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Reversible Inactivation of Superoxide-Sensitive Aconitase in Aβ1–42-Treated Neuronal Cell Lines

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Abstract: The activity of the superoxide-sensitive enzyme aconitase was monitored to evaluate the generation of superoxide in neuronal cell lines treated with β-amyloid (Aβ) peptide 1–42. Treatment of differentiated and undifferentiated rat PC12 and human neuroblastoma SK-N-SH cells with soluble Aβ1–42 (Aβ-derived diffusible ligands) or fibrillar Aβ1–42 caused a 35% reversible inactivation of aconitase, which preceded loss of viability and was correlated with altered cellular function. Aconitase was reactivated upon incubation of cellular extracts with iron and sulfur, suggesting that Aβ causes the release of iron from 4Fe–4S clusters. Aβ neurotoxicity was partially blocked by the iron chelator deferoxamine. These data suggest that increased superoxide generation and the release of iron from 4Fe–4S clusters are early events in Aβ1–42 neurotoxicity. **Key Words:** β-Amyloid 1–42—Oxidative stress—Aconitase—Iron—Superoxide—PC12 cells—SK-N-SH cells.
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The β-amyloid (Aβ) peptide is implicated in the complex neurodegenerative cascades associated with Alzheimer's disease (AD) (Mattson, 1997; Price et al., 1998; Selkoe, 1999). Aβ-dependent neuronal death in cell cultures can be blocked by the antioxidant vitamin E and by scavengers of hydrogen peroxide (H_2O_2), suggesting that oxidative damage mediates Aβ neurotoxicity in vitro (Behl et al., 1994; Behl, 1997; Mattson, 1997; Keller et al., 1998). Superoxide ($O_2^{\bullet -}$) is also implicated in Aβ toxicity. The overexpression of mitochondrial Mn superoxide dismutase prevents neuronal apoptosis induced by Aβ25–35 in PC6 cells (Keller et al., 1998), whereas treatment of rat organotypic hippocampal cultures with EUK-8, a scavenger of both superoxide and hydrogen peroxide, protects against Aβ1–42 toxicity (Bruce et al., 1996). However, these studies did not investigate the effect of Aβ1–42 on the generation of specific reactive oxygen species during the early phases of exposure. Consequently, the time course of the generation of specific oxygen species in Aβ1–42-treated neuronal cells remains poorly understood.

The 4 iron–4 sulfur (4Fe–4S) cluster contained in certain enzymes, for example, mitochondrial aconitase (EC 4.2.1.3), is a major target of superoxide in prokaryotic and eukaryotic cells (Fridovich, 1995). Upon exposure to superoxide, the 4Fe–4S cluster in aconitase is partially disrupted by the reversible oxidation-dependent release of iron from this cluster (Flint et al., 1993). This selective and reversible disruption of 4Fe–4S clusters can be used to estimate the superoxide concentration in mammalian cells (Gardner et al., 1995; Patel et al., 1996). Aconitase is also inactivated by H_2O_2 and peroxynitrite ($ONOO^-$), formed by the reaction of $O_2^{\bullet -}$ with nitric oxide (NO), but not by NO alone (Castro et al., 1994; Hausladen and Fridovich, 1994). However, a 50% inactivation of aconitase in mammalian cells is estimated to require 50–200 p*M* endogenous superoxide, but it requires 100 μM extracellular hydrogen peroxide (Gardner et al., 1995). Thus, aconitase activity can be used to study the early effects of Aβ on the intracellular generation of superoxide, when the cells are viable and functional.

Monomers of the 42-amino acid Aβ (Aβ1–42) rapidly self-aggregate into fibrillar forms that resemble the heterogeneous extracellular amyloid deposits of senile plaques in AD brains. However, the brain regional load of aggregated Aβ does not show strong correlation with the clinical stage of dementia (Katzman et al., 1988; Hyman et al., 1993), whereas degenerating neurons are found in regions that are plaque-free (Einstein et al., 1994). Moreover, the concentration of soluble Aβ inversely correlates with synaptic loss (Lue et al., 1999). Studies of mouse AD models also demonstrate that the disruption of neural circuits and synaptotoxicity can occur independently of plaque formation (Hsia et al., 1999;

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Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's disease; ADDL, Aβ-derived diffusible ligand(s); DFO, deferoxamine; DTT, dithiothreitol; FAS, ferrous ammonium sulfate; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor.

Mucke et al., 2000). Recently, soluble Aβ1–42 oligomers [Aβ-derived diffusible ligands (ADDL)] were found to be potent neurotoxins (Oda et al., 1995; Lambert et al., 1998). ADDL are oligomers of Aβ that form when monomeric Aβ1–42 is incubated in the presence of substoichiometric amounts of the protein clusterin (ApoJ). Clusterin is found at high concentration in senile plaques (McGeer et al., 1992; Zhan et al., 1995) and is elevated in AD brains (May et al., 1990). The experimentally generated ADDL may be related to the water-soluble oligomers of Aβ that increase by 10-fold in AD brains (Kuo et al., 1996) and to the stable dimeric and trimeric components of Aβ peptides associated with neuritic and vascular amyloid deposits (Roher et al., 1996).

We now test further the hypothesis that intracellular superoxide generation is induced in cells treated with Aβ with experiments to elucidate the mechanisms that link Aβ to loss of cellular functions and cell death. For this purpose, we studied the effect of both fibrillar and soluble Aβ1–42 (ADDL) on aconitase activity, cytotoxicity, and viability in rat PC12 cells and human neuroblastoma SK-N-SH cells.

