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Amyloid $A\beta_{1-40}$ preconditions non-apoptotic signals *in vivo* and protects fetal rat brain from intrauterine ischemic stress

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Abstract

The dualistic activities of the amyloid beta (Aβ) peptide as a pro-oxidant and ubiquitous constituent of amyloid deposits in Alzheimer's disease plaques and as an antioxidant of purported physiological function has been suggested but the mechanisms are far from being understood. In this report we measure several oxidative stress parameters and signaling cascades in brains of fetal rats subjected to global ischemia in order to evaluate the putative bifunctional properties of the $A\beta_{1-40}$ peptide. Intraperitoneal injection of 6 μg $A\beta_{1-40}$ into 18-days-old rat fetuses (approximately 3 g body weight) resulted after 24 h in the appearance of the peptide in various fetal organs including brain where it enhanced the levels of glutathione (GSH), glutathione reductase, glutathione peroxidase, and stimulated the levels of pro-survival signaling activities such as Akt serine/threonine kinase, extracellular signal-regulated kinase (ERK) and protein kinase C enzymes. Moreover, pretreatment with $A\beta_{1-40}$ reversed the consequences of a transient hypovolemic/hypotensive oxidative stress by restoring GSH levels via its recycling enzymes and by lowering the production of lipid peroxides presumably by activating the aforementioned pro-survival signaling cascades. It also caused a reduction in the number of DAPI-enhanced reactive cells and a decrease in p38 kinase phosphorylation and caspase-9 and -3 activity. These data suggest that pre-exposure to $A\beta_{1-40}$ stimulates fetal tolerance to ischemia via regulation of GSH metabolism and as such may be considered as neuroprotective.

Keywords: Alzheimer's disease, brain development, ischemia, neuronal cell death, oxidative stress, signal transduction.
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Although a major constituent of the senile plaques in Alzheimer's disease (AD), the mechanism of action of the amyloid-β (Aβ) peptide prior to its entrance into a pathogenic spin of harmful consequences still remains a puzzle (Turner *et al*. 2003). Spontaneous or transition metal ion-promoted peptide aggregation has been suggested as an initial event whereby Aβ, a peptide cleavage product normally secreted and processed by cells, turns into a toxic oligomeric and/or fibrillary form. The aggregation process is still unclear, however, some evidence exists that an essential part of Aβ toxicity is due to its capacity to generate free radicals (Hensley *et al*. 1994; Huang *et al*. 1999; Kontush 2001).

Addition of synthetic Aβ peptides causes toxicity and apoptotic death in cultured cells (Behl *et al*. 1994), a phenomenon exacerbated by the presence of transition metal ions (Bush 2003). Chronic infusion of Aβ in rodents has been shown to result in learning and memory deficits, impairment in spatial and non-spatial memory formation and neurodegeneration in brain areas related to cognitive functions (Turner *et al*. 2003). Other studies have shown beneficial effects following Aβ peptide administration. *In vivo* injection of the $A\beta_{1-42}$ peptide for example was reported to suppress iron-induced toxicity (Bishop and Robinson 2003) and to cause an increase in production of amyloid precursor protein and cytotoxic Aβ fragments in animal models following mild ischemia (Baiden-Amissah *et al*. 1998). The latter phenomenon has been considered as an adaptive, possibly neuroprotective mechanism of the precursor protein (Mattson 1997; Campbell 2001; Kontush 2001). Additional indications attributing a protective role to $A\beta_{1-40}$ peptide, notable through enhancement of anti-apoptotic signaling molecules, was found in cerebral cortex

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Abbreviations used: Aβ, amyloid beta; GPx, glutathione peroxidase; GR, glutathione reductase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PPB, potassium phosphate buffer.

cultures, in contrast to facilitation of cell death induced by divalent iron (Kuperstein and Yavin 2003).

There has been virtually no information about the signaling transduction cascades induced by Aβ following its administration in brains of fetuses during the late gestation periods. During this critical period, the fetal brain undergoes marked structural, cellular, molecular and functional changes that are accompanied by massive programmed cell death (Oppenheim 1991). Based on our recent work using dissociated neuronal cell cultures that differentiate *in vitro* (Kuperstein and Yavin 2003) we hypothesized that introduction of the Aβ peptide may affect neurogenesis *in vivo*. Furthermore, because ischemic and traumatic episodes elicit oxidative stress and enhance cell death (Ratan *et al*. 1994) it was of interest to subject fetuses to a mild *in utero* stress in the presence of the Aβ peptide. The stress elicited by a transient global ischemic episode consisted of a short removal of a portion of the maternal blood after an exogenous Aβ supplement. To minimize local damage resulting from intraventricular or intracerebral peptide injection, the Aβ was administered via the fetal intraperitoneal cavity. The data shown below suggests that preadministration of Aβ exerted a marked effect on neurogenesis by enhancing anti-apoptotic signaling cascades and by elevating the antioxidant capability of the developing brain. Furthermore, Aβ prevented pro-apoptotic markers following ischemic stress from being expressed. In that regard it is suggested that the Aβ peptide serves as a conditioning stimulus to activate signaling cascades in the fetal brain, thus rendering the stressed brain resistant to more severe damage.

