2-Deoxyglucose induces β-APP overexpression, tau hyperphosphorylation and expansion of the trans-part of the Golgi complex in rat cerebral cortex

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Abstract. The effects of a single intraperitoneal injection of a non-metabolizable glucose analog 2-deoxyglucose (2-DG, 500 mg/kg) on the levels of β-APP expression, and phosphorylated and unphosphorylated tau protein in the rat cerebral cortex were investigated. The effects of 2-DG on the ultrastructure of cortical neurons with particular emphasis on the morphology of the Golgi apparatus, and on brain bioenergetics assessed by in vivo ³¹P-MRS technique were also evaluated. Seven and a half hours after injection of 2-deoxyglucose a significant increase in brain cortex β-APP expression, increased tau phosphorylation, and a marked relative expansion of the trans- part of the Golgi intracellular secretory pathway in cortical neurons has been found. The changes of β-APP expression and tau phosphorylation appeared within 1 h after 2-DG application and continued for at least 24 h. However, brain ³¹P resonance spectra remained unchanged for up to 7.5 h after 2-DG. It is suggested that the increase of β-APP expression represents a response of brain tissues to 2-DG-evoked biochemical stress, while tau hyperphosphorylation and the change in Golgi morphology may be secondary phenomena.

Key words: 2-deoxyglucose, beta-amyloid precursor protein, tau protein, Golgi apparatus, phosphorous magnetic resonance spectroscopy, brain cortex
INTRODUCTION

The "amyloid cascade" hypothesis of Alzheimer's disease (AD) assumes that formation of insoluble, neurotoxic amyloid deposits resulting from imbalance between amyloid beta peptide (Abeta) production and catabolism is the primary and central pathogenetic event. The other phenomena characteristic of the Alzheimer's brain such as formation of neurofibrillary tangles, cholinergic dysfunction, brain hypoperfusion, hypometabolism and oxidative stress, and finally neurodegeneration, are considered secondary to the amyloid neurotoxicity (Hardy and Higgins 1992, Hardy and Selkoe 2002). Although currently the most popular, the "amyloid cascade" hypothesis is not universally accepted. For example, Swaab and coauthors (2002) pointed out that neuropathological hallmarks of AD (amorphous and neuritic plaques, pretangles and neurofibrillary tangles, and neuronal death) cannot form a single pathogenetic cascade because they may occur independently.

One of the numerous alternative hypotheses of AD is the "insulin resistance" hypothesis (Hoyer 1998, 2002) which assumes that the primary and central pathogenetic event in the AD (sporadic type) is the desensitization of brain insulin receptors, i.e., AD is brain-specific analog of type 2 diabetes mellitus (IDDM). According to this concept, brain glucose metabolism is controlled by insulin receptors, and their desensitization results in progressive metabolic and functional failure. The other phenomena characteristic for the Alzheimer's brain, including formation of insoluble amyloid deposits, result from the cerebral hypometabolic state. This hypothesis is supported by experimental data showing that intracerebroventricular injections of a diabeticogenic toxin streptozotocin are followed by a fall in brain glucose consumption (which is more pronounced than the concomitant fall in oxygen uptake), by a drop in the cellular energy pool, cholinergic deafferentation, learning and memory deficits and changes in membrane phospholipids. All these phenomena are similar to those which occur in sporadic AD (reviewed by Hoyer 2002). Streptozotocin, known to inhibit the phosphorylation of insulin receptor tyrosine kinase (Kadowaki et al. 1984), upon injection into the brain ventriculocisternal system is assumed to cause dysfunction of neuronal insulin receptors and impair insulin signal transduction across the brain. The "insulin resistance" hypothesis may explain some clinical, as well as experimental data, which does not fit well into the frame of the "amyloid cascade" hypothesis. These include, first of all, the early decrease of glucose metabolism in AD, which precedes not only the onset of dementia, but also the decrease of brain oxygen uptake (Fukuyama et al. 1994, Minoshima et al. 1997, Ogawa et al. 1996, Pieri et al. 1996).