MATERIALS AND METHODS

Materials

Rat pheochromocytoma PC12 cells and human neuroblastoma SK-N-SH cells were obtained from ATCC (Rockville, MD, U.S.A.). Horse and fetal bovine serum (FBS), RPMI-1640, penicillin, streptomycin, *cis*-aconitate, L-malate, ferrous ammonium sulfate (FAS), sodium sulfide (Na_2S), dithiothreitol (DTT), Aβ25–35, protease inhibitor cocktail P8340, and deferoxamine mesylate (DFO) were from Sigma (St. Louis, MO, U.S.A.). Aβ1–42, Aβ1–40, and Aβ40–1 were from U.S. Peptide (Rancho Cucamonga, CA, U.S.A.). NuPage bis-tris-4-12% sodium dodecyl sulfate gradient gel was from Novex (San Diego, CA, U.S.A.). Bio-Rad dye reagent was from Bio-Rad (Hercules, CA, U.S.A.).

Cell culture and treatment

PC12 cells were grown at 37°C in RPMI 1640 medium containing 10% horse serum, 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 12-well collagen-coated plates before treatment. SK-N-SH cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown to confluence (undifferentiated) or were differentiated before treatment by the removal of serum and incubation with 50 ng/ml nerve growth factor (NGF) for 10 days. SK-N-SH cells, at the time of treatment with Aβ, were transferred to RPMI-1640 medium containing 0.5% FBS.

Clusterin, Aβ1–42, ADDL, and Aβ25–35

Clusterin (ApoJ) was isolated from human plasma by immunoaffinity chromatography (Oda et al., 1995). Aβ1–42, Aβ1–40, and Aβ40–1 were aggregated in 0.1 *M* sodium phosphate buffer (pH 7.8) at 340 μg/ml at 37°C for 2 h. ADDL were prepared by incubation of clusterin and Aβ1–42 at equal mass [340 μg/ml Aβ1–42 (4 kDa) and clusterin (80 kDa); molar ratio, clusterin: Aβ1–42, 1:20] in 0.1 *M* sodium phosphate buffer (pH 7.8) at 37°C for 2 h. Centrifugation (16,000 *g*, 10 min) yielded the supernatant ("ADDLs") (Lambert et al., 1998), which contains both clusterin and soluble Aβ1–42. ADDL were frozen in presiliconized tubes and thawed immediately before treatment. Toxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was similar in freshly used and frozen ADDL or Aβ1–42 (stored frozen for 30 days and thawed). The concentration of Aβ1–42 and clusterin in the supernatant was approximately half of the initial concentration (i.e., 4 μ*M* Aβ and 0.2 μ*M* clusterin), as determined by measuring the total protein after removing clusterin by G7 affinity chromatography. Clusterin removal was checked by immunoblot using mouse monoclonal clusterin antibodies (not shown). Aβ25–35 stock was prepared by incubation in water for 24 h. Aliquots were stored frozen in presiliconized tubes until treatment.

Treatment with Aβ, assay of neurotoxicity, and preparation of cell extracts

Confluent undifferentiated PC12 or SK-N-SH cells grown in 12-well plates were treated with either 20 μ*M* Aβ25–35, 20 μ*M* Aβ1–42, 8 μ*M* Aβ1–40, 8 μ*M* Aβ40–1, or 4 μ*M* ADDL for indicated times. Differentiated PC12 cells were treated with 4 μ*M* ADDL for indicated times. Cells incubated in 12-well plates containing 0.5 ml of medium/well were detached by scraping. Aliquots were removed and were transferred to 96-well plates for the MTT assay (100 μl) and to Eppendorf tubes (20 μl) for viability measurements by trypan blue dye exclusion. For the MTT assay (Hansen et al., 1989), 10 μl of a 5 mg/ml MTT stock was added to each well containing PC12 cells. After 1–2 h, cells were lysed with 150 μl of a lysis solution containing 15% sodium dodecyl sulfate, 50% *N,N*-dimethylformamide, and 5% acetic acid. Viability was measured after mixing cells with an equal volume of a 0.4% trypan blue solution. Two independent cell counts were obtained for each well.

Cell extracts for aconitase assays were prepared as follows: After scraping cells from 12-well plates, a third aliquot (380 μl) was centrifuged at 1,500 *g,* washed with ice-cold phosphate-buffered saline, and lysed (Gardner et al., 1994). The cell pellet was resuspended in 50 m*M* Tris-HCl (pH 7.4), 0.6 m*M* $MnCl_2$, and 20 μ*M* fluorocitrate in an Eppendorf tube and was disrupted by sonication. The lysate was either used immediately for aconitase assays or was snap-frozen in dry ice/ethanol and stored at −70°C.

Light microscopy analysis of Aβ-treated cells

Aβ1–42 toxicity in differentiated PC12 cells was measured by plating cells on collagen-coated coverslips (10 μg/ml; Upstate Biologicals) at a concentration of 15,000 cells/12-mm coverslip. PC12 cells were differentiated in F-12K medium containing 100 ng/ml NGF (Alomone Labs) for 6 days with medium being changed on the third day. On the sixth day, medium was removed and replaced with fresh medium containing NGF and either ApoJ control, ADDL (4 μ*M*), or fibrils (8 μ*M*). The cells were allowed to incubate up to 5 days, with images of the cells collected at several different time points along the way. At 5 days, the cells that remained were fixed and mounted for higher-magnification imaging.

Iron chelation

For iron chelation experiments, cells were pretreated with 20 μ*M* DFO 10 min before Aβ treatment.

Enzyme assays

Aconitase was assayed spectrophotometrically by two methods: (1) by following the formation of NADPH (340 nm) at 25°C in a mixture containing 50 m*M* Tris-HCl (pH 7.4), 5 m*M* sodium citrate, 0.6 m*M* $MnCl_2$, 0.2 m*M* $NADP^+$, 1 U of

isocitric dehydrogenase, and 20 μl of the cell extract, as previously described (Gardner et al., 1994); and (2) by following the decrease in absorbance at 240 nm in a mixture containing 0.1 *M* NaCl, 0.02 *M* Tris-HCl (pH 7.4), and 1 m*M* *cis*-aconitate. Optical density change was monitored for 1 h. Due to a lag in NADPH formation, aconitase activity was calculated using the slope recorded after 10 min from the beginning of the assay. Aconitase was reactivated either by incubating 20 μl of lysates with 0.05 *M* DTT, 0.2 m*M* Na_2S, and 0.2 m*M* FAS for 30 min at 37°C or by adding 1 m*M* Na_2S and 1 m*M* Fe^{3+} directly to the assay mixture. Fumarase was assayed by measuring the optical density change at 240 nm at 25°C in a mixture containing 30 m*M* potassium phosphate, 0.1 m*M* EDTA, and 5 m*M* L-malate (pH 7.4). Protein concentration was measured using the Bio-Rad dye reagent.