Materials and methods

Surgery procedure and ischemic stress

Time-pregnant Wistar rats (250 g body weight) at 18 days gestation, were purchased from Harlan Laboratories at the Weizmann Institute (Rehovot, Israel). Dams anesthetized by 2% halothane (Rhodia Organique Fine Ltd, UK) in oxygen, were subjected to an abdominal midline incision and uterine horns exposed. About 6 μg of freshly dissolved $A\beta_{1-40}$ peptide (Bachem, Babendorf, Switzerland) in 3 μL double-distilled water was injected through the uterine wall, via the amniotic sac into the peritoneal cavity of 4–6 individual fetuses (approximately 3 *g* body weight) per dam. Control fetuses were injected with water only. The uteri were returned to the abdominal cavity, sutured and dams returned to cages. After 24 h designated dams were re-anesthetized and subjected to a hypovolemic/hypotensive (hVhT) stress following basically a procedure detailed elsewhere (Apak *et al*. 2001). Briefly, the femoral artery was dissected by a vertical incision and inclusion of the artery was made to insert a 0.6 nm diameter plastic catheter containing heparin in phosphate-buffered saline (PBS). Heparin, (1250 units in 0.25 mL) was injected into the artery to prevent coagulation and after 1 min, 2.5–4 mL of blood was slowly drawn into the syringe. A blood pressure device connected to the catheter by a stopcock was used to verify blood pressure that was kept at about 50 mmHg for 12 min. At the end of the global stress, blood was reinfused, the catheter removed and the femoral artery irreversibly ligated. Rats were given a single intramuscular injection of finadyne (3.0 mg/kg body weight) and after halothane removal, they promptly regained normal cage activity and were housed singly in cages. Sham animals were subjected to the entire procedure except for the hVhT stress. At times designated (reperfusion) dams were killed and fetuses delivered by caesarean section and killed by decapitation. Brain tissue was dissected out and processed depending on the protocol. The procedure has been approved by the ethical committee for animal experiments at the Weizmann Institute (Rehovot, IL, USA).

Aβ immunohistochemistry

Freshly dissected brains were fixed in 4% formaldehyde in PBS for 2–3 days and transferred to 70% ethanol for 24 h. Tissue was processed routinely, embedded in paraffin and sections of 7-μm thickness were performed on a Leika microtome. Deparaffinization and rehydration of sections was done according to established procedures. Briefly, slides were placed for 10 min in xylene for three consecutive times, followed by serial 5-min washes in ethanol (100, 95 and 70%) and finally rinsed three times in PBS. Sections were placed in a Coplin jar with diluted antigen retrieval solution (10 mM citrate acid, pH 6) and were heated in a microwave to 95–100°C for 10 min. Blocking of the endogenous peroxidase activity was done by incubating slides in 0.3% H_2O_2 and 1% HCl in 50% methanol in PBS for 20 min at room temperature. After rinsing three times with PBS, slides were blocked with 20% normal horse serum in PBS for 1 h at 37° C and subsequently stained at room temperature with a monoclonal antibody for human Aβ (anti-Aβ (6E10) antibody (Senetek, USA) at 1 : 100 dilution using 2% normal horse serum. After three washes with PBS, biotinylated (AP)-conjugated secondary antibody (Vectastain ABC kit, Vector lab, Burlingame, CA, USA) was added for 1 h at room temperature. Slides were washed with PBS (three times) followed by two rinses with phosphate buffer (pH 7.4). Labeling was detected using the avidin-biotinylated HRP complex (ABC) reagent (Vectastain ABC kit) coupled to diaminobenzidine (DAB) reaction. The counter stain was performed with hematoxylin. Slides were visualized under light microscope and frames taken with a high-resolution camera (E-800, Nikon, Kawasaki Japan) connected to a desk computer.

Nuclear staining

Tissue sections were deparaffinized, rehydrated and washed with PBS as described above. For cell nuclei visualization sections were stained for 5 min with 0.5 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO, USA) dye at room temperature followed by three times rinsing with PBS. Slides were visualized under Y-FL microscope (Nikon) equipped with appropriate filter settings for fluorescence. Photography was assisted with a DXM 1200F digital camera (Nikon) and frames were collected using Nikon ACT-1 software program. Counting of the stained cell nuclei was performed with a NIH Image 1.63 software program.

Lipid peroxide measurements

Thiobarbituric acid reactive substances (TBARS) released into the medium after a 15-min incubation of fresh fetal brain slices was determined according to a published procedure (Glozman *et al*. 1998).

Glutathione determination by HPLC

Frozen preweighed brain samples were homogenized with a Teflon pestle and the dry powder transferred into an ice-cold homogenization buffer (1 : 4 w/v) containing 50 mM H_3PO_4 (pH 1.8) and 0.1 mM EDTA following basically the procedure by Rose and Bode (1995). After vigorous mixing, the extract was centrifuged at 10 000 *g* for 20 min at 4° C. The supernatant was passed through a 20 μm sterile filter (Schleicher & Schuell, Dassel, Germany) and aliquots of 20 μL injected into a PU 1580 HPLC (Zasco, Japan) to determine the levels of reduced glutathione (GSH). The standard curve for GSH was prepared in order to determine the most suitable mobile phase and detector setting conditions.

Glutathione reductase and glutathione peroxidase activity assays

Fresh cerebral hemispheres cleared off the meninges were washed with ice-cold PBS, weighted and homogenized in ice-cold homogenization buffer (1 : 3 w/v) composed of 20 mM potassium phosphate buffer (PPB, pH 7.0) using a Polytron homogenizer. After centrifugation for 10 min at 10 000 *g* in 4° C, supernatant was used for immediate determination of glutathione reductase (GR) and glutathione peroxidase (GPx) activities using a 940-Uvicon spectrophotometer (Kontron Instruments, Switzerland). For GR activity the reaction mixture containing PPB (100 mM), 1 mM EDTA and 0.2 mM NADPH (pH 7.0) and 0.15 mL of tissue extract in 1 mL final volume was initiated at 30°C by the addition of glutathione disulfide (GSSG, 1 mM final) as per the original procedure (Gutterer *et al*. 1999). For GPx activity, the reaction mixture containing PPB (50 mM), 0.5 mM EDTA, 1 mM GSH, 1 mM sodium azide, 0.5 units GR, 0.2 mM NADPH (pH 7.0) and 0.15 mL extract in 1 mL final volume was initiated at 30°C by the addition of H_2O_2 (0.1 mM final concentration) as per the original procedure (Dringen and Hamprecht 1997). A decrease in the absorbance due to oxidation of NADPH was recorded at 340 nm. The activities of GR and GPx were calculated using the NADPH extinction coefficient (e = 6.22 mM^{-1}/cm^{-1}). Protein concentration was determined according to Bradford (1986).