Abeta is a product of amyloidogenic proteolysis of beta-amyloid precursor protein (β-APP) (Mattson 1997). This protein, which has a relatively short half-life of 20-30 min (Weidemann et al. 1989), is translocated into the endoplasmic reticulum and posttranslationally modified within the Golgi system. Later it undergoes either nonamyloidogenic or amyloidogenic proteolysis which yields soluble non-amyloidogenic peptides or amyloidogenic Abeta, respectively. These peptides are released into the extracellular space. In normal conditions both proteolytic pathways are active and certain quantities of Abeta are released from cells (Selkoe 2001, see also the references cited therein). However, Abeta is effectively catabolized and no amyloid deposits are formed.

The equilibrium between Abeta formation and clearance may be disturbed when the production of β-APP is increased. Various patterns of β-APP overexpression in brain cells have been reported after several types of cerebral injury including focal and global ischemia, trauma, excitotoxicity (for references see Mattson 1997), hyperglycemic hypoxia (Pedersen et al. 1999), hypoglycemia (Shi et al. 1997) and simulated subarachnoid hemorrhage (Ryba et al. 1999). Altered expression and phosphorylation of β-APP have also been found in heat-shocked neuronal PC12 cells in vitro along with the induction of heat shock protein hsp72 (Johnson et al. 1993, Wallace et al. 1993). Interestingly, stimulation of the amyloidogenic pathway of β-APP processing following glucoprivation of cultured hippocampal cells by a combination of 2-deoxyglucose (2-DG) and sodium azide has been found by Gabuzda and coauthors (1994) who attributed this effect to the inhibition of cellular energy metabolism. 2-DG is a non-metabolizable glucose analog which enters the cells and is phosphorylated to 2-DG-6-phosphate (2-DG-6-P), but is not metabolized further and acts as the competitive inhibitor of glucose-6-phosphoisomerase (Wick et al. 1957). Several investigators have used 2-DG as a non-specific stressor and employed it, for example, to define stress effects on immune responses (Dreau et al. 2000).
In the experiments described herein we attempted to find out whether a biochemical stress evoked in vivo by a single systemic injection of 2-deoxyglucose involves changes in brain bioenergetics and whether it is followed by the β-APP overexpression, tau protein hyperphosphorylation and ultrastructural changes in the morphology of the Golgi apparatus resembling those characteristic for the Alzheimer’s brain.

**METHODS**

The protocol of the study was approved by the First Ethical Committee of the City of Warsaw. Chemicals, antibodies and standards were purchased from Sigma (St Louis, MO, USA), unless stated otherwise. The experiments were performed on outbred male Wistar rats, 230-350 g body weight, bred in the Animal House of the Mossakowski MRCPAS. All experimental animals were injected intraperitoneally with 500 mg/kg 2-DG (prepared as 1g/ml solution in physiological saline). Control animals received solvent injection.

For Western blotting of β-APP- and tau-related immunoreactivity the animals were sacrificed by decapitation at different times after 2-DG injection (0 and 7.5 h in the first, and 0, 1, 2, 3, 6, 12 and 24 h in the second series of the experiments, 4-8 animals per each time point). The brains were immediately snap-frozen in liquid nitrogen and stored at -70°C until further processed. The cortex (grey matter) from each hemisphere was dissected out on a coated glass surface and homogenized (1:10, w/v) in 20 mM MES/Na buffer pH 6.8 containing 5 mM EGTA, 1 mM MgCl2, 1 mM PMSF, 50 μg/ml leupeptin, 25 μg/ml pepstatin A and 0.1 mg/ml aprotinin. For β-APP the following antibodies raised against synthetic peptides corresponding to the amino acid residues of (697 aa) isoform were used: mAb which reacts with the near-end N-terminal domain of β-APP (46-60 aa) (1:1 000); mAb 6E10 recognizing 1-17 aa residues of Abeta corresponding to 597-613 aa of β-APP, further referred to as the M-domain (1:1 000); pAb RAS57 against the C-terminal domain of β-APP (672-695 aa) (a gift from Prof. Henry Wiśniewski, IBR, Staten Island, NY, USA, 1:1 000). For phosphorylated and unphosphorylated forms of tau protein mAb AT-8 (from Innogenetics, Ghent, Belgium, 1:10) and mAb Tau-1 (from Boehringer Mannhein Germany, 1:200) were used, respectively. Tau-1 recognizes a non-phosphorylated and AT-8 a phosphorylated epitope of tau.