PC12/BV2 co-incubation and nitrite assay

PC12 cells were plated on 24-well plates and grown to confluence. On the day of the treatment, 1-μm-pore 24-well inserts (Costar) containing immortalized mouse microglial BV2 cells (200,000 cells/well) were inserted into the 24-well plates. BV2 and PC12 cells were incubated in Dulbecco's modified Eagle's medium/F-12 10% serum during the treatment. ADDL or fibrillar Aβ1–42 was added to both the insert medium (top) and the medium covering the PC12 cells (bottom). Nitrite concentration in the medium, an indirect measurement of nitric oxide, was quantified after 24 h of exposure to Aβ by the Griess reaction (Follett and Ratcliff, 1963). Fifty microliters of Griess reagent was mixed with 50 μl of supernatant of PC12 or BV2 cells. Absorbance was measured by spectrophotometry at 550 nm. Absorbance was compared with that of nitrite standards from a 200 μ*M* $NaNO_2$ stock.

RESULTS

Neurotoxicity of soluble ADDL at low micromolar concentration

Cells were treated with 4 μ*M* of the soluble ADDL for consistency with our previous studies (Oda et al., 1995) and with 20 μ*M* fibrillar Aβ1–42 and Aβ25–35, which is the lowest concentration consistently toxic to neuronal cells (Behl et al., 1994; Estus et al., 1997). To compare the toxicity of ADDL with that of another form of slowly sedimenting Aβ, cells were also treated with Aβ1–40 and with the inactive control Aβ40–1 (Jarrett et al., 1993; Snyder et al., 1994).

In rat PC12 cells, 4 μ*M* ADDL had approximately the same toxicity of 20 μ*M* fibrillar Aβ1–42 (Fig. 1). Exposure of PC12 cells to Aβ25–35, fibrillar Aβ1–42, or ADDL for 24 h did not alter cell viability (trypan blue exclusion) but caused a 30–40% decrease in MTT reduction (MTT assay, $p < 0.05$) (Fig. 1A and B). The reduction of MTT was originally proposed to occur mostly in the mitochondria at the level of ubiquinone and cytochrome oxidase (Slater et al., 1963). Recent studies suggest that Aβ can inhibit MTT reduction in two ways: (1) an oxidation-dependent cytotoxicity (Abe and Saito, 1999) or (2) an oxidation-independent MTT formazan exocytosis (Liu and Schubert, 1997). The relative contributions of oxidative cytotoxicity and exocytosis are determined by the concentration of Aβ and by the time of incubation in the presence of MTT. Based on the study by Abe and Saito (1999), the MTT assay is a reliable measure of oxidative cytotoxicity of micromolar Aβ, but incubations of ≥2 h cause the exocytosis of MTT formazan, which can inhibit further MTT reduction independently of oxidative cytotoxicity. This work was performed on rat hippocampal neurons, whereas Liu and Schubert (1997) performed most of their studies with B12 cells. In our experiments, PC12 cells were incubated with MTT for both 30 (Fig. 1C) and 120 (Figs. 1B and 3B) min with similar results. Thus, considering the concentration of Aβ used in our experiments (4–20 μ*M*), MTT reduction was most likely affected by both oxidative cytotoxicity and formazan exocytosis.

Cell viability at 48 h (n = 9, three experiments, trypan blue exclusion) decreased by 20% for PC12 cells treated with 20 μ*M* fibrillar Aβ1–42 ($p < 0.05$) and 30% for cells treated with 4 μ*M* ADDL ($p < 0.05$) (Fig. 1A). Aggregated Aβ1–40, but not Aβ40–1, caused a 20% decrease in MTT reduction at 48 h ($p < 0.05$). Neither Aβ1–40 nor Aβ40–1 decreased viability at either 24 or 48 h (Fig. 1A). The concentration of ADDL in the medium of undifferentiated PC12 cells (0.4–40 μ*M*) correlated with a decrease in MTT reduction at 48 h ($r^2 = -0.91$) (Fig. 1C). The smaller effect of ADDL compared with the experiment shown in Fig. 1B was probably caused by the reduced incubation time in the presence of MTT (from 120 min in Fig. 1B to 30 min in Fig. 1C).

ADDL were also toxic to human neuroblastoma SK-N-SH cells. These cells were sensitive to a 24-h treatment with 4 μ*M* ADDL but not with 20 μ*M* aggregated Aβ1–42 (Fig. 1D). Viability was decreased by 25% after a 24-h treatment with ADDL ($p < 0.05$) and by 30% after treatment with Aβ25–35 ($p < 0.05$).

Toxicity of ADDL, Aβ fibrils, and ApoJ control to differentiated PC12 cells was assessed using colony counting after 5-day exposure. Coverslips (n = 8) were examined at ×4, and the colonies were counted and sized (small colonies = <20 cells/colony, large colonies = >20 cells/colony). This examination showed that after a 5-day treatment, both forms of Aβ were toxic. ADDL showed slightly more toxicity and had fewer and smaller colonies than cells treated with Aβ1–42 fibrils. The largest difference between the conditions was the size and number of the colonies that remained on the coverslip (Fig. 1E and F). Control-treated cells had an average of 10 colonies/coverslip, with an average colony size of >50 cells and no colonies with <20 cells (Fig. 1E). ADDL-treated cells had an average of 4.5 colonies with similar numbers of large and small colonies. Cells treated with Aβ1–42 fibrils had an average of 6 colonies/coverslip and similar numbers of large and small colonies (Fig. 1E). Morphologically, the subpopulation of cells that remained attached to the coverslips in the treated cultures looked relatively similar to the untreated cells (Fig. 1F). In agreement with the decrease in colony number and viability (Fig. 1E), MTT reduction in differentiated PC12 cells treated with ADDL and fibrillar Aβ1–42 for 24 h was reduced by 40% (data not shown).