Measurements of caspase-9 and -3 enzymatic activities

Fresh cerebral hemispheres cleared off the meninges were washed with ice-cold PBS, weighted and homogenized in ice-cold homogenization buffer (1 : 3 w/v) composed of 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin and 25 μg/mL aprotinin and 250 mM sucrose, using a Polytron homogenizer. Tissue was then centrifuged for 15 min at 15 000 × *g* and the supernatant collected. Samples were frozen at −70° C and protein was determined. Caspase-9 activity was measured in the supernatant following cleavage of p-nitroaniline conjugated LEHD (LEHD-pNA) (Calbiochem, Schwalbach, Germany). Caspase-3 enzymatic activity was measured following cleavage of *p*-nitroaniline conjugated DEVD (DEVD-pNA) substrate (Bachem, Babendorf, Switzerland). To aliquots of the supernatant, 40 mM LEHD-pNA or DEVD-pNA in caspase assay buffer consisting of 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), 10 mM dithiothreitol, 1 mM EDTA and10% glycerol was added and final solution incubated for 3 h at 37°C. The released pNA chromophore was quantified by spectrophotometry at 405 nm and caspases activity normalized to milligrams of protein.

Western blot analysis

Fetal brains were homogenized with five volumes (w/v) of a buffer consisting of 1% Triton-X 100, 1 mM benzamidine, 2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin and 25 μg/mL aprotinin using a Polytron homogenizer. The lysate was incubated for 10 min at 4°C and centrifuged at 15 000 × *g* for 15 min.

Aβ peptide detection

Aliquots of the lysate were loaded on a 14% Tris/Tricine/SDS polyacrylamide gel electrophoresis (PAGE) and the resulting proteins and low molecular weight peptides transferred to Immobilon P membranes (Millipore). After blocking with PBS containing 0.05% Tween 20 (Tween/PBS) and 10% skimmed milk and rinsing, the paper was immersed for 1 h in Tween/PBS containing the monoclonal antibody 6E10 (1 : 250 dilution), at room temperature. Following incubation, the first antibody was removed and the paper blot rinsed three times, 10 min each, with a Tween/PBS solution. A second peroxidase-linked goat anti-mouse antibody (Promega, Madison, WI, USA) diluted 1 : 10 000 in Tween/PBS buffer was added for 1 h at room temperature. Then the second antibody was removed and the paper blot rinsed three times, 10 min each, with Tween/PBS. Immunolabeled proteins were visualized with a chemiluminescence (ECL) kit from Amersham according to the manufacturer's specifications.

PKC identification

Aliquots of the lysate (30 μg protein) was separated by sodium dodecylsulfate (SDS)−10% polyacrylamide gel electrophoresis (PAGE) as detailed elsewhere (Kuperstein *et al*. 2001). After separation, protein bands were transferred from the gel onto nitrocellulose paper (BA 83, Schleicher & Schuell) and stained with polyclonal antibodies for various PKC isoforms (1 : 750 dilution, Santa Cruz Biotech, Santa Cruz, CA, USA). A second peroxidase-linked goat anti-rabbit antibody (Sigma) diluted 1 : 16 000 was used and immunolabeled proteins visualized with a chemiluminescence (ECL) kit from Amersham (Piscataway, NJ, USA) according to the manufacturer's specifications.

Identification of phosphorylated proteins

The protocol for analysis of several phosphorylated proteins mentioned in this work was described recently (Kuperstein and Yavin 2003). Briefly, activated ERKs were detected using an anti dually phosphorylated ERK antibody (Promega, 1 : 10 000 dilution in Tween/PBS). Activated p38 MAPK was detected using an antiactive p38 MAPK antibody (Promega, 1 : 5000 dilution in Tween/PBS) for 1 h. Activated Akt was detected using an antiactive Akt antibody (Promega, 1 : 2000 dilution in Tween/PBS) while phosphorylation of BAD at Ser136 was detected by probing blots with anti-phospho-BAD antibody (Oncogene, Boston, MA, USA; 1 : 1000 dilution in Tween/PBS). After washing, blots were stained with a second peroxidase-linked goat-anti-mouse antibody (Promega, 1 : 10^4 dilution in Tween/PBS) and developed with ECL kit (Amersham, Buckinghamshire, UK). For detection of total (activated and nonactivated basal level of proteins) blots were restained with polyclonal rabbit antibodies for ERKs, p38 MAPK

and Akt, respectively, and immunoreactivity revealed with AP-conjugated goat anti-rabbit antibody (Jackson Immuno-Research, West grove, PA, USA; 1 : 7500 dilution) and BCIP/NBT AP substrate as detailed elsewhere (Kuperstein and Yavin 2002).

Scanning

Computer-assisted image analysis was performed on a Silver scanner II (LaCie, USA) and values processed with NIH Image 1.63 software program.

Statistics

Values are expressed as average ± SEM from at least triplicate experiments. Variance analysis of all groups was performed using ANOVA followed by Tukey's *post hoc* test. Levels of significance ranged from $p < 0.001$ to $p < 0.05$.

Results

Incorporation of $A\beta_{1-40}$ into fetal tissues.