Aliquots of brain tissue homogenates containing 40 μg protein for β-APP and 0.5-5 μg protein for tau were electrophoresed on linear SDS-PAGE 10% polyacrylamide gels (Laemml 1970), and on trycine–SDS-polyacrylamide gels (Schagger and von Jagow 1987) using 4% T 3% C stacking gel 10%, using II-Protean Gel Apparatus and Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA), respectively. The separated proteins were electrotransferred onto nitrocellulose membranes (Towbin et al. 1979). Non-specific binding sites were blocked with 5% skim milk in TBS for 2 h at 25°C. The membranes were incubated with primary antibodies for 4 h and the complexes were exhibited to appropriate secondary antibodies and visualized with an ECL Western Blotting Detection Reagent Kit (Amersham, UK), or an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, USA).

The intensity of immunoreactivity was quantified on nitrocellulose membranes, or ECL X-ray films by densitometric scanning using Gel Expert Software and Analysis System Apparatus Nucleo Vision 920 (NucleoTech Corporation, USA). To cover the range of isoforms detected by the antibodies, scanning was performed within 112-116 kD and 63-68 kD limits (according to the high MW standard), for uncleaved β-APP and tau, respectively. Quantitative data are presented in the figures as means ± SD. Statistical significance was tested with t-test for independent data, or one-way ANOVA followed by post-hoc Sheffe’s test, as appropriate.

To assess the effects of 2-DG on brain energy metabolism 31P MR localized spectra were acquired in vivo from rat cortex using a research MRI/MRS system (located at the H. Niewodniczañski INPPAS), equipped with a 4.7T/31 cm horizontal bore superconducting magnet (Bruker Germany), actively shielded gradient coil (Magnex Scientific, UK) and a digital console MARAN-DRX (Resonance Instuments, UK). A home-made dedicated probe with a double tuned surface coil (1H at 200.121 MHz, 31P at 81 MHz) placed over the skull was used to acquire MR signals from the brain cortex. The rats, anesthetized with a 2% halothane in oxygen/air 40/60 mixture and maintained with 1-1.5% halothane in the same mixture, were placed in the magnet. Before each experiment RF pulses were optimized to obtain maximum S/N ratio from the cortex. B0 homogeneity was corrected by manually protons over the whole brain to obtain final linewidth of 0.3 ppm. 31P spectra were acquired using the excitation-acquisition
method just prior to, and then every 6 minutes after 2-DG injection, up to 7.5 h (or prior to death). A glass pellet of 3 mm inner diameter containing 0.5 mM PPA water solution, placed on the skull surface in the centre of the surface coil, served as an external standard. The MRUI graphical user interface package and the VARPRO non-linear fitting algorithm were used to process the acquired FID signals.

Brain tissue sampling for electron microscopy was performed under ketamine (70 mg/kg) and xylazine (20 mg/ml) anesthesia 7.5 h after injection of 2-DG or vehicle. The animals (4 2-DG treated and 3 vehicle-treated) were perfused via the left heart ventricle with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 20°C. Tissues sampled from the fronto-parietal lobe of cerebral cortex were fixed in the same solution for 20 h, postfixed in a mixture of 1% OsO4 and 0.8% K4[Fe(CN)6], and processed for transmission electron microscopy. After dehydration in series of ethanol and propylene oxide, tissue specimens were embedded in Spurr resin. Serial ultrathin (50 nm) sections were examined with a JEM 1200EX electron microscope. In representative populations of neuronal cells, the average area of the whole Golgi apparatus as well as its -cis (proximal) and -trans (distal) parts was also determined, as described in detail in the accompanying paper (Grieb et al. 2004). Significance of differences between morphometric data for neurons from control and 2-DG-treated animals were evaluated with the Mann-Whitney U-test.

RESULTS

Seven and a half hours following subcutaneous 2-DG injection immunoreactivity detected in cortical homogenates by each of the three antibodies directed toward different domains of uncleaved β-APP revealed marked increases (Fig. 1). Interestingly, however, the effects on the immunoreactivity detected by the three antibodies recognizing different epitopes on uncleaved β-APP molecules were of different magnitude: the C-terminal immunoreactivity was increased by 59%, whereas that of N-terminal by 108%, and that of the middle domain of β-APP chain by 84%.