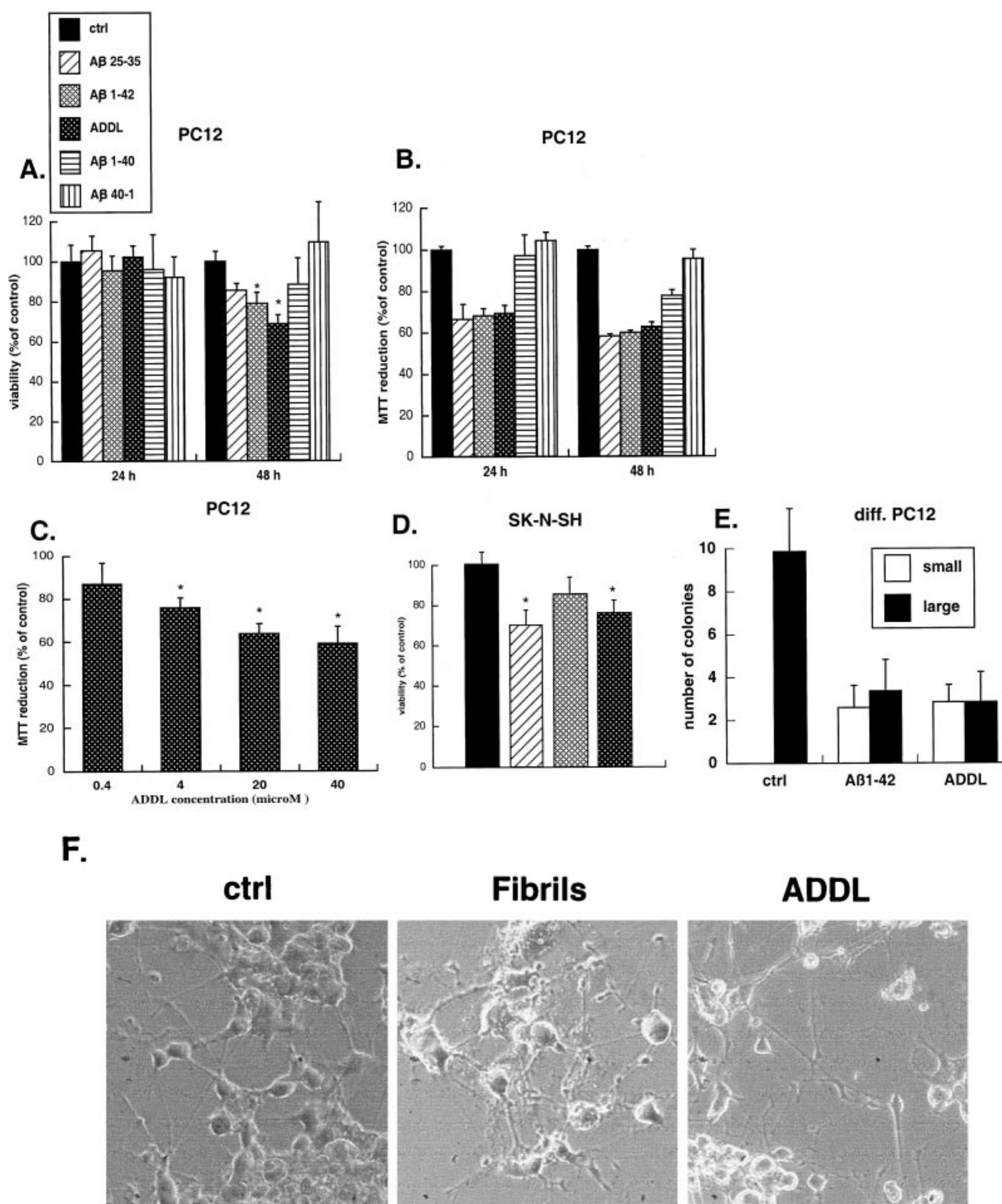

FIG. 1. Cytotoxicity of soluble and fibrillar Aβ. Confluent rat pheochromocytoma-derived PC12 and human neuroblastoma SK-N-SH cells were treated with 4 μM ADDL (clusterin + Aβ1–42), 20 μM Aβ1–42, 20 μM Aβ25–35, 8 μM Aβ1–40, and 8 μM Aβ40–1 for 24 or 48 (only PC12) h. Viability, reported as percentage of buffer-treated control values, was measured by counting the cells using the trypan blue exclusion method. MTT reduction, used to measure cytotoxicity, is reported as percentage of buffer-treated control values. Shown are means ± SEM. The *p* values were calculated by the Scheffé method for multiple-comparison procedure for one-way ANOVA. **A:** Viability of PC12 cells treated for the indicated time. *$p < 0.05$ vs. control. n = 9. **B:** MTT reduction for PC12 cells treated for the indicated time. *$p < 0.05$ vs. control. n = 6. **C:** MTT reduction for PC12 cells treated with the indicated concentration (μM) of ADDL for 48 h. *$p < 0.05$ vs. control. n = 3–6. **D:** Viability of SK-N-SH cells treated for 24 h. *$p < 0.05$ vs. control. n = 10. **E:** Number and size of differentiated PC12 colonies remaining on coverslips after 5-day exposure to the indicated form of Aβ. Coverslips (n = 8) were examined at ×4, and the colonies were counted and sized (small colonies = <20 cells/colony, large colonies = >20 cells/colony). **F:** Representative light microscopy images of differentiated PC12 cells treated with fibrillar Aβ1–42 or ADDL for 5 days. All images are of cells attached to the coverslips. Cells that floated off during the exposure could not be imaged, which is the most likely explanation for the differences in colony size and number seen in the three conditions.