Using a selective antibody against the human Aβ peptide (clone 6E10), a robust immunostaining reactivity was revealed in fetal brain slices after 48 h following intraperitoneal injection of 6 μg soluble $A\beta_{1-40}$ into 18-day-old fetuses (Fig. 1, right panel). The distribution of $A\beta_{1-40}$ in various brain areas was uneven and most of the stain appeared to reside within the cortex and hippocampus regions in both extracellular and intracellular domains. Notable, little or no endogenous rat Aβ was noticed when the 6E10 antibody was added to untreated control sections (left panel).

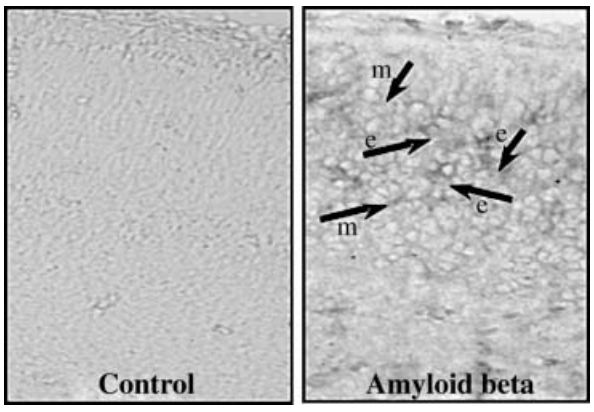

Fig. 1 Immunohistochemical visualization of $A\beta_{1-40}$ in fetal brain. Wistar rat dams at 18 days gestation were anesthetized and selected fetuses injected into the peritoneal cavity with either 6 μg of $A\beta_{1-40}$ peptide freshly dissolved in water to prevent aggregation (right panel) or water only (sham, left panel). After 48 h fetuses were killed by decapitation and brain tissue stored in liquid nitrogen. Slices from brain tissue were stained with a selective antibody against the human amyloid beta peptide (clone 6E10) as detailed under Materials and methods. Immunostaining of membrane (m) and extracellular (e) domains were recorded with light microscopy.

Further evidence for the accumulation of $A\beta_{1-40}$ in various fetal organs 24 h post injection of the peptide is illustrated in Fig. 2. Immunoblot analysis using the antibody specific to human $A\beta_{1-40}$ demonstrated an $A\beta_{1-40}$ immunoreactive band in three representative fetal organs. A similar distribution of the peptide can be seen after an ischemic episode generated by the reduced blood pressure following removal of 3–4 mL blood from the maternal circulation. There was no statistical difference between ischemic and control animals (data not shown). The degree of damage to the fetuses following a transient hVhT stress to the dams can be well appreciated by the high number of apoptotic cells seen in sections of the cortex of the affected fetuses stained with DAPI (Fig. 3, lower left frame). The nuclear morphology and the intensity of the DAPI stain above the background lend support to the possibility that these are cells in various stages of damage. Over fivefold increase in the number of DAPI-enhanced reactive cells was observed compared to the controls. In contrast, fetuses injected with $A\beta_{1-40}$ prior to hVhT stress showed a significant lower number of DAPI-enhanced reactive cells (Fig. 3, lower right frame), suggesting a possible neuroprotective effect of the Aβ peptide.

Effect of hVhT and Aβ on oxidative stress markers

The increase in the number of DAPI-enhanced reactive cells in brain tissue 24 h following hVhT stress prompted us to evaluate several oxidative stress parameters that may predate cell damage in fetal brain tissue. One such index is the production of TBARS a measure previously shown by us to be a good indicator for the degree of oxidative stress in a model of global ischemia (Glozman *et al*. 1998). In this work, freshly dissected slices from brains of fetuses subjected to various treatments were allowed to accumulate TBARS over a period of 15 min at 37°C. As illustrated in

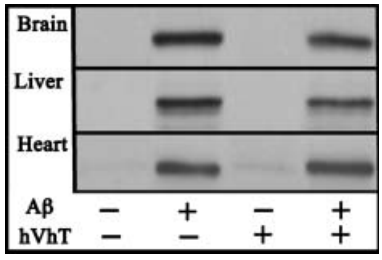

Fig. 2 Distribution of $A\beta_{1-40}$ in various fetal organs. Fetuses were injected with $A\beta_{1-40}$ as detailed in Fig. 1. After 24 h rats were re-anesthetized and subjected to a hypovolemic/hypotensive (hVhT) stress as detailed under Materials and methods. After 24 h of reperfusion, fetuses were killed by decapitation, fetal organs collected and immediately frozen in liquid nitrogen. Tissue aliquots were homogenized and total protein extract subjected to SDS gel chromatography followed by transblotting and immunovisualization with a selective antibody against the human Aβ peptide (clone 6E10).

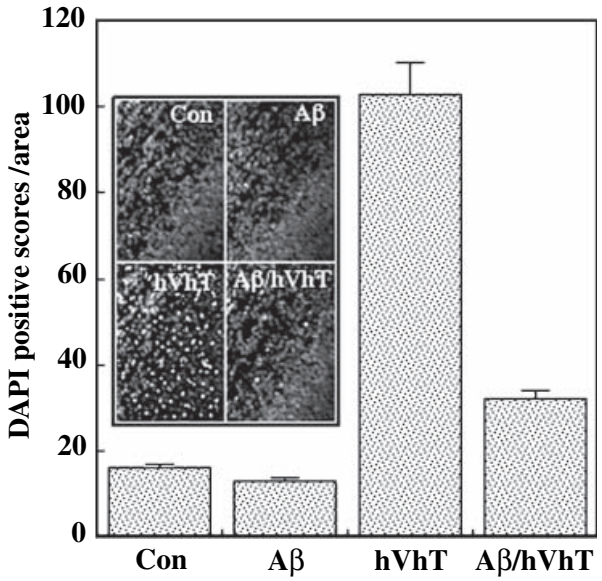

Fig. 3 Effect of $A\beta_{1-40}$ on hVhT-induced cell death. Fetuses were injected with $A\beta_{1-40}$ and after 24 h were subjected to a hypovolemic/hypotensive (hVhT) stress as detailed in Fig. 2. After 24 h fetuses were killed by decapitation and brain tissue immediately frozen in liquid nitrogen. Slices obtained from the cryostat were stained with DAPI dye. Slides were visualized and photographed under fluorescent microscope. Six different fields from two fetal cerebral cortex of different experimental dams were scored for the appearance of DAPI-positive stain. Values represent average of 12 scored fields ± SEM.

