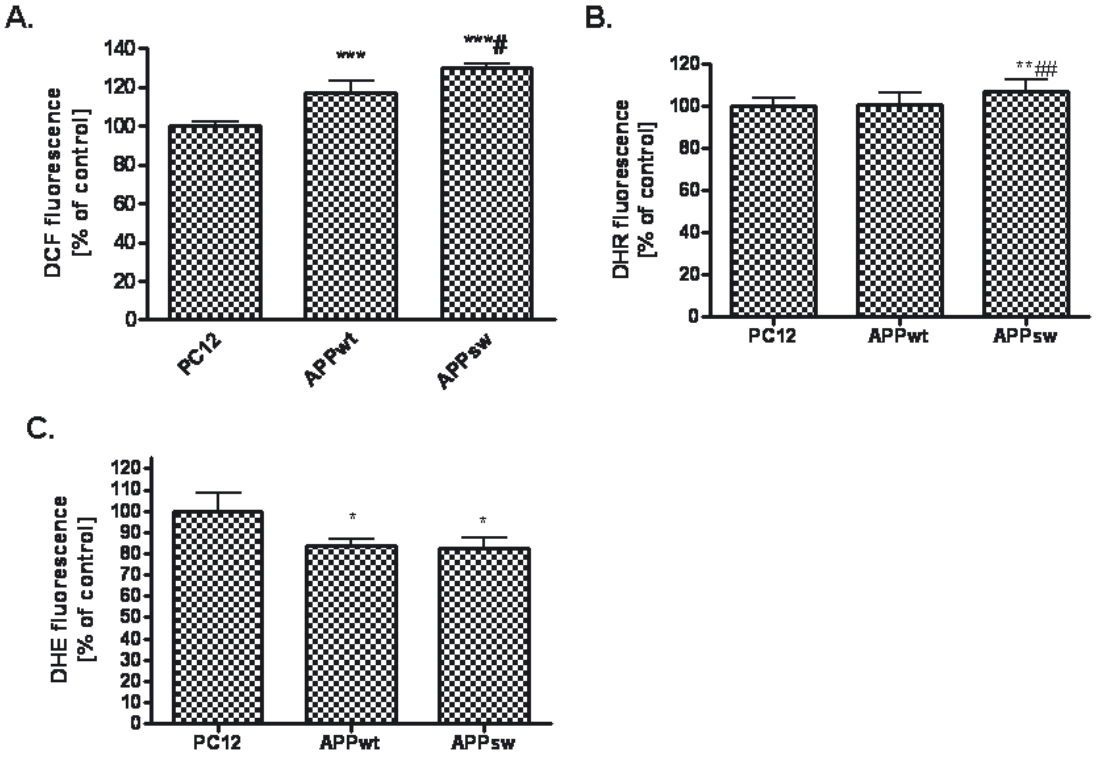


Figure 10. Cytosolic and mitochondrial ROS in APP-transfected and control PC12 cells.
(A) The level of free radicals in the cytosol was detected by using 2′,7-dichlorofluorescein (DCF) fluorescence assay. (B) The level of reactive oxygen species in mitochondria was determined by using dihydrorhodamine (DHR) fluorescent dye. C) Superoxide anions were detected by oxidation of dihydroethidium (DHE). Values are means ±S.E.M. from 5–8 experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* non-treated PC12 cells, $^{\#}P<0.05$ *versus* non-treated APPsw cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