Aconitase inactivation in Aβ-treated neuronal cells

We measured the activity of the $O_2^{\bullet-}$-sensitive enzyme aconitase in extracts of cells treated with different forms of Aβ. A 24- to 48-h exposure of undifferentiated PC12 cells to 4 μM ADDL caused a 35–45% inactivation of aconitase ($p < 0.05$) versus buffer-treated controls (Fig. 2A). Aβ1–42 (20 μM) caused a 20–25% inactivation at 24 and 48 h ($p < 0.05$). By contrast,

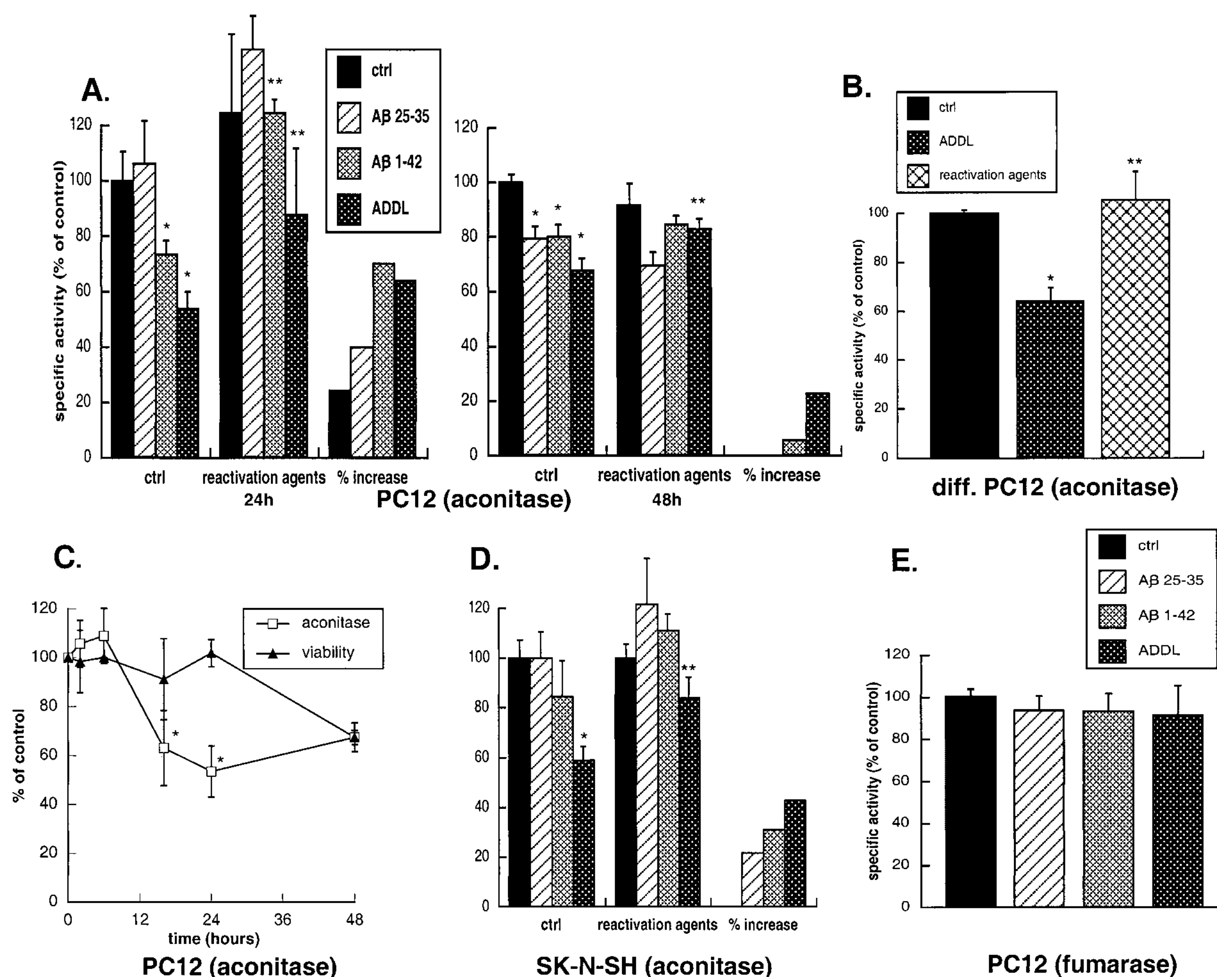

FIG. 2. Effect of Aβ on aconitase activity. Aconitase activity and reactivation in extracts of confluent PC12 and SK-N-SH cells exposed to 4 μM ADDL, 20 μM fibrillar Aβ1–42, and 20 μM Aβ25–35 are shown. The "% increase" represents the increase in aconitase specific activity after treatment of the indicated extract with reactivation agents. **A:** PC12 cells were treated with the indicated form of Aβ for 24 or 48 h. Activity was measured before and after incubation of whole-cell extracts with 4Fe–4S cluster reactivation agents (see Materials and Methods). Specific activity is reported as percentage of buffer-treated control value. *$p < 0.05$ vs. buffer-treated control; **$p < 0.05$ vs. Aβ-treated control. n = 3–6. **B:** Differentiated PC12 cells were treated with ADDL for 48 h. Activity was measured before and after incubation of whole-cell extracts with 4Fe–4S cluster reactivation agents (see Materials and Methods). *$p < 0.05$ vs. buffer-treated control; **$p < 0.05$ vs. Aβ-treated control. n = 6. Specific activity is reported as percentage of buffer-treated control value. The activity of aconitase in buffer-treated control extracts did not increase after incubation with reactivation agents. The viability of ADDL-treated cells was not different from that of buffer-treated controls at 48 h but decreased by 20% at 96 h (not shown). **C:** Viability and aconitase specific activity for PC12 cells treated with ADDL for 2, 6, 16, 24, and 48 h, reported as percentage of buffer-treated control value at the same time. *$p < 0.05$ vs. buffer-treated control. n = 3–9. **D:** SK-N-SH cells were treated with the indicated form of Aβ for 24 h. Aconitase activity was measured before and after incubation of whole-cell extracts with reactivation agents. Specific activity is reported as percentage of buffer-treated control value. *$p < 0.05$ vs. buffer-treated control; **$p < 0.05$ vs. Aβ-treated control. n = 5. **E:** Fumarase activity in PC12 cells treated with the indicated form of Aβ for 48 h. Fumarase activity was measured using whole-cell extracts (see Materials and Methods). Specific activity is reported as percentage of buffer-treated control value. n = 5.

aggregated Aβ25–35 decreased aconitase activity by only 20% at 48 h (Fig. 2A) ($p < 0.05$).