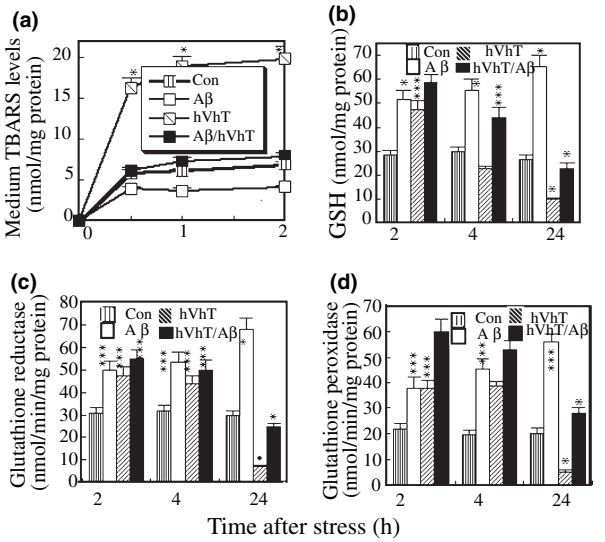

Fig. 4 Effect of $A\beta_{1-40}$ on the antioxidant status of the fetal brain after hVhT stress. Experimental conditions were similar to those detailed in Fig. 2. At times designated after hVhT stress (reperfusion) fetuses were killed by decapitation and fresh fetal brains slices were either incubated for 15 min at 37°C in DMEM in an atmosphere of 100% oxygen to generate TBARS (a) or subjected to extraction followed by HPLC for GSH analysis (b) as detailed under Materials and methods. Brain tissue homogenates were also prepared for GR (c) and GPx (d) enzymatic activity as detailed under Materials and methods. Values for thiobarbituric acid species (TBARS) obtained from triplicate measurements are expressed as nmol/mg protein released in the medium. Levels of GSH were obtained after pooling three fetal brains from one dam. Values are given in nmoles/mg protein from three independent experiments ± SEM. The specific enzymatic activities of GR and GPx detected were averages from two pooled fetal brains extract expressed as nmoles/min/mg protein. Four separate dams were used to obtain an average value ± SEM. Asterisks denote the range of significance: $^{*}p < 0.001$, $^{**}p < 0.01$, $^{***}p < 0.05$.

Fig. 4(a), the amount of TBARS measured in brain slices following the hVhT stress reached a level of 16.3 ± 1.3 nmol/mg protein/15 min. After 2 h reperfusion the values reached an apparent plateau at approximately 20 ± 1.5 nmol/mg protein/15 min. Brain slices from fetuses preinjected with either water (sham) or $A\beta_{1-40}$ in the peritoneal cavity, 24 h before killing, released significantly lower amounts of TBARS in the incubation medium. A basal plateau level between 3.7 and 7 nmol/mg protein/15 min was established for both slices from sham and Aβ-injected fetuses. Notably, fetuses preinjected with $A\beta_{1-40}$ 24 h prior to hVhT stress, showed significantly lower levels of TBARS (7.9 ± 0.4 nmol/mg protein/15 min after 2 h reperfusion) suggesting a protective role of $A\beta_{1-40}$ against the ischemic stress. These data strongly indicate that the $A\beta_{1-40}$ peptide may act *in vivo* in the developing brain as an antioxidant to abolish production of TBARS.

To investigate possible mechanisms through which the putative antioxidant property of the peptide may operate, we have measured levels of reduced glutathione. As evident in Fig. 4(b), after 2 h reperfusion, GSH levels in sham-treated brain slices (no hVhT), reached a level of 29.7 ± 1.9 nmol/mg protein and remained practically the same up to 24 h reperfusion. In contrast, 2 h after the hVhT stress, a 60% increase in GSH level in brain was noticed. By 4 and 24 h, these values were reduced by 24.2 and 68.0%, respectively, below the sham levels. A high level of GSH (51.6 ± 4 nmol/mg protein) was noticed in fetal brain extracts 2 h after $A\beta_{1-40}$ peptide administration. After 24 h, this value increased to 64.9 ± 5.3 nmol/mg protein. A partial and significant sustained level of GSH was noticed in brain extracts from fetuses pretreated with the peptide and subsequently subjected to hVhT stress. Under these conditions, the level of GSH detected in the brain extract after 24 h reperfusion was 22.9 ± 2.1 nmol/mg protein compared to 26.4 ± 1.6 and 9.5 ± 0.9 nmol/mg protein for the sham and hVhT treatments, respectively. These data clearly indicate that $A\beta_{1-40}$ peptide may play an active role in the maintenance of the antioxidant status of the fetal brain.