An elevated oxidative stress due to the NO-induced mitochondrial dysfunction (Keil *et al*., 2004) could be a possible explanation for the enhanced $cPLA_2$ activity in the transfected PC12 cells. This option is supported by the previous finding indicating an elevation of $cPLA_2$ activity following H_2O_2 stress (Chalimoniuk *et al*., 2006). In agreement with these assumptions, cytosolic ROS levels assayed by the DCF method were significantly higher in APPsw and APPwt PC12 cells compared to control PC12 cells (Fig. 10A). Similarly, mitochondria-derived ROS levels measured with the fluorescent dye DHR were also slightly, but significantly increased in APPsw cells compared to APPwt and control PC12 cells (Fig. 10B). By contrast, superoxide radicals assayed by the fluorescent dye DHE were significantly decreased in APPwt and APPsw cells compared to control PC12 cells (Fig. 10C), suggesting an elevated activity of antioxidant enzymes as a partial compensating mechanism (Schüssel *et al*., 2006). This was supported by our observations that Cu/Zn-superoxide dismutase and glutathione reductase activities were significantly increased in APPsw cells compared to APPwt and control PC12 cells, whereas glutathione peroxidase activity was significantly decreased in APPsw cells (Fig. 11).

DISCUSSION

There is a growing body of evidence suggesting that NO plays an important role in the pathomechanism of Alzheimer's disease (AD) within the cascade of events leading from enhanced intracellular Aβ accumulation to enhanced vulnerability to apoptosis and cell death (Luth & Arendt, 1998; Luth *et al*., 2001; Lahiri *et al*., 2003). In PC12 cells transfected with human wild type or mutated APP (APPwt, APPsw), an increased Aβ level is associated with an enhanced NO level, impaired mitochondrial function, and depletion of ATP (Eckert *et al*., 2001; Marques *et al*., 2003; Keil *et al*., 2004). The higher NO concentration activates AA release by $cPLA_2$ (Chalimoniuk *et al*., 2006). Elevated AA in turn might contribute to neuronal dysfunction and cell death as it has been suggested from the data of enhanced AA release in AD brain (Stephenson *et al*., 1996). Our results indicate a direct relationship between Aβ concentration, NOS activity and $cPLA_2$ level and AA release in cells. This sequence of events has significant consequences in cell degeneration and death. Since usually very high extracellular levels of Aβ are needed to induce oxidative stress and enhance NO levels in neuronal cells, we used two PC12-transfected cell