Immunoreactivity detected by the antibody recognizing the nonphosphorylated tau molecules revealed a marked decrease, while the opposite effect was seen with immunoreactivity directed toward the phosphorylated tau (Fig. 2). The effects of 2-DG on non-phosphorylated and phosphorylated tau were of comparable magnitude, namely a decrease of 55% and an increase of 45%, respectively, was found.

Electron-microscopic evaluation of the fronto-parietal cortex sampled 7.5 h after 2-DG application revealed enlarged endoplasmatic reticulum and markedly

Fig. 1. The effects of 2-DG on β-APP-related immunoreactivity detected by the three different antibodies. (0) homogenates of brain cortex of control animals; (7.5 h) homogenates of brain cortex of animals sampled 7.5 h after 2-DG injection. All differences are statistically significant ($P<0.01$).
expanded *trans*-part of the Golgi system in neuronal cells, while mitochondria were not affected (Fig. 3). For comparison, a micrograph of a cortical neuron from a vehicle-injected animal is also shown (Fig. 4). Quantitative morphometric analysis of the Golgi apparatus in neurons of control and 2-DG-treated animals showed that neither the average size of the whole Golgi complex, nor the average size of its *cis-* (i.e., proximal) part were significantly affected by the treatment (Fig. 5). However, in neurons from 2-DG-treated animals the size of the *trans-* (i.e., the distal) part of the Golgi was significantly increased (Fig. 5), and this resulted in markedly higher values of the *trans/cis* area ratio of the Golgi in most cells (Fig. 6).
MR spectroscopy evaluation of brain phosphorous compounds revealed no significant changes in the spectra for up to 7.5 h after subcutaneous injection of 2-DG (except when the condition of the animal deteriorated prior to death caused by anesthesia-related acidosis and hypotension). Following 2-DG application all spectral lines remained unchanged, except a transient increase of the phosphomonoesters (PME) signal at approx. 7 ppm (Fig. 7), reflecting accumulation of 2-DG-6P in the brain. According to Deuel and coauthors (1985) the chemical shift of 2-DG-6-P is 7.11 ppm.

The results of further immunoblotting experiments revealed that changes of all three β-APP-related immunoreactivities are highly significant and appear to be a bi-phasic phenomenon (Fig. 8). In the first phase there is a quick and massive increase in the M-domain-related immunoactivity of the uncleaved β-APP (to more than 250% of the control value 1 h after 2-DG application), whereas corresponding changes of the C-terminal and the near-N-terminal domains are of a much lower magnitude (to approx. 150%). In the second phase, which began later than 6 h after 2-DG injection, the increases in all three domains of uncleaved β-APP are of similar magnitudes compared to the control values.

The changes of unphosphorylated tau with time (Fig. 9, upper panel) were also highly significant, but did not show a bi-phasic characteristic. Instead, unphosphory-
Fig. 7. Brain $^{31}$P spectra acquired just prior to (upper panel) and approximately 2 h after the injection of 2-DG (lower panel). (PME) phosphomonoesters; (Pi) inorganic phosphates; (PDE) phosphodiesters; (PCr) phosphocreatine; (NTP) nucleoside triphosphates.
lated tau decreased sharply 2 h after 2-DG application (the difference between 0 and 2 h, and 1 and 2 h is highly significant by Sheffe’s test, \(P < 10^{-5}\)) and remained decreased afterwards for at least 24 h. The changes in phosphorylated tau (Fig. 9, lower panel) were also highly significant, but in the opposite direction, indicating a sharp increase 2 h after 2-DG injection (the difference between 0 and 2 h, and 1 and 2 h are highly significant by Sheffe’s test, \(P < 10^{-5}\)) and no changes afterwards.

Fig. 8. Changes of \(\beta\)-APP-related immunoreactivity detected by the three different antibodies. In all three cases the dependence of immunoreactivity on time is highly significant \((P < 10^{-5}\) by ANOVA). Different behavior of the three domains and a biphasic response is clearly seen.