Further confirmatory evidence to this possibility was obtained by measurement of the antioxidant enzymatic

activities namely GR (EC 1.6.4.2) and GPx (EC 1.11.1.9). GR is an essential enzyme for the glutathione-mediated detoxification of peroxides because it catalyzes the reduction of glutathione disulfide. At 2 h reperfusion there was a 1.6-, 1.5- and 1.8-fold increase in its activity when fetuses were treated with either $A\beta_{1-40}$, or subjected to hVhT stress alone or a combination of both, respectively (Fig. 4c). While these values remained unchanged after 4 h reperfusion, the activity of GR in the hVhT-treated group was reduced compared to that of the sham group. A sustained activity of GR due to treatment with the $A\beta_{1-40}$ peptide was also noticed. Similar to the activity of the glutathione reductase, the GPx activity (Fig. 4d) followed essentially the same kinetics with respect to time after reperfusion. Thus after 2 h reperfusion, the specific activities of GPx were 1.7-, 1.7- and 2.7-fold greater in fetuses subjected to either $A\beta_{1-40}$, hVhT stress or a combination of both, respectively. The remarkable loss of both enzymatic activities after hVhT stress and 24 h reperfusion indicates a strong disruption of the glutathione antioxidant system and is associated with massive cell losses as attested by the DAPI stain.

Pro-apototic and anti-apoptotic pathways after induction of hVhT stress and $A\beta_{1-40}$ administration

During the course of $A\beta_{1-40}/Fe^{2+}$-induced apoptotic death in cerebral cortex cells, we have recently reported changes in several cellular signaling cascades including a decrease in protein kinase C (PKC) isoforms expression, reduced Akt serine/threonine kinase activity, down-regulation of Bcl2-associated death promoter (BAD) phosphorylation and activation of p38 MAP kinase (Kuperstein and Yavin 2003). The notion that these activities are indicative of a pro-apoptotic cellular course, prompted us to examine these signaling cascades in fetal brain after hVhT stress. Furthermore, because $A\beta_{1-40}$ alone in cortical cultures increased PKC isoform levels and, furthermore, it elevated Akt activity and stimulated Ser136 BAD phosphorylation in line with an anti-apoptotic cellular course, it was of interest to evaluate the similarities between the cultures and *in vivo* Aβ administration.

Figure 5 depicts the results of such an experiment on the level of two representative Ca-dependent (α) and Ca-independent (ε) PKC isoforms, after dams were subjected to an hVhT stress. Notably, a significant reduction of approximately 82% in αPKC (Fig. 5a) and εPKC (Fig. 5b) protein levels relative to sham was attained 12 h after reperfusion. On the other hand, a 1.7- and 1.5-fold increase in the αPKC and εPKC, respectively, was observed following the intraperitoneal injection of $A\beta_{1-40}$. The latter turned out to be protective with respect to PKC isoforms down-regulation after the hVhT stress. Similar to elevated PKC levels, a nearly fourfold increase in Akt (Fig. 5c) and a two fold increase of BAD phosphorylation (Fig. 5d) were detected in fetuses 24 h after intraperitoneal administration

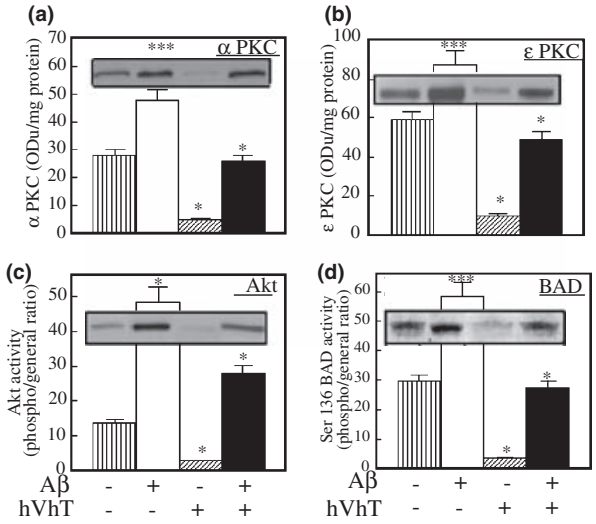

Fig. 5 Effect of $A\beta_{1-40}$ on anti-apoptotic pathways in fetal brain after hVhT stress. Experimental conditions were similar to those detailed in Fig. 2. After 12-h reperfusion, brains were removed and homogenized for western blot analysis as detailed under Materials and methods. Proteins were immunostained with polyclonal antibodies directed against α-PKC (a) and ε-PKC (b) and subsequently restained with an antibody recognizing β-actin for gel loading verification (not shown). Values obtained from X-ray films and expressed as arbitrary OD units normalized per mg protein. The phosphorylated form of Akt (c) and BAD (d) were detected by probing the blots with the appropriate antibodies and restaining with antibodies for general Akt and BAD, respectively (data not shown). OD values obtained from the X-ray film were expressed as relative ratio of phosphorylated to general levels. Values represent the average of three independent experiments ± SEM. Asterisks denote the range of significance: $^{*}p < 0.001$; $^{**}p < 0.01$; $^{***}p < 0.05$.

of the $A\beta_{1-40}$ peptide. In contrast, a marked reduction in the phosphorylation of these two proteins was seen after 15 min hVhT and 12 h reperfusion. Pretreatment with $A\beta_{1-40}$ retained the levels of both Akt and BAD high even though an hVhT stress was applied in accord with an overall enhanced tolerance induced by the peptide.

In accord with this possibility, and in line with our recent observations in cortical cultures (Kuperstein and Yavin 2002), a twofold increase in 42 kDa ERK/MAPK phosphorylation was noticed 24 h following intraperitoneal administration of the peptide (Fig. 6a). Under these conditions, the p38 MAP kinase was reduced by 50% from its basal levels (Fig. 6b). In contrast, the levels of p38 kinase increased by 11 fold in fetal brains of dams exposed to hVhT ischemic stress. In parallel, a substantial down regulation of 42 kDa ERK isoform was noticed after hVhT stress. Notably, fetuses pretreated with $A\beta_{1-40}$ and further exposed to hVhT stress, restored ERK phosphorylation to near control levels and also markedly reduced p38 kinase by nearly fivefold compared to stress levels.