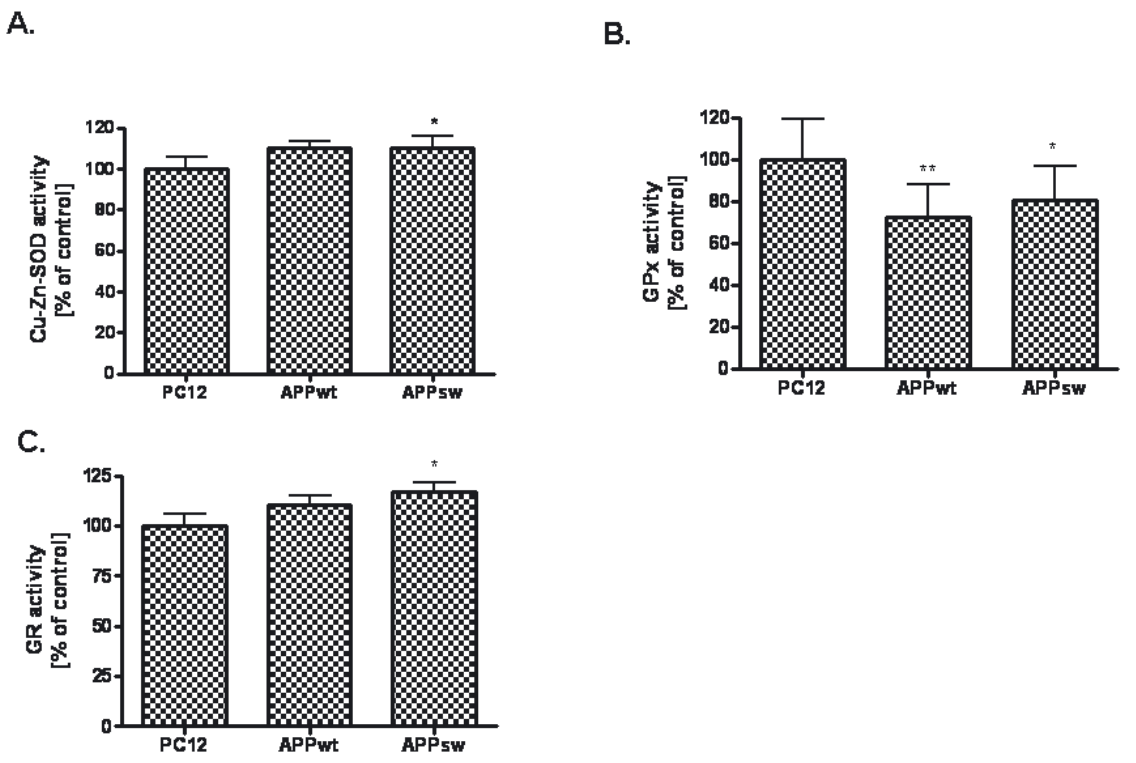


Figure 11. Antioxidative enzyme activities in APP-transfected and control PC12 cells.
Cu,Zn-SOD (A), GPx (B) and GR (C) activities were measured with assay kits as described in Material and Methods. Values are means ±S.E.M. from 6 experiments. $^{**}P<0.01$, $^{*}P<0.05$ *versus* control PC12 cells by one-way ANOVA followed by Newman-Keuls post-hoc test.

lines to clarify if elevation of Aβ concentration leads, *via* increasing NO, to enhanced $cPLA_2$ protein level and AA release.

In agreement with this assumption we found elevated NOS activity, increased NO level, and higher intra- as well as extracellular concentration of cGMP in APPwt cells as well as in APPsw *versus* PC12 control cells. Similarly to our findings using extracellular NO donors like SNP (Chalimoniuk *et al*., 2006), the elevated intracellular NO concentration was accompanied by increased $cPLA_2$ protein level, as well as AA release. Moreover, an increase of phospho-$cPLA_2$ was observed confirming the important role of protein kinases for $cPLA_2$ activation (Gijon *et al*., 2000; Xu *et al*., 2002; Evans *et al*., 2002; Chalimoniuk *et al*., 2006). The relationship between the elevated Aβ level and the enhanced $cPLA_2$ activity was confirmed by the always higher effects in the APPsw PC12 cells (higher Aβ level) in comparison to the APPwt PC12 cells (lower Aβ level). Moreover, our findings indicating that the γ-secretase inhibitor DAPT, which reduces Aβ generation (Keil *et al*., 2004), also reduced the elevated AA release supported the proposed mechanism.

Although many findings clearly show that $cPLA_2$ activity is regulated by $[Ca^{2+}]_i$, (Handlogten *et al*., 2001; Strokin *et al*.; 2003; Shimizu *et al*., 2004), several of our findings rule out alterations of $[Ca^{2+}]_i$ being of major relevance. Neither cytosolic nor mitochondrial $[Ca^{2+}]$ were different in the three PC12 cell lines which showed substantial differences in AA release under basal conditions. Thus, $[Ca^{2+}]_i$ is probably not the most important factor for the link of initial NO generation with AA release, but may contribute to the responses after an ER stress.

On the other hand, $cPLA_2$ activity is regulated by oxidative stress and by elevated intracellular ROS (Sapirstein *et al*., 1996; Samanta *et al*., 1998; Chalimoniuk *et al*., 2006). In our studies, increased cytosolic and mitochondrial free radical levels were observed. However, concomitantly a protective pathway(s) was stimulated that led to higher SOD activity and to decreased levels of superoxide radicals (O_2^-) in APPwt- and APPsw-transfected cells. However, all these events were not able to protect cells against higher ROS formation. On the basis of our results we suggest that the higher ROS formation in the APP-transfected cells compared to control PC12 cells may also be involved in the enhancement of $cPLA_2$ activity.