Fig. 9. Changes of non-phosphorylated (grey bars) and phosphorylated (black bars) tau immunoreactivity following 2-DG injection. In both cases the dependence of immunoreactivity on time is highly significant \((P < 10^{-5}\) by ANOVA).
DISCUSSION

The results of the present study indicate that systemic application of 2-DG produces a quick and significant increase in the uncleaved β-APP-related immunoreactivities which may be interpreted as the evidence for increased β-APP expression at the protein level. However, the changes of the immunoreactivity detected by each of the three antibodies recognizing different epitopes on the uncleaved β-APP molecule were of different magnitudes. Similar disparity between the increases of different domains of β-APP has been observed previously in response to simulated subarachnoid haemorrhage (Ryba et al. 1999). This phenomenon may reflect differences in posttranslational modifications of the amino acid residues in the domains detected by particular antibodies. β-APP undergoes numerous modifications affecting side amino acid residues, including phosphorylations at various sites (Alpin et al. 1996, 1997, Gandy et al. 1988). All the antibodies used in the present study have been raised toward synthetic peptides. If posttranslationally modified amino acid residues are located within, or close to the epitopes detected by the antibodies, modified (e.g., phosphorylated) protein molecules may not be detected, or sensitivity for their detection may be decreased. It is worth noting that the increase of M-domain-related immunoreactivity was always higher compared to the other two, which is in agreement with the fact that the 597-613 aa epitope of uncleaved β-APP, referred to as the M-domain, is not posttranslationally modified.

As mentioned in the introduction, increased β-APP expression in brain tissue has been found following a variety of insults. Physiological function of this protein remains poorly understood despite tremendous interest stimulated by the “amyloid cascade” hypothesis of AD etiology. There is, however, some evidence that β-APP and some of its nascent peptides may positively influence neurons. β-APP was able to enhance viability of the neurons in vitro (Perez et al. 1997), and soluble products of β-APP cleavage displayed neuroprotective activity in the in vivo brain ischemia model (Smith-Swintosky et al. 1994). Interestingly, following posts ischemic reperfusion the overexpression of β-APP and heat-shock proteins have been reported in the same brain regions, although mostly in distinct neurons (Tomimoto et al. 1994). In that study β-APP accumulated in neurons on the margin of the regions destined to die, but the majority of these neurons seemed to survive after ischemic insult (Tomimoto et al. 1994). On the other hand, 2-DG induced an increase in the level of the stress protein heat-shock protein 70 (HSP 70) in striatal cells in vivo, and 2-DG treatment induced HSP70 in cultured neurons (Yu and Mattson 1999). Repeated injections of 2-DG (dose 100 mg/kg/day) induced increased levels of the stress-responsive proteins GRP78 and HSP70 in brain neurons (Lee et al. 1999), and (dose 150 mg/kg/day) enhanced ischemia-evoked brain tolerance to seizures (Rejdak et al. 2001). The results of the present study add 2-DG-induced biochemical stress to the list of insults which result in β-APP overexpression in brain tissues. Increased β-APP may reflect yet another aspect of an unspecific response of brain tissues to stressful conditions. One may speculate that overexpression of this protein may help neurons survive and be involved in the development of brain tolerance to noxious stimuli.

The effects of 2-DG on brain tissue β-APP expression observed in the present experiments cannot be attributed to the failure of cellular bioenergetics. While such an explanation certainly holds true in the case of the in vitro study of Gabuzda and coauthors (1984), it doesn’t seem to be of value as an explanation of the results obtained in vivo. Although the effects of repeated administration of lower doses of 2-DG described in some studies were similar to those of dietary (caloric) restriction (Yu and Mattson 1999) or intermittent fasting (Wan et al. 2003), this can be attributed to the systemic stress response evoked by 2-DG rather than to insufficient glucose supply and metabolism in the brain. Our results show that a single dose of 500 mg/kg 2-DG given intravenously does not significantly affect in vivo brain tissue 31P NMR spectra. This is in agreement with the data of Horton and coauthors (1973) who have found no fall in brain ATP following a single dose of 3 g/kg 2-DG, although these authors have noted a fall in brain phosphocreatine. Apparently, although 2-DG enters brain and is phosphorylated, and 2-DG-6-P (which is not metabolized by glucose-6-phosphoisomerase) may transiently restrict glucose utilization, this effect is short lasting and, as far as the energy balance of brain tissue is concerned, appears to be fully compensated either by some other available sources of energy, or by decreased energy expenditure of brain cells.