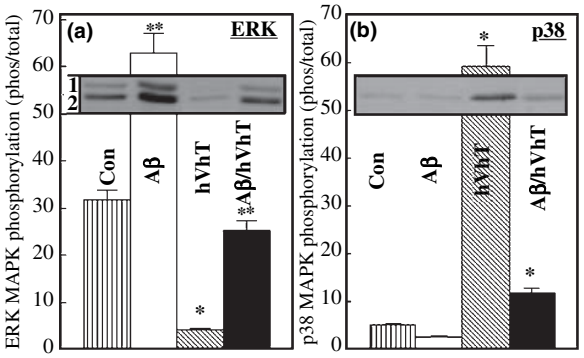

Fig. 6 Effect of $A\beta_{1-40}$ on MAPK pathways in fetal brain after hVhT stress. Experimental conditions were similar to those detailed in Figs 2 and 5. After 12-h reperfusion, brains were removed and homogenized for western blot analysis as detailed under Materials and methods. Proteins were immunostained with monoclonal antibodies directed against ERK (a) or p38 (b) and restained with polyclonal general anti ERK and anti p38 antibodies, respectively (data not shown). OD values obtained from the X-ray film were expressed as the relative ratio of phosphorylated to general levels. Values are expressed as average ± SEM of three independent experiments. Asterisks denote the range of significance: $*p < 0.001$; $**p < 0.01$; $***p < 0.05$.

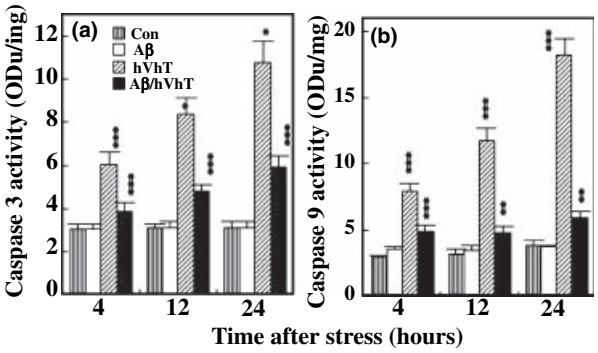

Fig. 7 Effect of $A\beta_{1-40}$ on caspases activity in fetal brain after hVhT stress. Experimental conditions were similar to those detailed in Fig. 2. At times designated after hVhT stress homogenates were prepared for caspase-3 (a) and -9 (b) activities as detailed under Materials and methods. Activities were obtained from three brains from each dam. Values are expressed as arbitrary OD units normalized per milligram of protein from three independent dams ± SEM. Asterisks denote the range of significance: $*p < 0.001$; $**p < 0.01$; $***p < 0.05$.

Cell death signaling via caspases after hVhT stress and $A\beta_{1-40}$ administration

Activation of the caspase family is unquestionably the hallmark of neuronal cell damage and the irreversible course towards apoptosis in a variety of pathophysiological conditions including oxidative stress (Love 2003). Figure 7 depicts the time course of caspase-3 and -9 activations over a 24-h period after hVhT stress in fetal brain after various pretreatments. As anticipated, a gradual increase in both caspases was seen in animals following hVhT treatment. In contrast, only a moderate elevation was noticed in caspase-3 (Fig. 7a) above control level after pretreatment with $A\beta_{1-40}$. The levels of caspase-9 (Fig. 7b) remained basically unchanged under these conditions, even 24 h after reperfusion. Notable $A\beta_{1-40}$ alone had no significant effect on caspase activity.

Discussion

The main objective of the present study was to evaluate the consequences of *in utero* administration of the $A\beta_{1-40}$ peptide on signal transduction pathways in the developing brain given the beneficial effects we have observed after administration of $A\beta_{1-40}$ to cultured cortical neurons at similar developmental stages (Kuperstein and Yavin 2003). Although in the present experiments we have employed a single dose of Aβ, several limited conclusions emerge with respect to a possible physiological role of the peptide in the prenatal brain.

First, we have shown that after intraperitoneal administration, $A\beta_{1-40}$ could reach the fetal brain where it elevated GSH levels and enhanced both GPx and GR enzymatic activities within 24 h (Fig. 4). GSH is the predominant low molecular weight thiol reductant and a key determinant in maintaining the intracellular redox state in the brain (Dringen and Hirrlinger 2003). Sustained levels of cellular antioxidant levels including GPx and glutathione transferase were recently shown in the adult brain after intraventricular infusion of $A\beta_{1-40}$ (Kim *et al*. 2003). Likewise, in PC12 cells a marked resistance to Aβ toxicity has been attributed to components of the glutathione recycling system (Sagara *et al*. 1998; Barkats *et al*. 2000).

Second, $A\beta_{1-40}$ peripheral administration enhanced Akt and BAD activities in the fetal brain, in line with enhancement of cellular signaling that promote cell survival. Activation of the PI3K/Akt signaling pathway has been shown in neuronal cultures exposed to subtoxic concentrations of Aβ (Zhang *et al*. 2001; Kihara *et al*. 2001). In addition to the PI3/Akt pathway, we also show a significant elevation of ERK2, a known member of the larger MAPK superfamily. Both PI3K/Akt and ERK/MAPK pathways have been implicated in protective response after stress (Xue *et al*. 2000). Although Aβ has been shown to activate MAPK cascades in cultured cells (McDonald *et al*. 1998; Daniels *et al*. 2001), to the best of our knowledge, this report provides for the first time evidence that $A\beta_{1-40}$ activates the ERK/MAPK cascade in the developing brain.