Decreased O_2^- levels could be caused by increased Cu,Zn-SOD and glutathione reductase activ-

ity in APPsw cells. However, the lower activity of glutathione peroxidase in APPwt and APPsw PC12 cells may be responsible for the increased mitochondrial and cytosolic H_2O_2 levels in these cells. The activity of glutathione peroxidase might be reduced by increased NO levels in APPsw PC12 cells (Dobashi *et al*., 2001; Miyamoto *et al*., 2003). Recently, it has been shown that H_2O_2 can activate PKC, MAPK and the enzymes involved in phospholipid metabolism, e.g. phospholipase D (Oh *et al*., 2000), phospholipase C (Shasby *et al*., 1988) and $cPLA_2$ (Samanta *et al*., 1998; Birbes *et al*., 2000) resulting in stimulation of AA release (Sapirstein *et al*., 1996; Xu *et al*., 2003). The data of Xu *et al*. (2003) demonstrated that the response of astrocytes to oxidant compounds such as H_2O_2 stimulated signaling pathways leading to the activation of $cPLA_2$ and $iPLA_2$ and to an enhancement of AA release. H_2O_2 upregulated the MAPK pathway and phosphorylation of ERK1/2 and PKC. All these kinases are also involved in phosphorylation and activation of $cPLA_2$ (Xu *et al*., 2002; Six & Dennis, 2000; Evans *et al*., 2002; Chalimoniuk *et al*., 2006).

An increased level of free AA can exert several neurotoxic effects. For example, AA itself can affect mitochondrial function inducing oxidative stress that may activate PKC, PKA, NADPH oxidase, GTPase-activating protein and may increase intracellular calcium ion concentration and apoptotic processes in neurons. The *sn*-2 position of phospholipids of mammalian cells is enriched in arachidonic acid. AA is a substrate for cyclooxygenases and lipoxygenases that synthesize several eicosanoids which play a significant roles in physiology and neurodegenerative diseases (Teismann *et al*., 2003; Sun *et al*., 2004). Thus, activation of $cPLA_2$ can increase the production of reactive oxygen species by supplying AA to cyclooxygenases and lipoxygenases (Kukreja *et al*., 1986). Reactive oxygen species lead to lipid, protein and DNA damage. Superoxide generated by a $cPLA_2$-mediated process can activate mitochondrial PLA_2, leading to the release of free fatty acid and concomitant formation of lysophospholipids (Madesh & Balasubramanin, 1997). The enhancement of AA release can subsequently lead to an increase of prostaglandin synthesis, oxidative stress and apoptotic processes in astrocytes (Xu *et al*., 2003). Thus, the increased arachidonic acid concentration through conversion into eicosanoids could also be involved in the observed significant increase of free radical levels in APPwt and APPsw PC12 cells.

Moreover, enhanced oxidative stress-induced AA release can be blocked by antioxidants (Chalimoniuk *et al*., 2006). Since cytosolic ROS were elevated in APPsw and APPwt PC12 cells, an important role of oxidative stress in the mechanisms of Aβ toxicity leading to mitochondrial dysfunction and increased AA release due to enhanced PLA_2 activity seems to be possible. In addition, the increased AA release leads to the generation of multiple eicosanoid products which can play a key role in the initiation and progression of inflammatory brain damage, thereby linking the two important features of AD neuropathology, namely oxidative stress and neuroinflammation (Stephenson *et al*., 1996; Rosenberger *et al*., 2004; Lukiw & Bazan, 2000; Bazan *et al*., 2002; Sun *et al*., 2004).

In conclusion, our data showed that Aβ peptide enhanced the $cPLA_2$ protein level and phosphorylation and AA release. Moreover, our results demonstrated that NO plays an important role in the Aβ-evoked AA alteration in PC12 cells.

Acknowledegment

This study was supported by grant PBZ-MIN 001/P05/16.

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