To test whether aconitase inactivation was due to superoxide-mediated iron release from the 4Fe–4S cluster in the active site, we incubated PC12 cellular extracts with "reactivation" agents that reconstitute the cluster (Na_2S, FAS, DTT) (Patel et al., 1996). The reactivation agents increased activity by 60% in extracts of PC12 treated with either 4 μM ADDL or 20 μM Aβ1–42 ($p < 0.05$) for 24 h (Fig. 2A) ($p < 0.05$) but did not significantly increase aconitase activity in controls or Aβ25–35-treated cells. At 48 h, aconitase could be reactivated by 20% in whole-cell extracts of ADDL-treated PC12 cells ($p < 0.05$) but not in extracts of PC12 cells exposed to fibrillar Aβ1–42 or 25–35. Aconitase activity increased in the presence of reactivation agents in extracts of both PC12 and SK-N-SH cells treated with Aβ25–35. Although this increase is not significant, it may reflect a slight induction of the expression of the aconitase gene by Aβ25–35, considering that aconitase

activity in the absence of reactivation agents is comparable to that in untreated controls.

We also extended these experiments to NGF-differentiated PC12 cells. A 48-h treatment of differentiated PC12 cells with ADDL caused a 35% inactivation of aconitase (Fig. 2B). Aconitase activity in these extracts was completely restored by incubation with reactivation agents (Fig. 2B). At 48 h, the viability of ADDL-treated differentiated PC12 cells was comparable with controls, but it was 80% of controls at 96 h. Thus, aconitase was inactivated by 35% in both differentiated and undifferentiated PC12 cells treated with ADDL at a time preceding loss of viability.

ADDL-dependent loss of aconitase activity was detected by 16 h (Fig. 2C), suggesting that the increase of superoxide concentration and iron release from the 4Fe–4S cluster precedes loss of viability by 8–32 h. These results were also performed using the human neuroblastoma SK-N-SH cell line (Fig. 2D). A 24-h treatment with 4 μM ADDL caused a 45% inactivation of aconitase ($p < 0.05$). Incubation of SK-N-SH cells with 20 μM Aβ1–42 did not decrease aconitase activity ($p > 0.05$). The release of iron from aconitase in ADDL-treated SK-N-SH cells was confirmed by the ability of reactivating agents to increase activity by 40% ($p < 0.05$). Reactivation agents also increased aconitase activity in extracts of aggregated Aβ1–42-treated cells, but the reactivation was not significant.

Mammalian cells express a cytosolic aconitase (iron regulatory protein-1; IRP-1) in addition to mitochondrial aconitase. For this study, it was not feasible to measure the subcellular activity of aconitase due to the large number of cells necessary to measure aconitase activity in isolated mitochondria (60–100 million cells) (Gardner et al., 1995) and considering the amounts of Aβ1–42 and of purified clusterin needed to treat 100 million cells.

The specificity of ADDL toxicity on aconitase was demonstrated by measuring the activity of the mitochondrial enzyme fumarase, which does not contain a 4Fe–4S cluster. The activity of fumarase in ADDL-treated cells did not decrease compared with controls (Fig. 2E).

Role of iron in Aβ1–42 toxicity

We tested the effect of Aβ in the presence of the iron chelator DFO. The cells were tested at confluence to minimize cell growth and to separate the role of DFO as a cell cycle blocker (Farinelli and Greene, 1996) from its role in preventing the reactivity of redox-active iron. DFO had no effect on cell growth (PC12 cultures, three experiments, nine independent samples, data not shown) but prevented the decrease in viability and MTT reduction in PC12 cells exposed to ADDL ($p < 0.05$) and fibrillar Aβ1–42 compared with controls (Fig. 3A and B). As expected, incubation of PC12 cells with DFO alone decreased aconitase activity by 40% (data not shown). In fact, DFO limits the availability of the iron needed to assemble iron–sulfur clusters. Treatment with ADDL or Aβ1–42 for 48 h in the presence of 20 μM DFO did not further decrease aconitase activity versus DFO alone (data not shown). Similar results were obtained using SK-N-SH cells. Incubation of SK-N-SH cells with DFO blocked the effect of 4 μM ADDL on viability (Fig. 3C).

Nitric oxide generation in cells treated with Aβ1–42

Peroxynitrite, formed by the reaction of superoxide with nitric oxide, can rapidly inactivate aconitase (Castro et al., 1994; Hausladen and Fridovich, 1994). To determine whether nitric oxide may be involved in the inactivation of aconitase in Aβ1–42-treated PC12 cells, we measured the concentration of nitrite (a metabolite of nitric oxide). Nitrite concentration in the medium of PC12 cells treated with Aβ1–42 for 24 h was in the low micromolar range and did not increase compared with untreated controls (Fig. 4). As a positive control for nitrite generation, PC12 cells were co-incubated with BV2 mouse microglial cells separated by filters. Co-incubation of PC12 cells with BV2 microglial cells increased the concentration of nitrite by >10-fold. Nitrite generation was further stimulated by treatment with fibrillar Aβ1–42 (Fig. 4).