Third, $A\beta_{1-40}$ peripheral administration activated two principal members of the PKC family, namely the Ca^{2+}-dependent α-PKC, and the Ca^{2+}-independent ε-PKC isoforms. Enhanced levels of both enzymes are in accord with work of other laboratories showing that ischemia preconditioning caused translocation/activation of PKC (Katsura *et al*. 2001) and that stimulation of PKC activity protected

from Aβ-dependent and stress-induced apoptosis (Behrens *et al*. 1999; Maher 2001; Cordey *et al*. 2003). It is also in line with the marked increase in both the enzymatic activity and absolute levels of PKC isoforms noticed by us in cortical cultures after $A\beta_{1-40}$ treatment (Kuperstein *et al*. 2001). Along with the observation that Aβ-pretreated fetuses subjected to hVhT stress maintain adequate levels of PKC we strongly suggest that the peptide renders the fetal brain resistant and more tolerant to the more severe stress.

A second objective of the present study was to determine the outcome of a global *in utero* ischemic stress on fetal brain in the absence or presence of the $A\beta_{1-40}$ peptide. It is well established that cellular responses to brain insults such as ischemia or stroke are multifaceted and may involve perturbations of membrane ionic properties, enhanced Ca^{2+} influx, impaired mitochondria activity, generation of reactive oxygen species, energy depletion, and ultimately activation of intracellular signaling cascades leading to apoptotic cell death (White *et al*. 2000; Love 2003).

A note in advance is needed in order to establish the feasibility of the present ischemic model that basically involves a transient decrease in both volume and pressure of the entire maternal blood flow and consequently, the trans-placental blood flow. As clearly shown by us, a number of parameters that are considered typical oxidative stress markers have been altered in the fetal compartment. Among these, the ability of brain slices from hVhT-treated fetuses to produce TBARS was profoundly increased in accord with a free fatty acid release following ischemia/reperfusion stress (Siesjo and Katsura 1992). Furthermore, a severe disturbance in glutathione antioxidant cycle as demonstrated by a significant decline in levels of GSH-regulating enzymes and in GSH (Fig. 4) was detected. Additionally, a marked decrease in the phosphorylation of several kinases including Akt and ERK, as opposed to an increase of p38 MAPK, indicative of activation of pro-apoptotic signaling cascades, was noticed. Most striking, however, was the accelerated cell death as attested by enhanced caspase-9 and -3 activities (Fig. 7) and by an increase in the number of DAPI-enhanced reactive cells (Fig. 3). Adding up these changes, the increase in p38 MAPK activity is also in agreement with cell damage (Hull *et al*. 2002; Wang *et al*. 2003). Interestingly, increased p38 MAPK was identified in Aβ deposits in a transgenic AD mouse model (Savage *et al*. 2002). p38 MAPK was elevated after ischemia injury in an APP over-expressing mutant mice (Koistinaho *et al*. 2002). Thus p38 MAPK is an important indicator for tissue deterioration following stress. Finally, the levels of two PKC isoforms are markedly down-regulated following the hVhT stress. There is a general agreement that serine/threonine kinases are down-regulated in severe conditions of ischemia (Domanska-Janik 1996; Baines *et al*. 1999). Because losses of PKC and subsequent cell death (Fiorani *et al*. 1995; Kuperstein *et al*. 2001) could be prevented by treatment with antioxidants (Gopalakrishna and Jaken 2000), an oxidative stress component (i.e. free radicals) causing this process is strongly indicated. Down-regulation of PKC has been documented in a variety of neurological disorders including AD where it has been associated with memory deficits and dementia (Cole *et al*. 1988; Olariu *et al*. 2002; Clark *et al*. 1991 Matsushima *et al*. 1996).

After establishing this model of prenatal ischemia we posed the question as to whether the *in vivo* Aβ peptide administration may be beneficial to the fetal brain. Indeed, one dose preadministration of $A\beta_{1-40}$ appeared neuroprotective as attested by (i) a partial recovery of the GSH antioxidant apparatus; (ii) a shift from pro-apoptotic to anti-apoptotic signaling cascades activities; and (iii) a reduction in the number of DAPI enhanced reactive cells. The reduction in TBARS levels and the restoration of GSH defense mechanisms strongly indicated that the peptide may have exerted its neuroprotective effect either by acting directly as an antioxidant and free radical chain breaker (Hou *et al*. 2002; Zou *et al*. 2002) or indirectly, by activating pro-survival signaling cascades including kinases and antioxidant enzymes. At this time, whether the neuroprotection acquired via $A\beta_{1-40}$ is of temporary or of long lasting nature, remains unknown. Our studies did not go beyond a total of 48 h time frame and it is possible that delayed effects of ischemia may be notable at later times. Although the newborn pups appear grossly normal, as their non-hVhT counterparts, differences in biochemical and behavioral properties warrant further investigations.

In summary, the fetal brain undergoes dynamic organizational changes during intrauterine and early postnatal life. Acute episodes of oxidative stress may render growing neuronal populations irreversibly damaged with severe consequences for the adult life (Hankins and Speer 2003). Based on this study we would like to postulate that the presence of Aβ or Aβ-like peptides may be crucial in the acquisition of tolerance to free radicals by accelerating the effectiveness of the endogenous antioxidants system. Could it possibly be that the reason for the remarkable resistance to neurodegeneration, accompanied by activation of cell survival signaling cascades in transgenic mice mutants over-expressing the amyloid precursor protein (Stein and Johnson 2002) is acquired via a continuous release of APP or APP cleavage products which enhance the tolerance to stress? That possibility remains to be examined.

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