We have also found a significant and sustained increase of the phosphorylated form of tau protein following 2-DG injection, which was counterbalanced by a decrease of unphosphorylated tau. The link between β-APP overexpression and tau phosphorylation may be provided by glycogen synthase kinase 3βeta.
(GSK3beta), the enzyme implicated in a variety of intracellular receptor-mediated processes including transduction of insulin signaling (Grimes and Jope 2001). GSK3beta activity has been implicated in virtually all aspects of Alzheimer’s pathology, including pro-amyloidogenic phosphorylation of the carboxy-domain of β-APP (Alpin et al. 1996), tau hyperphosphorylation (Lee et al. 2003, Lovestone et al. 1994) and disassembly of neuronal Golgi apparatus (Elyaman et al. 2002). Its unique feature is that it is constitutively active in resting conditions. One may consider the possibility that 2-DG produces hypoinsulinemia which activates GSK3beta and hyperphosphorylates tau. Indeed, prolonged treatment of rats with lower doses of 2-DG has been recently found to cause a marked decrease in plasma insulin (Wan et al. 2003). However, Pascoe and coauthors (1989), using an experimental design identical to that of the present study, found that i.v. infusion of 500 mg/kg 2-DG to male Wistar rats resulted in the approximately 3-fold increase in plasma insulin level.

A recent finding that Abeta is a direct competitive inhibitor of insulin binding to its receptor binding and insulin receptor autophosphorylation (Xie et al. 2002) may link overexpression of β-APP directly to the activation of GSK3beta. One may speculate that overexpression of β-APP, which is a primary brain cellular response to 2-DG-evoked stress, shifts the balance between Abeta production and removal toward the increase of Abeta concentration outside brain cells. The next step would be the competition of Abeta with insulin at the insulin receptors, impaired transduction of the insulin signaling and the activation of GSK3beta, to create a vicious cycle.

The changes in Golgi morphology which we have found following 2-DG injection may also be driven by the same mechanism. It is worth noting that a similar morphological picture (i.e., the apparent relative expansion of the trans-part of the Golgi complex) has been previously found in our lab in brain neurons of rats subjected to acute insulin-induced hypoglycemia (Wierzba-Bobrowicz 1980, 1984 – unpublished doctoral thesis). These observations are in agreement with findings of the others. Agardh and coauthors (1981) have noted that the pathogenesis of cell damage in hypoglycemia is different from that in hypoxia-ischemia and indicated that other mechanisms than energy failure must contribute to neuronal cell damage in the brain. Also, according to Simon and coauthors (1986), the neuropathology of rat hippocampal neurons at the EM level following hypoglycemia was markedly different from that seen following status epilepticus and ischemia, the main difference being that microvacuoles were rarely seen and, when present, their ultrastructural correlate was swollen Golgi apparatus, not dilated mitochondria.

Neither the changes in Golgi morphology produced by intravenous injection of 2-DG, nor similar changes reported previously following hypoglycemia, seem to have a direct relation to brain tissues energy failure. It is also unlikely that they are related to hyperinsulinemia which would rather be expected to dephosphorylate tau protein in the brain. It is well known that starvation decreases insulin in blood, and Yanagisawa and coauthors (1999) have shown that starving induces tau hyperphosphorylation in the mouse brain. On the other hand, similar but even more pronounced enlargement of the trans compartment of the Golgi has been found following intracerebroventricular injections of streptozotocin, a toxin which desensitizes brain insulin receptors (Grieb et al. 2004). It is tempting to speculate that all these functional and ultrastructural effects may be related either to stimulation of β-APP synthesis and Abeta formation, modulation of GSK3beta activity, or an interplay between these two mechanisms at the level of brain insulin receptors.

**CONCLUSION**

A single dose of 2-deoxyglucose (500 mg/kg i.p.) induces in brain cortex of rats a bi-phasic β-APP overexpression, a hyperphosphorylation of tau protein, and a marked expansion of the trans part of the neuronal Golgi apparatus, but does not appreciably influence brain energy compounds.

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