DISCUSSION

The complex mechanisms of Aβ toxicity are poorly understood even in neuronal cell lines. We provide evidence for the role of superoxide, aconitase inactivation, and iron in the toxicity of Aβ1–42. The superoxide-sensitive enzyme aconitase was reversibly inactivated in viable neuronal cells exposed to soluble (ADDL) and to fibrillar Aβ1–42. Cell dysfunction and the consequent loss of viability in Aβ-treated cells were partially blocked by the chelation of redox-active iron.

The increased generation of reactive oxygen species in neuronal cells exposed to various forms of Aβ is well documented (Behl, 1997; Mattson, 1997). However, evidence for the increase in superoxide production and toxicity in Aβ1–42-treated cells is less conclusive, as it is based on measurements using fluorescent dyes that are not selective for superoxide (Mark et al., 1995; Mattson, 1997). Our results with two neuronal cell types show that both soluble (ADDL) and fibrillar Aβ1–42 cause the selective and reversible inactivation of aconitase, validated as an indicator for intracellular generation of superoxide in primary neurons (Patel et al., 1996) and in other cell types (Gardner and Fridovich, 1991; Gardner et al., 1994; Longo et al., 1999). Gardner et al. (1995) estimated that in mammalian epithelial cells, the concentration of superoxide ranges between 50 and 200 pM under stress conditions, when aconitase is 50% inactive, and between 8 and 30 pM under normal conditions, when aconitase is 14% inactive. Thus, considering that in control PC12 and SK-N-SH cells, aconitase is not significantly reactivated (Fig. 2), our results suggest that exposure to soluble Aβ1–42 increases the concentration of superoxide by at least 10-fold between 8 and 32 h preceding loss of viability. The oxidation of the 4Fe–4S cluster of aconitase by superoxide is likely to cause the release of iron from the cluster (Flint et al., 1993; Fri-

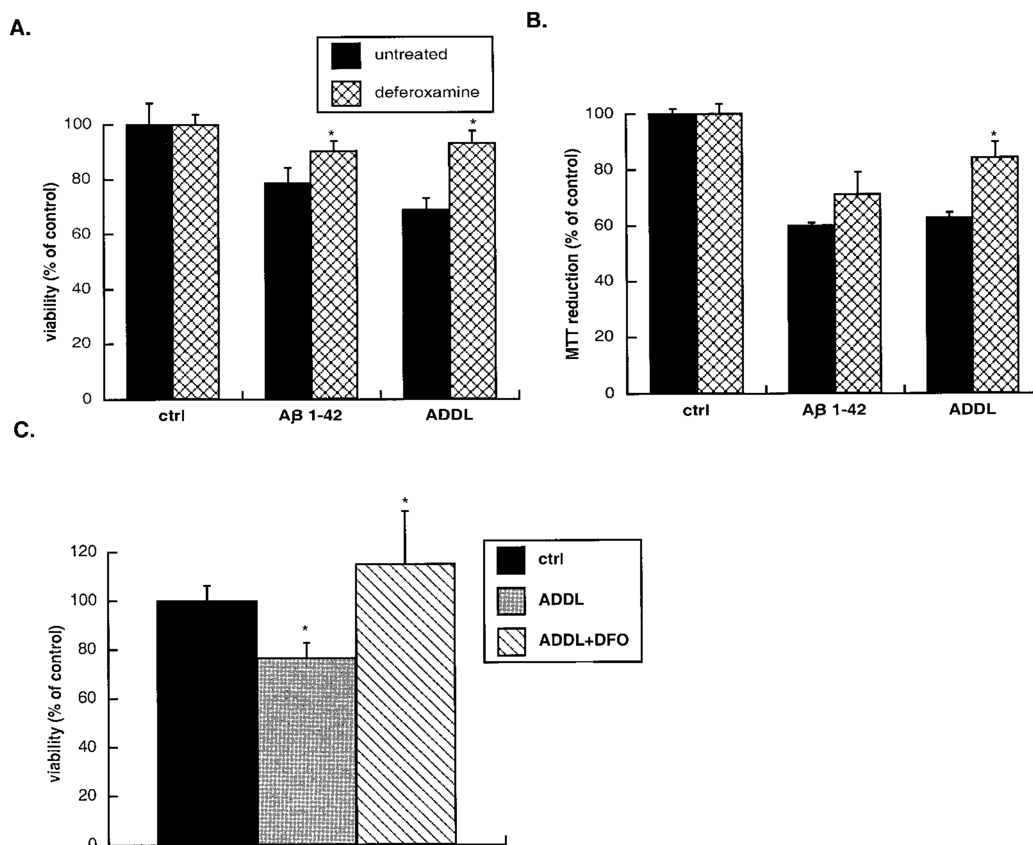

FIG. 3. Effect of DFO on the toxicity of Aβ1–42. Confluent cells were exposed, in the presence or absence of 20 *μM* DFO, to 4 *μM* ADDL or 20 *μM* fibrillar Aβ1–42 for 48 h. Viability was measured by the trypan blue dye exclusion method. DFO did not reduce the number of viable cells after a 48-h treatment (data not shown). MTT reduction for DFO + Aβ-treated cells is reported as percentage of DFO-treated control values. Shown are means ± SEM. The *p* values were calculated by one-way ANOVA. **A:** Viability of PC12 cells treated with the indicated form of Aβ for 48 h ± 20 *μM* DFO reported as percentage of buffer-treated or DFO-treated control values. *$p < 0.05$ vs. ADDL- or Aβ1–42-treated controls. n = 9. **B:** MTT assay for PC12 cells treated for 48 h with the indicated form of Aβ ± 20 *μM* DFO, reported as percentage of buffer-treated or DFO-treated control values. *$p < 0.05$ vs. ADDL- or Aβ1–42-treated controls. n = 6. **C:** Viability (trypan blue) in whole-cell extracts of SK-N-SH cells treated for 24 h with 4 *μM* ADDL ± 20 *μM* DFO reported as percentage of buffer-treated or DFO-treated control values. *$p < 0.05$ vs. ADDL-treated controls. n = 3.

dovich, 1995), which can in turn generate the highly reactive hydroxyl radical by the Fenton reaction. These results are consistent with recent evidence that the hydroxyl radical scavenger indole-3-proprionic acid protected PC12 and SK-N-SH cells from Aβ1–42 toxicity (Chyan et al., 1999).

Treatment of PC12 cells with either soluble or fibrillar Aβ1–42 did not increase nitrite generation compared with untreated controls, suggesting that nitric oxide does not play a major role in the inactivation of aconitase. However, we cannot exclude that the inactivation of aconitase in neuronal cells exposed to Aβ1–42 may be caused by peroxynitrite rather than by superoxide. The available nitric oxide (Fig. 4) may be sufficient to generate the low micromolar concentration of peroxynitrite required for aconitase inactivation (Hausladen and Fridovich, 1994). It is improbable that hydrogen peroxide is responsible for the early effect of Aβ1–42 on aconitase, as this would require a micromolar concentration of this oxygen species (Gardner et al., 1995). Such concentration is at least a thousandfold higher than that required for superoxide-mediated inactivation of aconitase and would be expected to cause cell death by 24 h (Fig. 1). Nonetheless, hydrogen peroxide plays a major role in the iron-catalyzed generation of hydroxyl radical and may therefore be required in the terminal steps of Aβ1–42-dependent neurotoxicity (Behl et al., 1994). As aconitase activity in most mammalian cell lines tested is markedly higher in the mitochondria than in the cytosolic fraction (Gardner et al., 1995), it is likely that mitochondrial aconitase is inactivated in Aβ1–42-treated cells. However, further studies are necessary to determine whether Aβ1–42 may affect the activity of cytosolic aconitase (IRP-1), which is encoded by a different gene.

Iron concentration is increased in several brain regions affected by AD above that seen in elderly control samples (Ehmann et al., 1986; Connor et al., 1992; Loeffler et al., 1995). Iron associated with senile plaques and neurofibrillary tangles was shown to catalyze the oxidation of H_2O_2 (Smith et al., 1997). In *Escherichia coli,* the overexpression

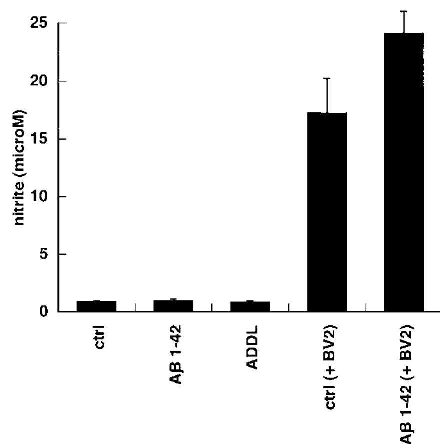

FIG. 4. Indirect measurement of nitric oxide concentration by the assay of nitrite in the medium of PC12 cells treated with Aβ1–42. Undifferentiated PC12 cells were treated with fibrillar Aβ1–42 or ADDL. The concentration of nitrite, a metabolite of nitric oxide, in the medium of PC12 cells was measured after a 24-h treatment. As a positive control for nitrite generation, PC12 cells were co-incubated with BV2 mouse microglial cells separated by filters.

of enzymes containing 4Fe–4S clusters dramatically increases the content of "free iron" in the cells, which in turn causes DNA damage (Keyer and Imlay, 1996). The 4Fe–4S-containing enzymes are the major target of superoxide in bacteria (Fridovich, 1995), yeast (Longo et al., 1999), and mammalian cells (Gardner et al., 1995; Li et al., 1995). Taken together with the evidence for increased superoxide generation in neuronal cells exposed to Aβ, these studies suggest that iron is released from intracellular 4Fe–4S clusters in AD brains and that it participates in the catalysis of oxidative reactions leading to neurodegeneration. However, the relative contribution of redox-active iron by 4Fe–4S clusters and by other possible sources of iron such as ferritin or heme oxygenase-1 is not known. The iron chelator DFO prevents loss of viability in PC12 and SK-N-SH cells and partially blocks the Aβ-dependent decrease in MTT reduction in PC12 cells (Fig. 3). These results may be explained by the ability of DFO to limit the availability of redox-active iron released from aconitase and consequently to prevent the Fe^{2+}-dependent generation of the highly reactive hydroxyl radical (•OH). Alternatively, DFO may protect cells from Aβ1–42 toxicity by other mechanisms such as, for example, blocking the generation of hydroxyl radical by the extracellular iron associated with Aβ peptides (Smith et al., 1997). Further studies using primary neurons and CNS slice cultures are required to determine the relevance of these findings to AD.

The neurotoxicity of Aβ1–42 and NMDA may share common mechanisms. Treatment of rat cortical cultures with NMDA or kainate causes selective and reversible superoxide-dependent inactivation of aconitase, which correlates with cell death (Patel et al., 1996). These agents activate glutamate receptors and induce excitotoxic death through mechanisms that require influx of extracellular Ca^{2+} (Choi, 1985). Fibrillar Aβ also causes an elevation of intracellular Ca^{2+} in primary neurons (Mark et al., 1995) and PC12 cells (Zhou et al., 1996). The increase of intracellular Ca^{2+} induced by Aβ1–40 in substantia nigra/neuroblastoma hybrid cells and the consequent neurotoxicity were reduced by treatment with the NMDA receptor antagonist MK-801 and increased by treatment with NMDA (Le et al., 1995). Therefore, the activation of the NMDA receptor, Ca^{2+} influx, and the inactivation of aconitase prior to cell death are events shared by cells treated with Aβ and NMDA.

In conclusion, these data suggest that both soluble (ADDL) and fibrillar Aβ1–42 increase the generation of superoxide, which in turn inactivates aconitase and releases iron from the 4Fe–4S cluster preceding loss of viability (Oda et al., 1995; Lambert et al., 1998). Our results also suggest that the toxicity of Aβ1–42 is mediated in part by redox-active iron. Further studies in neuronal cell lines and primary neurons are necessary to identify the sources of superoxide in Aβ-treated cells and to determine how the presence of microglia may affect aconitase inactivation and iron toxicity.

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